Advancing molecular macrobenthos biodiversity monitoring: a comparison between Nanopore and Illumina based metabarcoding and metagenomics.

Karlijn Doorenspleet[1]*, Amalia Aikatarini Mailli[2]*, Berry van der Hoorn[3], Kevin Beentjes[3], Annelies De Backer[4], Sofie Derycke[4], Tinka Murk[1], Henning Reiss[2], Reindert Nijland[1].

* Contributed equally.

[1]Marine Animal Ecology group, Wageningen University and Research, Wageningen, The Netherlands

[2]Faculty of Biosciences and Aquaculture, Nord University, Bodø, Norway

[3]Naturalis Biodiversity Center, Leiden, The Netherlands

[4]Flanders Research Institute for Agriculture, Fisheries and Food – Marine Research, 8400 Oostende, Belgium

Corresponding Author:

Reindert Nijland

Email address: reindert.nijland@wur.nl

Abstract

DNA-based methods and developments in sequencing technologies are integral to macrobenthos biodiversity studies, and their implementation as standardized

monitoring methods is imminent. Evaluating the efficacy and reliability of these technological developments is crucial for bulk macrobenthos biodiversity assessments.

In this study, we compared three DNA-based techniques for assessing the biodiversity of bulk macrobenthos samples from the Belgian North Sea. Specifically, we compared the results of Illumina MiSeq metabarcoding, Oxford Nanopore metabarcoding and Illumina NovaSeq metagenomic shotgun sequencing. The 303 bp COI Leray region served as the target region for the metabarcoding analysis.

Our results indicate that Nanopore and MiSeq metabarcoding performed comparable in terms of alpha and beta diversity, revealing highly similar location-specific community compositions. The NovaSeq metagenomics method resulted in lower alpha diversity and different community compositions compared to the metabarcoding approach. Despite these differences, location specific community compositions were maintained across all platforms.

Notably, read counts of the NovaSeq metagenomics showed the weakest correlation to morphological abundance and half of the species found using morphological identification were undetected with DNA based methods, primarily due to insufficient reference sequences.

Our findings underscore the critical importance of database completeness prior to implementing DNA-based techniques as standardized monitoring method standardized monitoring method. Nevertheless, our findings emphasize that Oxford Nanopore metabarcoding proves to be a viable alternative to the conventional Illumina MiSeq metabarcoding platform for macrobenthos biodiversity monitoring.

Introduction

The European Union established the Marine Strategy Framework Directive (MSFD, 2008/56/EC), alongside the European Water Framework Directive (WFD, 2000/60/EC) and the European network for protected areas for conserving the most valuable North Sea species and habitats (Natura 2000 network, Habitat Directive). These directives form the basis of ecosystem management and use a variety of monitoring-based assessments (e.g., Borja et al., 2010) that aim to inform (non-)governmental decisions on marine environmental health. However, taxonomy-based monitoring practices rely on great taxonomic expertise, are time-consuming and limited in taxonomic resolution (Danovaro et al., 2016; Pawlowski et al., 2018), particularly when identifying understudied taxonomic groups and species at different life stages (Ammon et al., 2018; Gleason et al., 2023). Consequently, DNA-based technological developments have attracted particular interest in study of marine biodiversity (Bucklin et al., 2011; Cordier et al., 2019; Elbrecht et al., 2017; Lanzén et al., 2017; Leray & Knowlton, 2016). A reduction in labour time and the growing reference sequence libraries demonstrate DNA based methods to be a promising alternative for monitoring (Baird & Hajibabaei,

2012; DeSalle & Goldstein, 2019; Gostel & Kress, 2022; Leray & Knowlton, 2015). Different DNA-based monitoring studies have demonstrated correspondence with morphological findings (Derycke et al., 2021; Mauffrey et al., 2021). As a result, DNA-based methods are repeatedly proposed as a suitable routine biodiversity assessment strategy to inform policy (Aylagas et al., 2020; Goodwin et al., 2017; Hering et al., 2018; Hinz et al., 2022; Pawlowski et al., 2018; Thalinger et al., 2019).

Generally, DNA-based biodiversity assessments are based on DNA metabarcoding, which allows for the identification of species from environmental samples based on a short DNA fragment that is amplified using universal primer pairs. This amplified DNA is then sequenced with next-generation sequencing platforms to identify the taxa found in samples (Taberlet et al., 2012). It is important to consider the biases that can be introduced at each stage (van der Loos & Nijland, 2021). This includes decisions on the sampling method and the appropriate use of preservation techniques (Gaither et al., 2011; Ransome et al., 2017), DNA extraction methods, using sufficient replicates (Van den Bulcke et al., 2021), and using appropriate primer pair(s) (Creer et al., 2016; Devloo-Delva et al., 2018; Leray & Knowlton, 2016). Bioinformatics pipelines are also crucial to take into consideration as the pipeline choice, and the processing settings greatly influence the output (Antich et al., 2021).

Current developments in DNA-based methods have resulted in the availability of several different third-generation sequencing platforms, such as Illumina, Ion Torrent, Oxford Nanopore sequencing and Pacific Biosciences (Hu et al., 2021). These platforms provide exciting opportunities to study the environment in convenient ways that include obtaining abundance data (Klunder et al., 2022), epigenetic modifications (Zhao et al., 2023) and population genetics (Jahnke et al., 2022). For metabarcoding of gene fragments shorter than 500 bp, Illumina MiSeq is currently the standard platform because of its high accuracy (M. Meyer & Kircher, 2010). In comparison to Illumina MiSeq, the Oxford Nanopore sequencing platform measures an electrical current that is produced when the nucleotides of a sequence pass through a transmembrane nanopore, allowing for real-time sequencing and base calling (Bleidorn, 2016; Y. Wang et al., 2021). The advantages of Nanopore sequencing include lower costs, the sequencing of long fragments and its suitability for real-time in-field experiments (Krehenwinkel et al., 2019). However, the error rates of raw Nanopore sequences are currently higher (87-99% accuracy) compared to Illumina platforms, therefore different bioinformatics processing pipelines have been used to circumvent this problem (Baloğlu et al., 2021; Doorenspleet et al., 2023; Egeter et al., 2020). Short read Nanopore sequencing has been shown to have consensus with Illumina MiSeq in low-diversity samples (Egeter et al., 2020; van der Reis et al., 2023). However, comparisons of the sequencing platforms also showed the lack of several bacterial taxa when comparing

high diversity samples (Heikema et al., 2020; Stevens et al., 2023). Thus, it remains unclear to what extent short read Nanopore sequencing is directly comparable to Illumina data.

Recently, metagenomics and shotgun sequencing have gained interest as alternatives to metabarcoding for community analysis (Bernatchez et al., 2024; Theissinger et al., 2023). Shotgun sequencing can bypass some methodological disadvantages that are inherent to metabarcoding such as PCR (Zhou et al., 2013) and primer amplification bias (Leray & Knowlton, 2015), because DNA is directly processed for sequencing. Moreover, shotgun sequencing has been previously suggested to cover the full spectrum of biota in a sample and provide a correlation with morphological biodiversity studies. This method is seen as a viable contestant to metabarcoding methods to monitoring genetic biodiversity (Bista et al., 2018; Lopez et al., 2022; Monchamp et al., 2022; Stat et al., 2017). However, metagenomics can be limited by i) the sequencing depth, which has become more cost-efficient with the advent of current sequencing platforms such as NovaSeq, and ii) the availability of reference sequences within databases, given that the shotgun sequencing process is non-selective. Thus, it is inconclusive whether shotgun sequencing is currently more useful for both diversity detection and relative abundance data for macrobenthos studies.

In this study, we compare different DNA-based methods: pair ended Illumina MiSeq metabarcoding, nanopore MinION metabarcoding and Illumina NovaSeq Metagenomics shotgun sequencing. We used bulk macrobenthos community samples collected from different soft-bottom habitats along the Belgium North Sea. By using different DNA-based methods and sequencing platforms for metabarcoding, we assessed the suitability of these methods for monitoring benthic community composition and diversity. We hypothesized that the metabarcoding data from both sequencing platforms and the shotgun metagenomics method are robust and resemble the morphologically identified community both in alpha and beta diversity, despite incomplete reference databases.

Materials and methods

Sample collection

Sampling was conducted at four locations in the Belgian North Sea that contained different macrobenthos communities with high, medium, and low diversity (see Breine et al., 2018) (Figure 1). The bulk samples have been previously used to optimize the metabarcoding protocols and to test the method reproducibility (Derycke et al., 2021; Van den Bulcke et al., 2021, 2023). Samples were collected from a coastal muddy fine-sand habitat with a high taxa diversity of sessile tube-forming organisms and high bioturbation (location 120 - *Abra alba* community). Samples were also collected from a medium sand habitat with a medium taxa diversity of mobile organisms (location 330 - *Nepthys cirrosa* community), a coarse sand habitat with high taxa diversity for sessile interstitial species (location 840 -

Hesionura elongata community) and lastly, a muddy habitat with low taxa diversity (location ZVL - *Macoma balthica* community) (Breine et al., 2018). A Van Veen grab was used to collect three biological replicates per location (A, B, C). All sediment samples were sieved using a 1 mm sieve, and the remaining material (for example, shells and rocks) was fixed using absolute ethanol and stored at -20 °C prior to further processing.

Morphological identification

The morphological identification followed the protocols described by Derycke et al. (2021) and van den Bulcke et al. (2021; 2023). Organisms from one replicate per location (120 - B, 840 - C, 330 - C, ZVL - A) were identified to species level and juveniles to genus level, except for specimens belonging to Nemertea, Anthozoa and Oligochaeta, which were identified up to phylum, class, and order level, respectively. The complete list of species identified in each location is available (Table S1). Species were recorded per individual hence no biomass information was available for this dataset. To correct for the lack of biomass data, the count data were multiplied by the average size (from each each size class). This was done to compare whether there was a correlation between the morphology abundance data and the read count of each DNA based method for the identified species.

DNA extraction

For molecular comparison, all specimens isolated from each field replicate were retained to obtain a bulk sample. Bulk samples were homogenized with a blender or, if the sample was < 100 ml, with a mortar and pestle. Subsamples of 2 ml were taken and stored in Eppendorf tubes at -20 °C before processing at three institutes (Table S2). Samples used for Illumina MiSeq and Oxford Nanopore sequencing were extracted at Naturalis Biodiversity Centre (Leiden, The Netherlands) and used by Wageningen University and research (Wageningen, The Netherlands). Samples used for Illumina NovaSeq were processed at Nord University (Bodø, Norway). DNA was extracted from all samples according to Van den Bulcke et al. (2021). In short, the Eppendorf tubes were centrifuged for 3 min at 10,000 RPM, and the supernatant was removed. Samples were incubated at 50 °C for 1 hr to evaporate the remaining ethanol. Three subsamples from each biological replicate (3X3) were incubated with 10 µL proteinase K overnight at 56 °C. DNA was extracted from each subsample using the DNeasy PowerSoil kit (Qiagen, USA) according to the manufacturer's protocol. After extraction, the DNA extracted from each subsample was pooled and cleaned using the Wizard DNA clean-up system (Promega, USA) and eluted in 50 µL TE buffer. After processing, samples were stored at -20 °C before amplification or shotgun sequencing (Figure 1).

PCR amplification

For amplification, part of the DNA barcode region, COI Folmer was used (Leray et al., 2013) with the nanopore extension sequence (Wageningen University) for the Nanopore metabarcoding results (Table S2). Amplification was performed on each sample in triplicate. Each reaction contained 8.5 µL nuclease-free water, 12.5 µL 2x KAPA HiFi HotStart ReadyMix (Roche, USA), 0.75 µL (10 µM) forward and 0.75 µL (10 µM) reverse primer and 2.5 µL of DNA template. For Nanopore sequencing, DNA template was diluted 10x prior to amplification. PCR conditions were 3 min at 95 °C, 35 cycles of 30 s at 98 °C, 30 s at 57 °C, 30 s at 72 °C and a final extension for 1 min at 72 °C. PCR replicates were pooled, and a clean-up was performed using a 2:1 mixture with AMPURE beads (Beckman Coulter Inc., USA) and >70% ethanol. Amplification was confirmed using gel electrophoresis (1% gel, ethidium bromide).

Illumina MiSeq metabarcoding

Index PCR

For the index PCR, 5 µL nuclease-free water, 12.5 µL 2X KAPA HiFi HotStart ReadyMix (Roche, USA) and 2.5 µL of each index primer (Nextera XT primer 1 and 2) was used with 2.5 µL of initial pooled PCR product. The PCR program was 3 min at 95 °C, 8 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C and a final extension for 3 min at 72 °C. Amplification was confirmed using gel-electrophoresis (1% gel, ethidium bromide). The purified Index PCR products were equimolarly pooled and sequenced using the Illumina MiSeq 2*300bp platform (sequenced by Baseclear BV.).

Bioinformatics of Illumina MiSeq reads.

After Illumina MiSeq sequencing, the quality of the demultiplexed reads was checked using *MultiQC* (Ewels et al., 2016), and primers were removed using *Trimmomatic* (Bolger et al., 2014). Amplicon sequence variants (ASV) were generated using the *DADA2* pipeline in the *Dada2* v1.17.0 package (Callahan et al., 2016) in R Studio v4.0.2 (R Core Team, 2020). Standard settings were used and an error rate of 3 mismatches was allowed. Reads with a quality score lower than 30 were removed. Unique paired-end reads were determined, merged, and filtered for chimeras for each sample. Taxonomy was assigned using the *assignTaxonomy* function in the *Dada2* package (Q. Wang et al., 2007) using the *Ribosomal Database Project* (RDP) (Q. Wang et al., 2007) with a minimum bootstrap confidence parameter of 80. A public reference database that contains 1992 COI sequences of 565 North Sea invertebrate species was used for taxonomic identification (dx.doi.org/10.5883/DS-GEANS1).

Nanopore metabarcoding

Nanopore sequencing

The PCR barcoding kit 96 (EXP-PCB096) was used for the barcoding PCR, and the sequence library was prepared with the SQK-LSK114 kit (Oxford Nanopore Technologies, UK). Several adaptations deviated from the manufacturer's instructions: barcoding PCR was achieved in a total volume of 10 µL using 0.3 µL 10 µM PCR barcode primer pair and 10-50 ng amplicon. The following PCR program was used: initial denaturation at 95 °C for 3 min, 15 cycles of 95 °C for 10 s, 62 °C for 15 s, 65 °C for 90 s, followed by a final extension at 65 °C for 180 s.

The concentration of the barcoded PCR products was measured using the Qubit HS kit (Thermo Fisher Scientific, USA) on the non-purified products, after which barcoded PCR products were pooled in equimolar ratios. The pooled amplicon sequence library was cleaned twice using AMPURE beads (Beckman Coulter Inc., USA). The first clean-up step used 70% ethanol and the second used Short Fragment Buffer (SFB) to enrich for the target size fragments. After end prep and adapter ligation, the library was again washed with SFB during the final clean-up of the protocol. A total of 5 µL library containing 98.5ng DNA was loaded onto an R10.4.1 flow cell (Oxford Nanopore Technologies, UK) mounted on a Minion Mk1C device. Sequencing continued until an average sequencing depth of 200 k reads was obtained per barcode.

Sequence read processing

Sequence read processing was performed according to the post-processing protocol as described by (Doorenspleet et al., 2023). Base-calling of the fast5 pass files was performed using *Guppy* (Version 6.5.7, Oxford Nanopore Technologies, UK) in super high accuracy (SUP) mode. The *Decona* pipeline was used (https://github.com/Saskia-Oosterbroek/decona) for trimming, clustering, and taxonomic assignment of the reads. Raw base-called reads were trimmed to between 250-400 bases for each sequence. A cluster similarity of 85% was set as the clustering threshold of the sequences. Medaka consensus sequences were generated from each cluster larger than 5 reads (Decona -f -q 10 -T 18 -l 300 -m 320 -c 0.85 -g "GGWACWGGWTGAACWGTWTAYCCYCC;max_error_rate=0.1;min_overlap=20...GRTTYTTYGGHCAYCCHGAR-GTHTA;max_error_rate=0.1;min_overlap=19" -n 5 -r 0.99 -R 500 -k 6 -M).

Taxonomic assignment

The consensus sequences were classified using *BLASTn* (NCBI, version 2.11.0) and the North Sea invertebrate species reference database was used for taxonomic identification (dx.doi.org/10.5883/DS-GEANS1). This database was the same as used for the Illumina MiSeq metabarcoding taxonomic assignment. Top hits were considered at species level when there was a minimal alignment length of 250 nucleotides with <4 bp mismatches and >98% identity.

Commented [RGS1]: Change formatting in this case to Doorenspleet et al. (2023)

Commented [RGS2]: In other parts of the manuscript you use basecalled as one word. It will be better to be consistent

Commented [RGS3]: I think you need to clarify a bit more what did you do with the BLAST results. How many alignments did you keep? I would assume there were many matches that aligned with each query sequence. Did you just kept the best match or did you calculate the LCA of the matches clearing a threshold?

Read abundance correction

After taxonomic assignments, a tag correction was performed on the Nanopore data to correct the tag jumping that had occurred. Tag jumping had occurred when both forward and reverse barcode tags were on sequences that did not belong to that barcode. After troubleshooting, most of the contamination could be alleviated by removing 1% of the total read count of each species from each barcode. This correction is intended to correct proportionally for the total read abundance of each taxon. Although the negative PCR control did not show a positive band after barcode PCR, contamination was detected in the sequence data.

First, the total read count was calculated for all species in all barcodes using the mutate() function in the *dplyr* v1.1.0 package (R Core Team, 2023). Second, 1% of the total read count was calculated and rounded to a whole number of reads using the round()and mutate() functions (R Core Team, 2023). Then 1% of the total read count of each species was subtracted from each barcode using mutate().

NovaSeq shotgun sequencing

Preparations for sequencing

After DNA extraction and clean-up, libraries were directly prepared using NEBNext® Ultra™ II DNA Library Prep Kit for Illumina (New England Biolabs, USA). Samples were indexed using NEBNext® Multiplex Oligos for Illumina (New England Biolabs, USA). The final quality check was conducted using the Agilent Tapestation system (Agilent Technologies, USA). Samples were pooled in equimolar concentrations and sent to the Norwegian Sequencing Center in Oslo to be sequenced using the NovaSeq S4 quarter flow cell.

Sequence read processing

The NovaSeq reads were trimmed using cutadapt (--minimum-length=100, q=30), and merged with *PEAR v 1.7.2* (Zhang et al., 2014) using the default parameters. In the first iteration of the data analysis, reads were assembled into contigs and aligned against the complete NCBI database for use in shotgun sequencing. Despite the large number of high-quality alignments, many were false positives. Therefore, this workflow was discarded and instead the merged sequences were directly aligned against the GEANS ref v4 database (>97 % identity, e-value set at -10, and alignment length over 100 bp). Single alignments were discarded for the data analysis.

Data analysis

Data analysis was carried out in RStudio v4.2.2 (R Core Team, 2022). A rarefaction curve (*Vegan* v2.6.4) was plotted to understand the effect of differential sequencing depths between samples. Each sample showed a flattening curve (Figure S1), which indicated that for each DNA-based method, an appropriate sequencing depth was achieved. Therefore, the data were not rarefied but normalized using a

Commented [RGS4]: Did you know this because there was a positive control of known sequence? I am curious as to how this tag jumping happened, as the samples were pooled after the barcodes were added and the ligation of adapters in Nanopore libraries happens afterwards. Would lab contamination rather than tag-jumping be an easier explanation?

Commented [RGS5]: I have a bit of trouble understanding what was done. Please correct me if I am wrong. You suspect that 99% of the reads of each species are in the right sample, and 1% might be in the wrong sample. So you calculate, for each species, what is 1% of their reads. And for each species-sample combination you substract the value for that species.

Commented [RGS6R5]: Also, the dplyr package is not from the R Core team. I think the usage of mutate is not adding information here - I would explain the process, add that you perform it in R, and add the script which does it to the repository or the supplemental

Commented [RGS7]: Rstudio was not made by the R Core Team. I think you mean in RStudio using R v4.2.2

log_{10} transformation. For alpha diversity, species level richness, and evenness (Shannon, log 10 transformed) of the read counts were calculated using the diversity() function (*Vegan* v2.6.4)) and visualized using boxplot (ggplot2, v3.4.0). Normality of the data was tested using Shapiro-Wilk for normal distribution, Q-Q plots, and a histogram. Based on these results a 2-way ANOVA using the aov() function (stats, v3.6.2) was carried out to determine the differences between sampling locations and the DNA-based method used and whether an interaction effect could be observed. For a pairwise comparison, a post hoc analysis was performed using the Tukey test (HSD) using the TukeyHSD() function (stats, v3.6.2). For beta diversity, non-metric multidimensional scaling ('bray') was performed on each dataset separately (Nanopore metabarcoding, Illumina MiSeq metabarcoding and Shotgun metagenomics sequencing) in combination with *betadisper* to check for homogeneity of variance. A PERMANOVA was used (~~Adonis2~~adonis2(), *Vegan* v2.6.4) to analyse which locations were significantly different from each other, within each dataset. A Spearman correlation was used (stats v3.6.2), to compare the size class corrected morphological abundance findings with each DNA-based method.

Results

Read processing comparison between DNA-based methods

From the Illumina MiSeq metabarcoding samples, a total of 2,538,798 sequences were obtained with 2,153,086 used after processing. Of these, 1,724,841 reads were used as ASVs for taxonomy assignment. An overview is available of the sequencing output of each DNA-based method after sequencing and processing (Table S3). From the Oxford Nanopore metabarcoding dataset, 2,426,017 reads were basecalled of which 1,841,385 remained after clustering and consensus building. A total of 1,191,853 of the remaining reads were used as consensus sequences for taxonomy. From the 3,060,417,120 data obtained from the Illumina NovaSeq metagenomics run, 2,425,520,473 reads passed the quality values for direct taxonomic assignment of the reads. In total, 42,262 reads could be assigned to species level, which resembled 0.0017% of the total filtered reads. This illustrates that 100 times more reads were used for the taxonomic assignment of the metabarcoding data, indicating an imbalanced final read count between methods. This was however, not reflected in the rarefaction curves (Figure S1). All the curves showed flattening curves and indicated that for both methods, enough sequencing depth was reached.

Commented [RGS8]: Don't you think that this illustrates that rarefaction curves are not useful to determine whether enough sequencing depth was reached? I think it illustrates that is not worth sequencing each of the samples further, but not that you have an adecuate view of the diversity in the sample

Alpha diversity obtained with the three DNA-based methods

A two-way ANOVA showed a significant interaction effect between sequencing techniques (Illumina MiSeq, Nanopore, NovaSeq), and location (120, 330, 840 and ZVL) for species richness ($F = 8.68$, $p < 0.05$). Main effects were also observed for both sequencing techniques, ($F = 9.94$, $p < 0.05$) and location ($F = 98.79$ $p < 0.05$). Post-hoc analysis using a Tuckey's HSD test showed that there

were significant differences in richness between the NovaSeq metagenomics and MiSeq metabarcoding ($p = 0.021$, Table S5) and between NovaSeq metagenomics and Nanopore metabarcoding ($p < 0.001$, Table S5) but not between Nanopore and MiSeq metabarcoding ($p = 0.31$). In addition, post-hoc analysis showed a significant difference between location 120 and all other locations (Table S5), where 120 had the highest richness (Figure 2a). A significant difference was also observed between ZVL and all other locations (Table S5), where ZVL had the lowest richness. No significant difference was found between location 330 and 840 ($p = 0.99$). As for the interaction effect, the NovaSeq had significantly lower richness in location 120 compared to the other techniques (Nanopore: $p < 0.01$; NovaSeq: $p < 0.01$, Figure 2a). A two-way ANOVA showed a significant interaction of Shannon indices between sequencing techniques and locations ($F = 4.89$, $p < 0.01$, Table S5). The main effects were only significant for the factor location ($F = 69.76$, $p < 0.01$, FS5) but not for the factor method ($F = 0.94$, $p = 0.403$). Post-hoc analysis using a Tuckey's HSD test showed significant differences in Shannon indices between location 120, and all other locations (Table S5), where 120 had the highest Shannon index (Figure 2b). A significant difference was also found between ZVL and all other locations (Table S5), where ZVL had the lowest Shannon index (Figure 2b). As for the interaction effect, the NovaSeq was significantly different from both Nanopore and MiSeq for location ZVL (Table S5, figure 2b), where ZVL was significantly higher than NovaSeq. Overall, these results highlight that all sequencing techniques similarly observe alpha diversity between locations, except for the richness in high diversity at location 120, where the NovaSeq shotgun metagenomics retrieved less species than the metabarcoding approaches. Shannon indices were higher for NovaSeq for the low diversity location ZVL.

Beta diversity obtained from the three DNA-based methods

The PERMANOVA demonstrated significant differences in macrobenthic community compositions between locations ($F = 26.03$, $p < 0.01$) and between techniques used ($F = 6.88$, $p < 0.01$) (Table S6, Figure 3). In addition, a significant interaction effect was observed between location and the DNA-based approach used ($F = 2.48$, $p < 0.01$), indicating that benthic community composition and location depend on the DNA-based method used and the other way around (Table S6). Post-hoc pairwise PERMANOVA tests showed that there was a significant difference in community composition between all locations ($p < 0.01$) except between locations 330 and 840 (Table S6, $p = 0.108$). Post-hoc analysis also showed a significant difference in community composition between Nanopore and NovaSeq ($p < 0.01$) but not between Nanopore and Illumina ($p = 0.99$) and Illumina and NovaSeq ($p = 0.08$). The NMDS plot showed a clear clustering for each location, except for locations 330 and 840 (Figure 3). The plot also illustrates that location is responsible for the biggest contrasts in community composition, indi-

cating that the location explains most of the variation between community compositions. Similarly, the NMDS plot also indicated that NovaSeq has a different community composition in some locations. An interaction effect was also observed, as the NovaSeq samples clustered closer to each other within each location compared to the other DNA-based methods. Clustering between DNA-based methods was not found in Illumina and Nanopore, as these approaches clustered together within each location.

Commented [RGS9]: I think this interpretation of the nMDS is confusing. An interaction effect would be that the NovaSeq behaves differently in a locality compared to others. I don't think you can conclude that from the nMDS

Comparison of DNA-based methods for assessing location-specific species composition

All three DNA-based methods (Figure 4) shared 27 species and an additional 27 species were shared between just the metabarcoding methods (Figure 4, S3). At location 120, 23 species were shared between the two metabarcoding methods whereas only 12 species were shared between all three DNA-based methods (Figure 4, S3). The Nanopore metabarcoding data had 6 unique species for all locations (Figure 4b, Figure S3), and found the most unique species for each location (Figure 4b, Figure S3). For example, several species such as, *Crepidula fornicate*, *Eumida mackiei* and *Magelona mirabilis*, occurred at location 330 using the Nanopore dataset but with less reads than in location 120 (Figure 4b). However, these species were only in location 120 using the MiSeq dataset. Nevertheless, all DNA-based methods detected unique species with each method. In general, the NovaSeq dataset had lower diversity across all the locations compared to the other DNA-based methods (Figure 2, Figure 4c). Interestingly, *Nypthys cirrosa* occured in a high relative abundance in the metabarcoding datasets and is a dominant species at location 330 but remained absent in the NovaSeq results. Nevertheless, the key species of location 840, *Hesionura elongata*, was only detected using the NovaSeq method.

Commented [RGS10]: Is Hesionura elongata present in your locus-specific database?

Comparison of species presence and abundance between DNA-based methods and morphology

For the morphological identification, one biological replicate was available (120B, 330C, 840C and ZVLA) for each location. This resulted in the identification of 56 species. A total of 39, 13, 10 and three species were identified at locations 120, 330, 840 and ZVL, respectively (Table S1). Of the 56 species identified, 25 species were identified using morphology only (Figure 5a). Of these 25 species, 19 did not have a reference sequence available in the chosen reference database (Table S4, colour red). In addition, 12 of the species that did not have a reference sequence available belonged to the phylum Annelida (Table S4, colour green), which are known to have low primer efficiency and are therefore harder to include in COI reference databases (Carr et al., 2011). This indicated that a total of 6 species were unique findings in the morphological dataset (Table S4, orange). Of the 32 species that were identified using the both the DNA-based methods and

morphology, 10 species were identified using all three DNA-based methods, 13 species were identified using the metabarcoding-based methods only. Lastly, 5 species were identified in only one of the three DNA-based methods (Figure 5a, Figure S4).

Three species were identified with all three DNA-based methods (Figure 5a), that were not identified with morphology and an additional six were identified using only the metabarcoding methods. These six species included *Scoliopsis bonnieri* and *Cylista troglodytes* which represented a substantial amount of the identified reads (Figure S2). This indicated that the DNA-based methods can identify species that are missed using morphological identification. Nanopore identified an additional nine species that were unique (Figure 5a) but that were found in low read count of only one read (Table S4).

A Spearman correlation between morphological size class corrected counts and relative read abundance between each DNA-based method across all locations showed the highest positive correlation with both the metabarcoding methods (Sp= 0.24 (MiSeq), Sp =0.27 (Nanopore), although this correlation was not significant (Figure 5b, c, d). However, the metagenomics method did not show a correlation with the size class corrected morphological counts (Figure 5b, c).

Discussion

We compared three different DNA-based methods: two metabarcoding approaches (MiSeq, Nanopore) and a metagenomics approach (NovaSeq). We assessed the robustness between techniques in terms of alpha and beta diversity to understand the suitability of these techniques for macrobenthos monitoring. We have demonstrated that the two metabarcoding methods showed a similar diversity richness and evenness as well as location-specific species compositions. The NovaSeq metagenomics method had lower macrobenthos richness and as a result, different community compositions than the metabarcoding methods. Interestingly, almost half of the species found in the morphological dataset were not identified with molecular techniques mostly due to a lack of references in the database. Nevertheless, all methods showed that most species were shared within each location. The NovaSeq metagenomics method correlated best with the morphological species count. Nevertheless, improvements can be implemented to optimize the suitability of DNA methods for macrobenthos monitoring, especially with regards to improving the reference databases.

Commented [RGS11]: Wasn't it possible to generate reference sequences from the morphological dataset? Because if the explanation of the differences relies on the un-completeness of the databases, doesn't that mean that in 2,3,5 years time the conclusion of your work would have been different?

Macrobenthos communities are highly similar using metabarcoding despite using different sequencing platforms

No difference was found between alpha and beta diversity when comparing a standardized lab protocol with Illumina MiSeq sequencing (Van den Bulcke et al., 2023) to Nanopore-based metabarcoding. Furthermore, both methods identified similar species composition and community structure at each location. Therefore,

this study clearly demonstrated that Nanopore and Illumina MiSeq metabarcoding performed evenly well for monitoring macrobenthos biodiversity. Previous research showed that the Nanopore technique can miss certain taxonomic groups and therefore does not perform as well as MiSeq (Heikema et al., 2020; Stevens et al., 2023) which contrasts our results. More recent comparisons ((Chang et al., 2023; van der Reis et al., 2023; Voorhuijzen-Harink et al., 2019) have shown that the Nanopore datasets are overall comparable with Illumina MiSeq results. Thus, the findings of this study also suggest that metabarcoding with both sequencing platforms performs similarly for macrobenthos biodiversity regardless of the expected richness of an area of interest.

Nanopore sequencing had the most unique species (9 vs 2, respectively; Fig S5). These unique taxa were found mostly in very low relative read abundance and did not influence the alpha and beta diversity. For this comparison, we used the same DNA extract; it is therefore likely that either the lab procedure, the sequencing platform or the bioinformatics method have resulted in slightly different results. When assessing the reproducibility of laboratories minor variations in detected species have previously been reported (Van den Bulcke et al., 2021). These detections could be explained as an effect of the stochastic nature of PCR that is observed in each dataset (van der Loos & Nijland, 2021) or further emphasize the influence of lab-specific factors.

Another potential explanation for these differences is that the Nanopore chemistry and its protocols might be more prone to introducing false positive detections of species. This is because the Nanopore dataset had some species with only several reads in one of the replicates, while in another location this species was abundant. Using the other methods, the same species was found in only one location. Even though a simple correction of tag leakage was used for this study (as presented in the materials and methods), it is possible that tag jumping, or barcode leakage was still a problem for this dataset. After routine testing, this problem was related to the temperature at which barcoded samples were pooled for the Nanopore sequencing library (personal communication). In addition, barcode leakage problems have been reported often in metabarcoding studies (Beentjes et al., 2019; van der Loos & Nijland, 2021). Therefore, it is important to consider protocols that minimize the possibility of barcode leakage. This includes using negative controls at each stage, minimizing the handling and amplification of tagged product, or correcting for a crossover of tags between samples (Beentjes et al., 2019). Similar other platforms, Nanopore uses two cycles of PCR, one for the amplification of the region of interest and a second PCR for the barcode attachment using Nanopore-specific protocols and kits for barcoding. The protocol used for this study could be optimized in such a way that individual samples could be amplified with already tagged initial barcodes to circumvent the second PCR step (Srivathsan et al., 2021) or by using amplicon-free barcode kits (Toxqui Rodríguez et al., 2023; van der Reis et al., 2023).

Commented [RGS12]: I don't see how a sequencing platform can perform better or worse in a particular taxonomic group.

Commented [RGS13]: Are "unique species" in this context species that were only found in one platform? I would say that the null hypothesis as to why this happened is the combined stochasticity of PCR and sequencing (What gets amplified in a sample is a combination of abundance, affinity with the primers and stochasticity - and what gets sequenced is a subsample of that PCR product: this will likely explain the uniqueness of some taxa to a method, and not some preference or likelihood of sequencing rare things)

Commented [RGS14]: I see that you mentioned this: I would move it forward

Commented [RGS15]: Hard to argue without looking at the raw data . I would rather sequence a positive control of known composition so you can have two tests of tag'jumping: do we see something besides the positive control there, and do we see positive control sequences inside the normal samples

The bioinformatics pipeline used in this study could also be responsible for the little observed difference in metabarcoding results. The newest 10.4.1 flow cells were used, which greatly improved the quality of the metabarcoding data (Zhang et al., 2023). Nevertheless, clustering and consensus building of the data with bioinformatics processing might also result in overlooking some elements of the biodiversity (Brandt et al., 2021). Therefore, the reads that were not included in a cluster for consensus building were also considered when they met the threshold for taxonomy assignment. Including this portion of the reads may have led to the detection of additional species, although this is unlikely since it did not result in obtaining extra species (data not shown).

Despite these minor differences between metabarcoding methods, this did not lead to significant differences in commonly used biodiversity indices. Therefore, this study suggests that Nanopore and Illumina MiSeq metabarcoding can be used equally well for macrobenthos monitoring.

NovaSeq Shotgun sequencing identified a reduced number of species compared to metabarcoding

The primer and PCR-free shotgun metagenomics method yielded lower richness compared to the metabarcoding methods and resulted in a significant but slightly different community compositions. These different community compositions between methods were mostly due to the reduced number of species, as most species that were detected by NovaSeq metagenomics were also detected by the two metabarcoding methods.

This study, therefore, does not reflect current environmental metagenomics studies that show equal or higher levels of biodiversity compared to amplicon-based approaches (Bista et al., 2018; Garlapati et al., 2019; Monchamp et al., 2022; Paula et al., 2022). However, in these studies, an environmental sample was taken that was not targeted to a certain taxonomic group and therefore, reflected a wider spectrum of biota. In contrast, this study aimed to test whether metagenomics is suitable for macrobenthos biodiversity monitoring and therefore the data presented are not directly comparable to other metagenomics studies.

For this comparison the reference database that was used contained the most North Sea macrobenthos and contained only COI rRNA sequences. On average, ~3,500 reads of the ~200,000,000 reads per sample had a hit within the database. Considering the relatively low amount of mitogenomic sequences in a DNA extract (Quiros et al., 2017) let alone sequences that represents the COI region, suggests that searching solely for COI is a needle in a haystack situation. Therefore, it is not surprising to have such a low retrieval of reads that align with a target species. These results suggest that with the current available references, a much higher sequencing depth is needed to improve the performance of this technique. Regarding costs, the present study's findings align with previous research that

suggest that shotgun sequencing metagenomics is hardly feasible for environmental studies (Ficetola & Taberlet, 2023; Quince et al., 2017). These results also indicate the necessity to improve reference databases, particularly for full (mito)genomes of macrobenthos, as the limited availability of references largely contributed to the reduced number of species found. Currently, reference databases are mainly focused on genetic regions that are popular for metabarcoding (Weigand et al., 2019) and references of full (mito)genomes are still in their infancy (Blasiak et al., 2020; Leray et al., 2022).

Similarly, of the species that were identified using the metagenomics approach, there was also no correlation with the, for size class corrected read count of the morphologically identified samples. Unfortunately, no biomass data was obtained from the morphologically identified samples, which would be a more appropriate representation of the actual abundance. Nevertheless, a better correlation was found with the metabarcoding data, which is in contrast with findings that suggest a better correlation between found metagenomics reads and biomass (Bista et al., 2018). Since in the study by Bista et. al., the mitogenomes of mock community was available, the lack of correlations in this study further suggests that the metagenomics method is presently not feasible for reliable DNA-based monitoring of macrobenthos biodiversity. In the future, focussing on reference databases and especially databases that contain complete (mito)genomes will greatly improve detection rates and may revolutionize DNA-based monitoring.

Morphology identification retrieved more species due to lack of annelid sequences in the reference database

Most taxa were detected using both morphology and a DNA-based technique, but there were also 25 species that were not identified using any DNA-based method. This was especially apparent in the high diversity samples. However, only 6 of those species were represented in the COI reference database for North Sea macrobenthos. The incompleteness of biodiversity databases, especially in relation to marine invertebrates has often been reported as a limiting factor (Aylagas et al., 2016; Günther et al., 2018; Steyaert et al., 2020; Willassen et al., 2022). Several efforts have been made to improve (Lavrador et al., 2023) and increase the coverage of these databases (Leray et al., 2018; Radulovici et al., 2021). Approximately 22-43% of all European marine species have reference sequences available in the widely used reference database BOLD (Weigand et al., 2019) and 50% in a pan-European gap analysis of aquatic invertebrates (Csabai et al., 2023). Multiple studies in the North Sea have emphasized the importance of enhancing these databases (Christodoulou et al., 2021), indicating a need for sustained long-term efforts.

There were also 15 species from the phylum Annelida that were not identified using DNA-based methods. Annelida, and especially certain Polychaeta, are known to be especially difficult to sequence using conventional markers for

Commented [RGS16]: You mention it later on for Annelida, but the other explanation is not database coverage but primer performance.

metabarcoding because this group has high variation within the COI region and therefore, lower primer efficiency (Carr et al., 2011). This also causes the COI reference databases to be biased towards a lower representation of Annelida. Using multiple markers that are more specific to certain taxonomic groups might therefore greatly improve the capability to detect species from metabarcoding methods. However, consequently this may increase laboratory time and costs (Cordier et al., 2019; Gielings et al., 2021; A. Meyer et al., 2021). Metagenomic methods can also improve the detection of annelids as this technique doesn't rely on the amplification of specific regions. Nevertheless, the metagenomics method in this study did not result in the retrieval of more annelids and this is probably due to the chosen reference database that is solely based on the commonly used region of the COI gene. Especially, the low percentage (0.002%) of reads eventually used for taxonomic identification suggests that the lack of a reference database for this method has strongly hampered the power of the metagenomics method used in the present study.

The DNA-based methods also found taxa with a high read count (>13000 reads) that were not reported during the morphological analysis. These taxa include soft bodied organisms such as the Anthozoa *Sagartia*, whose tissue becomes hard or impossible to recognize when not intact. It is, therefore, not surprising that these species are easier to identify with molecular methods while only classified to a high taxonomic rank during morphological analysis (Robinson et al., 2022). In conclusion, although DNA-based methods missed 25 species compared to morphological analysis, the DNA-based methods detected an additional 17 species that were otherwise undetected. This is in line with previous reports, also confirming that DNA-based methods and morphology should remain complementary (Cahill et al., 2018; Kelly et al., 2017; A. Meyer et al., 2021). However, improvements to the databases and the choice of multi-marker approach for specific taxonomic groups might further improve detection sensitivity and at some point, may surpass morphological identification.

Conclusions

In recent years, there has been increased interest in implementing DNA-based tools into routine biodiversity monitoring practices. To achieve this, standardized protocols are necessary to ensure reproducibility and data robustness across studies and regions, particularly as sequencing technology is evolving rapidly (Van den Bulcke et al., 2023).

In this study, we showed that similar alpha and beta diversity patterns were found regardless of the metabarcoding platform used. Thus, Illumina MiSeq and Nanopore sequencing results are, at this stage, highly similar and can both be used to monitor macrobenthos biodiversity. Incomplete reference databases still hamper detection, as most morphologically identified species did not have a representative sequence in the database. NovaSeq metagenomics has the potential for

Commented [RGS17]: It is here when I realice that the GEANS ref v4 database is a COI database. So it seems that the experiment is playing for metabarcoding advantages and not to metagenomics strengths. I see the reason is the rate of false positives, which I think should be addressed, but I think the experiment as it is now, could be summarised as: If you want to use COI for species detection, then amplify COI.

environmental monitoring especially for better representation of abundance data but is at this stage not ideal for macrobenthos monitoring of bulk samples as fewer species were identified compared when using the metabarcoding methods. These findings demonstrate that newer next-generation sequencing platforms are ready to be integrated for standard monitoring practices. We emphasize the importance of improving sequence reference databases to implement and enhance next-generation of sequencing methods for robust and harmonized monitoring practices.

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Author contributions

Karlijn Doorenspleet: Laboratory work, analysis, writing and reviewing with emphasis on writing the draft. Amalia Mailli: Laboratory work, analysis, writing and reviewing with emphasis on visualization of the results. Annelies de Backer Concept. Writing and reviewing, project administration, funding acquisition. Sofie Derycke concept, methodology writing and reviewing, project administration, funding acquisition. Berry van den Hoorn: writing and reviewing. Kevin Beentjes: laboratory work, writing and reviewing. Henning Reis: Writing, reviewing and supervision Albertinka Murk: Supervision, writing and reviewing Reindert Nijland: Concept, supervision, project administration writing and reviewing.

Data availability

The raw data as well as the processed data and the used scripts are available including descriptive metadata are available under DOIXXXXXX in the Marine data ~~Archieve~~Archive (MDA) https://marinedataarchive.org/ Customs R scripts used for the Data Analysis can be accessed at: https://github.com/amalia03/GEANS_Platform_Comparison_Scripts/blob/main/tax_geans.R.

The data generated from the metabarcoding approach with Illumina MiSeq, as well as the morphological identification have been previously used for the publication by van der Bulke et al. 2023. The data has been used to compare datasets generated from the same samples with the same protocol in different labs. This was to show whether the methods were robust between institutes. In our approach however, we use the dataset of one lab only and the morphology data to compare

675 this with other DNA based methods and sequencing platforms and therefore
676 serves another purpose.

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