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Marine benthic metabarcoding

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CHAPTER 2

Diversity of Wadden Sea
macrofauna and meiofauna
communities highest in DNA from
extractions preceded by cell lysis

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ABSTRACT

Metabarcoding of genetic material in environmental samples has increasingly been employed as a means to assess biodiversity, also of marine benthic communities. Current protocols employed to extract DNA from benthic samples and subsequent bioinformatics pipelines differ considerably. The present study compares three commonly deployed metabarcoding approaches against a morphological approach to assess benthic biodiversity in an intertidal bay in the Dutch Wadden Sea. Environmental DNA was extracted using three different approaches; extraction of extracellular DNA, extraction preceded by cell lysis of a sieved fraction of the sediment, and extraction of DNA directly from small amounts of sediment. DNA extractions after lysis of sieved sediment fractions best recovered the macrofauna diversity whereas direct DNA extraction of small amounts of sediment best recovered the meiofauna diversity. Extractions of extracellular DNA yielded the lowest number of OTUs per sample and hence an incomplete view of benthic biodiversity. An assessment of different bioinformatic pipelines and parameters was conducted using a mock sample with a known species composition. The RDP classifier performed better than BLAST for taxonomic assignment of the samples in this study. Novel metabarcodes obtained from local specimens were added to the SILVA 18S rRNA database to improve taxonomic assignment. This study provides recommendations for a general metabarcoding protocol for marine benthic surveys in the Wadden Sea.

INTRODUCTION

Benthic organisms play a crucial role in marine nutrient cycling and in primary and secondary productivity in the ocean and shelf seas (Austen et al., 2002; Covich et al., 2004; Levin et al., 2001; Snelgrove, 1997; Thrush et al., 2006). Anthropogenic stresses on the seafloor such as trawling, oil, gas and sand extraction but also warming and ocean acidification (Anadón et al., 2007; Halpern et al., 2008) are inducing changes in benthic ecosystems. Subsequent disruption of key ecosystem services and community stability from an accelerated loss of biodiversity is currently a major concern (Daily et al., 2000; Danovaro et al., 2008; Hooper et al., 2012; Solan, 2004). The ever-expanding economic exploitation necessitates implementation of policies to ensure habitat protection. This, in turn, requires regular monitoring of marine benthic ecosystems. Benthic biodiversity is a widely used indicator of ecosystem health (Snelgrove, 1997). Ideally the monitoring approach should assess biodiversity at the relevant temporal and spatial scales in a consistent and reliable way.

Current estimations of biodiversity are subject to high levels of uncertainty, especially in marine ecosystems (Costello, 2015; Hajibabaei et al., 2011; Hortal et al., 2015; May, 1988), suggesting that current methods are insufficient. Assessing the composition of the marine benthos by traditional methods such as morphological identification of individual specimens is time-consuming, labour-intensive as well as costly, and requires a taxonomic knowledge that is increasingly scarce, particularly for invertebrates (Bucklin et al., 2011; Cardoso et al., 2011; Cowart et al., 2015). Morphological identification is typically limited to large specimens and consequently the meiofauna and immature individuals usually remain unidentified (Balsamo et al., 2012; Boyd et al., 2000; Chariton et al., 2015; Compton et al., 2013; Danovaro et al., 2000; Spilmont, 2013; Zeppilli et al., 2015). During recent years, DNA sequencing has emerged as an alternative and efficient method for species identification, most recently in the form of next-generation sequencing (NGS) and metabarcoding (Taberlet et al., 2012a). In principle, metabarcoding facilitates the assessment of biodiversity in a consistent and replicable manner across different ecosystems (Baird and Hajibabaei, 2012). This potentially allows comparisons of in situ biodiversity studies (Bik et al., 2012; Cowart et al., 2015; Ji et al., 2013).

Several studies have successfully implemented metabarcoding approaches to assess marine benthic biodiversity (Brannock et al., 2014; Chariton et al., 2010, 2015; Fonseca et al., 2010; Guardiola et al., 2015). However, several aspects of metabarcoding-based assessments of benthic diversity potentially bias the outcome and this has been tested insufficiently. One aspect is the DNA extraction approach and the corresponding fractioning of sediment samples. Common DNA extraction methods for marine sediment samples can be divided in three categories: direct DNA extraction from small amounts of sediments, DNA extraction from fractioned

sediments, and extraction of extracellular DNA. Direct DNA extraction from small amounts of non-fractionated sediments has been applied for the identification of both macrofauna and meiofauna communities (e.g., Chariton et al., 2015; Sinniger et al., 2016). This method retrieves both the extracellular DNA present in the sample as well as the intracellular DNA through a lysis step. The DNA extraction of a particular size-fraction - obtained after a sieve or elutriation step - is restricted to intracellular DNA from faunal species (and their incidentally gut contents) within this size fraction (e.g., Fonseca et al., 2014; Leray and Knowlton, 2016). Recently, DNA extraction of only extracellular DNA in sediment samples, has been applied as an alternative approach (Bienert et al., 2012; Guardiola et al., 2015; Taberlet et al., 2012b). Extracellular DNA adsorbed on minerals has been shown to be protected against degradation and is therefore expected to reflect longer-term biodiversity (Dell'Anno and Corinaldesi, 2004). This approach, in principle, is therefore less susceptible to short-term temporal heterogeneity (Alawi et al., 2014; Taberlet et al., 2012a). The extraction of extracellular DNA has been applied for the identification of both macrofauna and meiofauna communities (Guardiola et al., 2015; Pearman et al., 2015). To date, no comparisons have been undertaken with regards to which of the three DNA extraction methods is best suited for the assessment of marine benthic biodiversity.

Another aspect that potentially biases the outcome of metabarcode based benthic biodiversity assessments is the taxonomic assignment of operational taxonomic units (OTUs) among the sequenced metabarcodes. Species identification, not just OTU diversity, relies heavily upon the completeness of reference DNA databases. Many studies solely rely on the DNA sequences that are available in public databases such as GenBank™ and SILVA. The DNA sequences, and hence the OTU composition, detected during a study are often interpreted without in-depth knowledge of the species diversity at the study site or the reference databases. However, the current databases are incomplete and strongly biased towards model organisms. Consequently, the identification is mainly confined to specimens belonging to well-known taxa (Pompanon and Samadi, 2015). Although some studies have compared the efficiency of different metabarcoding methods (Brannock and Halanych, 2015; Lekang et al., 2015), and some studies investigated the effectiveness of metabarcoding with artificial compiled samples (Dell'Anno et al., 2015; Leray and Knowlton, 2015), only a few studies verified different molecular approaches against morphological identification for marine benthic samples; usually due to a lack of the necessary taxonomic knowledge and time constraints (Creer et al., 2016). Positive controls, mock samples with known species composition, are typically absent in benthic biodiversity assessments, even though mock communities represent an excellent approach to validate the specific experimental and bioinformatics pipeline of a study (Creer et al., 2016).

This study compared three methods that are commonly employed in metabarcoding studies against a morphological approach to assess marine benthic macrofauna and meiofauna diversity in an intertidal area in the western Dutch Wadden Sea. Public reference databases were complemented with sequences obtained from morphologically identified local benthic species, representing all abundant macrofauna species known for the sampling area. The focus of this study was on the effectiveness of different DNA extraction methods; as employed in current biodiversity studies, to capture the benthic macrofauna and/or meiofauna diversity. Also, a comparison was conducted between two commonly used methods to assign OTUs to their nearest taxon (i.e., BLAST and the RDP classifier) in public and local reference databases. A mock sample of marine benthic biodiversity was analysed to assess the quality of taxonomic assignment (Leray and Knowlton, 2016).

MATERIAL AND METHODS

Reference library

Sampling

Benthic macrofauna species were sampled from the western part of the Dutch Wadden Sea during the period between February 2014 and March 2016 following Beukema and Cadée (1997). Specimens were identified by an experienced taxonomist according to Hartmann-Schröder (1996) and Hayward and Ryland (1995) based on morphological characteristics. Molluscs, crustaceans and polychaetes were identified to the species level, whereas oligochaetes and Nemertea were identified to the phylum level. After identification, up to three specimens per species were stored individually in separate tubes in 96% ethanol at room temperature.

Molecular analyses

Genomic DNA was extracted using the GenElute™ Mammalian Genomic DNA miniprep kit (Sigma-Aldrich Inc.) following the manufacturer's protocol, except for the length of the initial cell lysis step which was increased to 18 hours to enhance DNA yield. A 650 base pair (bp) part of the 18S rRNA gene was amplified using the oligonucleotides F-566 and R-1200 as primer pair (Hadziavdic et al., 2014). All polymerase chain reactions (PCR) were performed in a 50µl reaction volume, containing 0.5µM of each primer, 0.2µM dNTPs, 2U BioTherm™+ Taq DNA Polymerase (BioTherm™ Inc.), 1x PCR buffer (BioTherm™ Inc.) and 2µl of DNA extract. PCR reactions were subjected to five minutes at 95°C, followed by 35 cycles comprised of 45 seconds at 95°C, 60 seconds at 60°C and 60 seconds at 72°C, respectively, and one final extension step of seven minutes at 72°C. Subsample of the PCR products (5µl) were checked by electrophoresis through a 2% agarose gel at 75volt for 50 minutes after ethidium bromide staining. The size of the 18S rRNA PCR product matched the expected 650 bp for all species, except arthropod species.

In arthropods the 18S rRNA PCR products were ~1000 bp. The PCR products were Sanger sequenced in both directions with the ABI3730XL sequencer from Life Technologies by BaseClear (Leiden, Netherlands).

Alignment of Sanger sequences

Forward and reverse sequences obtained by the Sanger procedure were aligned using Geneious™ (version. R9, Kearse et al., 2012). Alignments were obtained using the default Geneious alignment function with a gap open penalty at 12 and gap extension penalty at 3. The cost matrix was set at a 65% similarity (5.0/4.0). The consensus sequence was obtained with a highest quality threshold. All sequences were supplemented with their taxonomic data and stored as a local reference database.

Mock sample

One mock test sample was composed by combining DNA extractions from ten local species, representing three different phyla (Table 2.1). The DNA extracts of the selected species were quantified on a Qubit 3.0 fluorimeter (Qiagen, Inc.) and were pooled in equimolar quantities. The mock sample served as a positive control throughout the 18S species identification process.

Field experiment

Sampling

Samples were collected during low tide at nine locations separated by less than 50 m in Mokbaai, an intertidal bay at the southern tip of the isle of Texel in the western part of the Dutch Wadden Sea (53°20'00"N; 4°46'50"E). Three sediment cores were collected at each location: two cores (termed A and B below) had a 177 cm² surface area and a 25 cm sampling depth (equivalent to roughly 4.5 liters of sediment). The third sample (termed sample C below) was collected using a smaller core at 5.60 cm² surface area and a 10 cm sampling depth (equivalent to 50ml of sediment). Samples A were stored in clean plastic buckets at 5°C. Samples B were sieved through a 1mm round mesh in the field and stored at 5°C in plastic bags. Samples C were stored at -80°C in clean plastic pots.

Molecular analyses

Three different DNA extraction methods were employed.

- 1) The extracellular DNA extraction method was adapted from Taberlet, et al., (2012b). The entire sampled sediment from sample A (4,5 L sediment; n=9) was dissolved in 5 L saturated phosphate buffer (Na₂HPO₄, 0.12M, pH ≈8) and mixed for 15 minutes. Two 50 mL aliquots of dissolved sediment were collected and centrifuged at 10 000 g for ten minutes. A volume of 400µL of supernatant was recovered and the DNA extracted using the Powersoil™ DNA

isolation kit (MoBio Inc.) following the manufacturer's instructions leaving out the initial lysis step.

- 2) For the sieved lysis method, the sieved fraction from sample B (4,5 L sediment; n=9) was cryodesiccated and ground in liquid nitrogen. Subsequently, DNA was extracted from the ground residue using the Powermax Soil™ DNA isolation kit (MoBio Inc.) following the manufacturer's instructions.
- 3) The direct DNA extraction method where all samples C (50 ml sediment; n=9) were cryodesiccated and ground in liquid nitrogen. DNA was extracted from 10 g of ground sediment following the procedure described in method 2.

The extracted DNA was quantified using a Qubit™ 3.0 fluorimeter (Qiagen, Inc.). The six samples with the highest DNA yields were selected among the nine extracts from each DNA extraction method. The DNA extracts from these 18 samples as well as the compiled mock sample, were used as template to amplify the 650 bp fragment of the 18S rRNA gene using the oligonucleotides F-566 and R-1200 as PCR primers. The F-566 oligo-nucleotide was extended at the 5'-end with a ten nucleotide multiplex identifier (MID) designed by 454 Roche Life Sciences (Corp.). Each sample was labelled with a unique MID. All PCRs were performed in triplet in a 25µL volume reaction, containing 0.5µM of each primer, 0.2µM dNTP, 800ng/µL BSA, 1U Phusion® High-Fidelity DNA Polymerase (Thermo Scientific Inc.), 1X Phusion® HF buffer (Thermo Scientific Inc.) and 3 µL of DNA extract. The thermal cycle programme included an initial cycle of 30 seconds at 98°C; followed by 29 cycles, comprised of 10 seconds at 98°C, 20 seconds at 68°C and 30 seconds at 72°C, followed by a single cycle of seven minutes at 72°C. The PCR products were run through a 2% agarose gel at 75volt for 50 minutes. The PCR products were visualized by ethidium bromide staining. Three out of the 18 samples failed during PCR and were discarded. The remaining amplification products were purified with the Qiaquick™ purification kit (Qiagen Inc.) and quantified with a Qubit™ 3.0 fluorimeter (Qiagen Inc.). All samples were pooled in equimolar quantities together with a positive control, the mock sample, and a blank PCR control. The pooled sample was then subjected to a final purification using a MinElute™ PCR Purification column (Qiagen Inc.) as described by the manufacturer. Pyrosequencing was performed on the pooled sample in a single lane using Roche 454 GS-FLX Titanium platform with the Lib-L kit, by Macrogen Inc.

Bioinformatics

Raw sequences were quality trimmed and filtered using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) using the fastq_quality_trimmer and fastq_quality_filter scripts. Reads shorter than 250 bps were discarded and bases with Phred quality scores less than 30 were end-trimmed. Reads with a quality score ≤ 30 at $>50\%$ of the positions were discarded. Quality filtered reads were de-multiplexed based on the MID sequences in

QIIME (Caporaso et al., 2010) using the `split_libraries.py` script. Reads were first dereplicated at a 100% similarity and the unique sequences were clustered using a 95% similarity cut off in VSEARCH (Rognes et al., 2016). Taxonomic assignments were performed against the SILVA 18S rRNA database (release 119, www.arb-silva.de; Pruesse et al., 2007), our local reference database or both, using two different assignment algorithms. Two alternative BLAST searches were performed, the first using the default `blastn` settings in which the e-value is 10, `word_size` is 10, `match/mismatch` scores are set on respectively 1 and -2 and gap costs are linear. A custom `blastn` search was performed using an extended `word_size` of 30, `match/mismatch` scores were set at 1 and -4 and the penalty for opening a gap was set at 12 and for the extension at 2. The second taxonomic assignment was performed using the RDP Classifier (Wang et al., 2007) using the `assign_taxonomy.py` script in QIIME with a minimum confidence of 0.5. Also, a random set of 10 OTU's were manually blasted as an empirical control. A neighbour-joining tree was built from the 18S rRNA V4-V5 barcodes of the species that were found in the mock sample.

Morphological analyses

Samples A were sieved through a 1 mm mesh sieve. Sieved fractions were preserved in a 4% formaldehyde solution and stained using Bengal rose. Species in the sieve residue were sorted by hand and identified while alive following the procedure described above.

Data analysis

OTUs assigned to either the Annelida and Mollusca phylum and the Arthropod class Malacostraca were categorized collectively as macrofauna. Although larvae or juveniles within these groups technically could be meiofauna, the adult stages were used as the reference point for this classification. OTUs from the phyla Gastrotricha, Gnathostomulida, Nematoda, Platyhelminthes and Xenacoelomorpha as well as the Arthropod classes Hexanauplia and Ostracoda were categorized as meiofauna. Presence/absence ratios for macrofauna were estimated at the genus level from both the morphological (samples A) and the molecular data (samples A, B and C). Species diversity at the intertidal Wadden Sea is extremely low and a 95% cut off in combination with identification of macrofauna at the genus level has been found reliable by analysing sequence variance from our reference database. As meiofauna species were not sampled for our reference data base, presence/absence ratios for meiofauna were not estimated beyond the family level from the molecular data.

RESULTS

Validation taxonomic assignment – reference library – mock sample

After sequence quality control, a total of 6,946 reads were assigned to the mock sample, resulting in 27 OTUs clustering at a threshold of >95%. Initial taxonomic assignment using the custom BLASTn search and the RDP classifier against the SILVA SSU rRNA reference database, respectively missed five and four out of the ten species present in the mock sample. After complementing the database with new 18S rRNA DNA sequences of macrofauna species that are common in the Wadden Sea, the RDP classifier recovered all mollusc and annelid mock species (Table 2.1). Also, the custom BLASTn search recovered these species. However, in addition five false positives were detected: the polychaetes *Abarenicola affinis*, *Platynereis dumerilii* and *Notomastus tenuis* and the molluscs *Phaxas pellucidus* and *Scissula similis*. The barcode sequences of these false positives were all similar but not identical to the mock sample species (Figure 2.1). The taxonomic assignment at the genus level of the 10 randomly picked OTU's, which were manually blasted, all corresponded to those assigned by the RDP classifier. However, one OTU was different at the genus level compared to both BLASTn searches, a match was found at the family level.

The arthropod species that were included in the mock community, *Urothoe poseidonis* and *Corophium arenarium*, were not recovered and only a few reads were assigned to *Bathyporeia sarsi*. In all cases, a few OTUs were assigned to the Siphonostomatoida, a group of parasitic copepods that might have been present in the macrofauna from which the mock sample was generated.

Table 2.1 | Species added to the mock sample and their presence in the molecular dataset after taxonomic assignment. The species indicated with an * where those added to the mock sample. The SILVA and SILVA + local column show the outcome of taxonomic assignment for either BLAST or the RDP-classifier based on respectively the SILVA SSU rRNA database or the same database complemented with sequences from local species. Species were either retrieved (+) or not found (-). The colours indicate if the species was correctly identified (green), misidentified (red).

Phylum	Class	Taxonomy		SILVA + local	
		Family	Species	Blastn	RDP
Annelida	Polychaeta	Arenicolidae	Arenicola marina*	+	+
Annelida	Polychaeta	Arenicolidae	Abarenicola affinis	+	-
Annelida	Polychaeta	Capitellidae	Heteromastus filiformis*	+	+
Annelida	Polychaeta	Capitellidae	Notomastus tenuis	+	-
Annelida	Polychaeta	Orbiniidae	Scoloplos armiger*	+	+
Annelida	Polychaeta	Nereididae	Hediste diversicolor*	+	+
Annelida	Polychaeta	Nereididae	Platynereis dumerilii	+	-
Arthropoda	Malacostraca	Bathyporidae	Bathyporeia sarsi*	+	+
Arthropoda	Malacostraca	Corophiidae	Corophium arenarium*	-	-
Arthropoda	Malacostraca	Urothoidae	Urothoe poseidonis*	-	-
Arthropoda	Hexanauplia	Siphonostomatoida		+	+
Mollusca	Bivalvia	Cardiidae	Cerastoderma edule*	+	+
Mollusca	Bivalvia	Pharidae	Phaxas pellucidus	+	-
Mollusca	Bivalvia	Tellinidae	Scissula similis	+	-
Mollusca	Bivalvia	Tellinidae	Limecola balthica*	+	+
Mollusca	Gastropoda	Hydrobiidae	Peringia ulvae*	+	+

Table 2.2 | Numbers of OTUs for each DNA extraction method. OTU numbers are calculated as mean value per sample for the extracellular DNA extraction method (n=3), the sieved lysis method (n=5) and the direct method (n=6).

Method	Eukaryota	Metazoa	Proportion metazoan
Extracellular	80 ± 27 SD	27 ± 17 SD	31%
Sieved lysis	18 ± 7 SD	17 ± 6 SD	91%
Direct extraction	82 ± 20 SD	41 ± 19 SD	48%

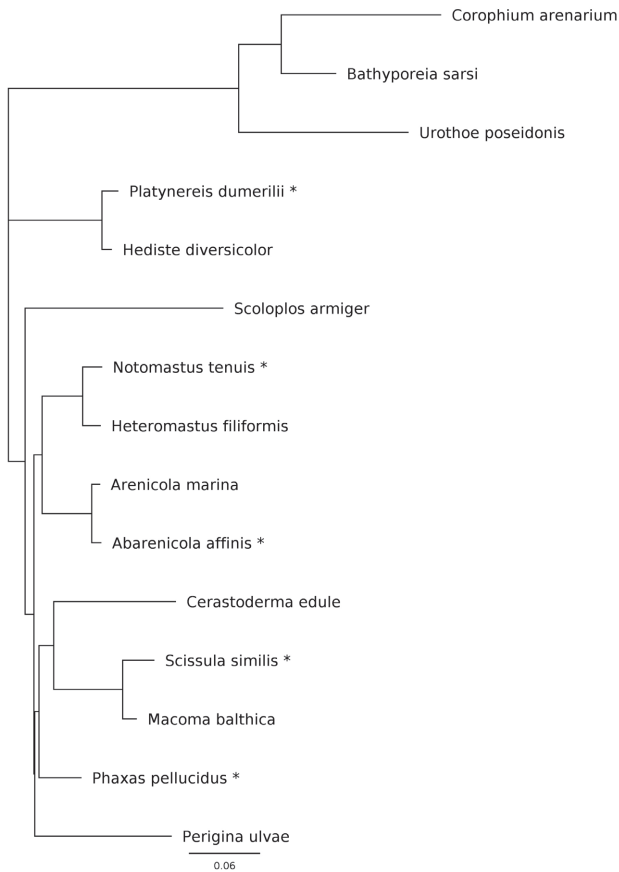


Figure 2.1 | Neighbor-Joining tree of species found in the mock sample. The neighbour-joining tree is based on the 18S rRNA barcodes from the V4-V5 region (Hadziavdic et al., 2014). Species indicated with* where present in the mock sample.

Comparison extraction methods

Taxonomic composition

The 454-based amplicon sequencing generated 142,118 raw reads of which 104,101 were retained after quality control. One sample out of the 15 was discarded since it yielded only 765 18S rRNA sequences; the according multiplex identifier (MID) was suspected to interfere during amplification (Berry et al., 2011). The number of recovered 18S rRNA sequences varied considerably among the remaining samples but did not differ significantly among methods (one-way Anova, $F_{2,11} = 1.47$, $p = 0.272$). The OTU diversity was highest with the direct DNA extraction method (method 3), with an average number of 82 (SD = 20) OTUs per sample (Table 2.2). The extracellular DNA extraction method (method 1) recovered 80 (SD = 27) OTUs per sample, whereas the DNA extraction method performed on sieved sediment (method 2) recovered on average 18 (SD = 7) OTUs. This was significantly lower than the number of OTUs recovered with

the two other DNA extraction methods (one-way Anova, $F_{2,11} = 19.3$, $p < 0.001$). The sieved lysis DNA extraction method recovered the highest percentage of metazoan OTUs, 91% versus 31% and 48% in case of the extracellular and direct DNA extraction method, respectively (one-way Anova, $F_{2,11} = 32.83$, $p < 0.001$).

Most metazoan OTUs were assigned to annelids within the extracellular DNA extraction method (32%) and the sieved lysis DNA extraction method (72%), whereas with the direct DNA extraction method most OTUs (36%) were assigned to nematodes (Figure 2.2). The sieved lysis DNA extraction method recovered four metazoan phyla; Annelida, Arthropoda, Mollusca and Nematoda. The two DNA extraction methods starting with unsieved sediment samples both recovered four additional meiofaunal phyla; Gastrotricha, Gnathostomulida, Platyhelminthes and Xenacoelomorpha. The extracellular DNA extraction method also recovered the phylum Cnidaria.

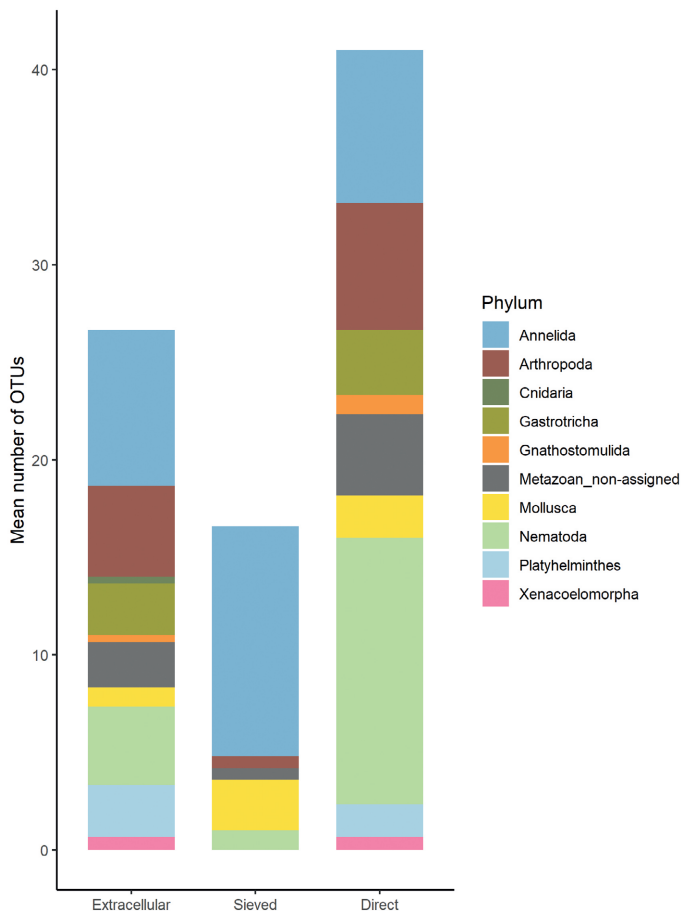


Figure 2.2 | Taxonomic composition. For each extraction method, the average number of OTUs per phylum is shown.

Macrofauna

The three DNA extraction methods recovered similar macrofaunal diversity (Figure 2.3). Annelids were the most diverse group with 88%, 81% and 75% of the OTUs for the extracellular DNA extraction method, the sieved lysis method and the direct DNA extraction method respectively. Only few arthropod OTUs were recovered with the sieved lysis method and none with the unsieved methods. The traditional classification method, based on morphology, identified 16 different macrofaunal genera, belonging to three different phyla (Figure 2.4). Annelids were most diverse as ten genera were recovered whereas three mollusc and arthropod genera were recovered in each phyla. The molecular methods recovered most annelid genera except the *Marenzelleria* sp. which were not detected by any of the DNA extraction methods. Most mollusc genera identified with the traditional morphological methods were also recovered with the molecular methods. However, *Limecola* sp. was not recovered with the direct DNA extraction method and *Peringia* sp. was not recovered with the extracellular DNA extraction method. Among the three arthropod genera detected using traditional morphological methods, only *Bathyporeia* sp. was recovered, with the sieved lysis DNA extraction method.

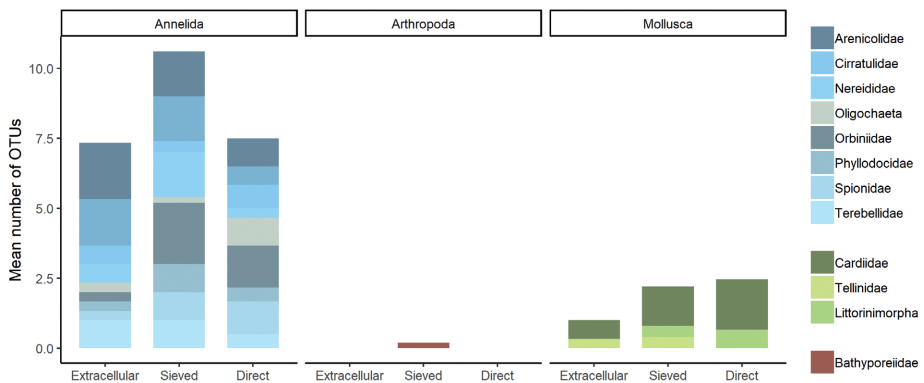


Figure 2.3 | Order and family diversity for macrofauna. The number of OTUs for macrofaunal orders from the phyla Annelida, Mollusca and Arthropoda are shown for the different extraction methods. The mean number of macrofauna OTUs per sample were 8, 14 and 10 for respectively the extracellular, sieved and direct DNA extraction method.

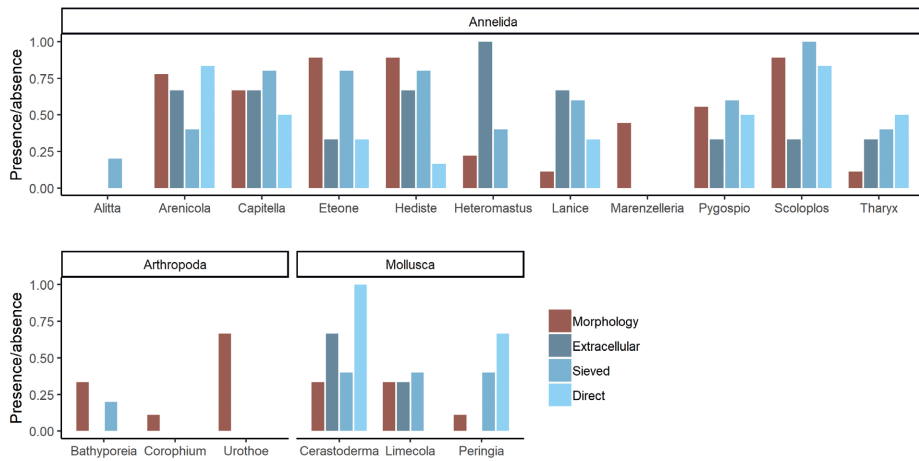


Figure 2.4 | Presence/absence ratios for macrofauna genera. Presence/absence ratios are reported for macrofauna genera in the Annelida, Arthropoda and Mollusca phyla. Ratios are calculated as detection rate within the samples for either the extracellular DNA extraction method, sieved lysis method, direct DNA extraction method or morphological method.

Presence/absence estimations obtained with the sieved lysis method correlated significantly (Pearsons, $r = 0.54$, $p = 0.014$) with presence/absence estimations based on morphological identifications (Figure 2.5). The extracellular and direct DNA extraction methods both underestimated the presence/absence ratios of the genera *Eteone* sp. and *Hediste* sp. whereas the presence/absence ratios of the genera *Lanice* sp. and *Cerastoderma* sp. were overestimated. Remarkable differences were found in the presence/absence ratios for *Heteromastus* sp., which was recovered in all samples with the extracellular DNA extraction method but in none of the samples with the direct DNA extraction method.

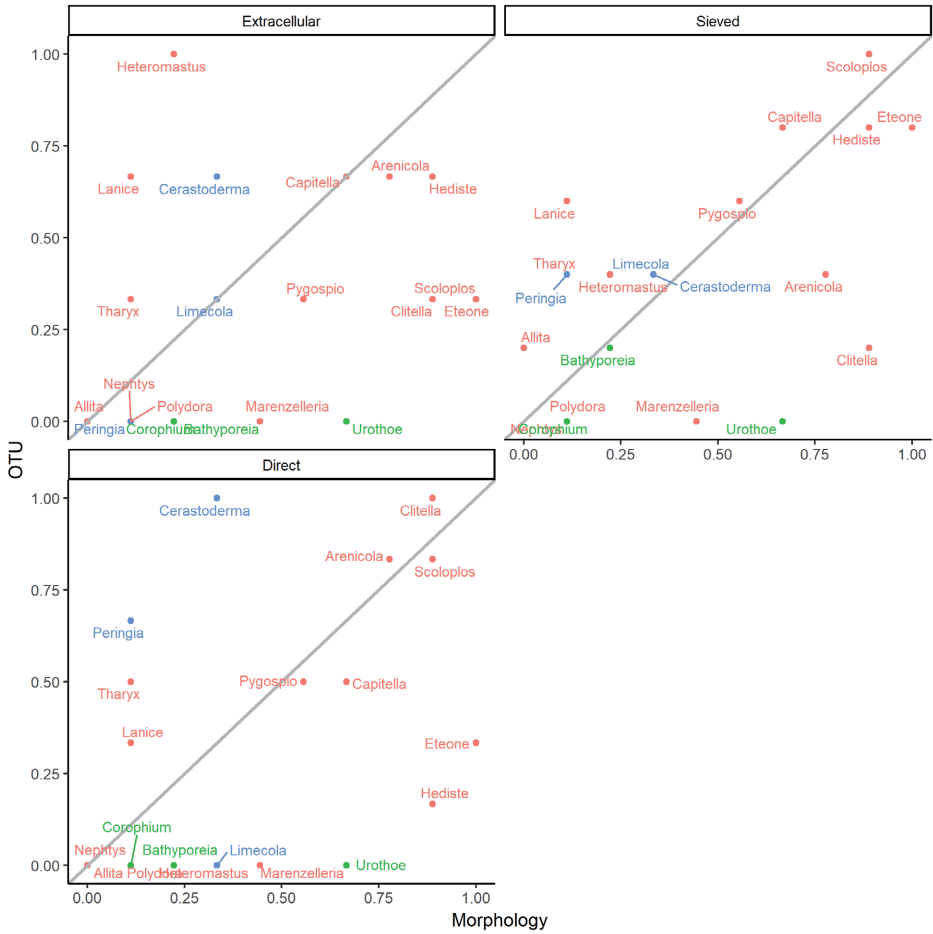


Figure 2.5 | Scatterplots of presence/absence ratios. Presence/absence ratios for macrofauna genera assessed by either classical taxonomy versus the extracellular DNA extraction method, the sieved lysis method or the direct DNA extraction method. Presence/absence ratios for the morphological approach are calculated as detection rates from all morphological identified samples (n=9). The presence/absence ratios for the molecular approaches are calculated as detection rates within the samples of the particular molecular method. The colours represent different phyla; red = Annelida, blue = Mollusca, green = Arthropoda.

Meiofauna

The meiofaunal taxa recovered with the extracellular DNA extraction method and the direct DNA extraction method belonged to the arthropods, nematodes, flat-worms, gnathostomulids and gastrotrichs (Figure 2.6). The total number of OTUs for all meiofaunal orders did not differ between the two methods (t-test, $t_6 = 2.202$, $p = 0.07$) but the number of nematode OTUs differed significantly (t-test, $t_6 = 3.594$, $p = 0.011$). Compared to the direct DNA extraction method, the extracellular DNA extraction method recovered only one-third of the nematode OTUs and entirely failed to recover OTUs from the order Araeolaimida. Presence/absence estimations for all meiofaunal orders obtained with the extracellular and the direct DNA extraction methods were highly different (paired-t, $t_{13} = 2.939$, $p = 0.012$) (Figure 2.7). Presence/absence estimations for meiofauna orders obtained with the direct DNA extraction method were overall higher. This was especially true for nematode orders that were detected in 57% of the sediment samples with the direct DNA extraction method and in 16% of the samples with the extracellular DNA extraction method. Presence/absence ratios for the nematode orders differed significantly (paired-t, $t_4 = 4.489$, $p = 0.011$) between these two DNA extraction methods.

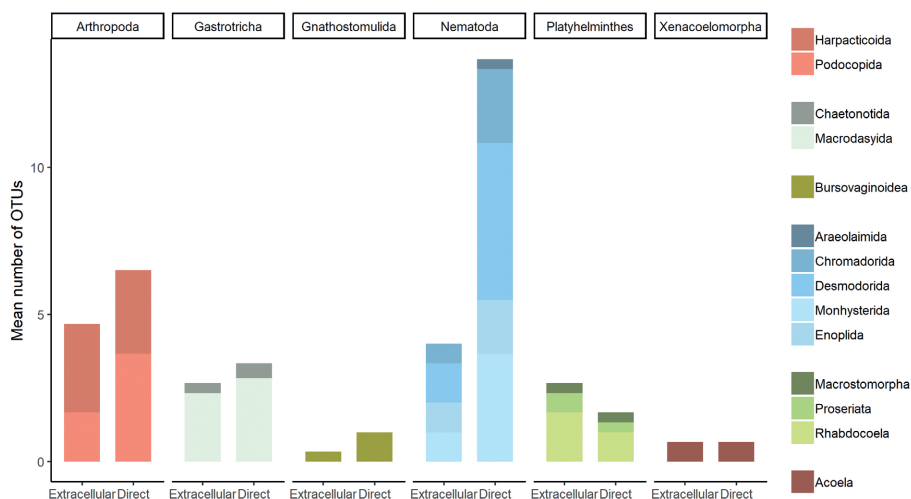


Figure 2.6 | Phylum and order diversity for meiofauna. The number of OTUs for the meiofaunal orders from the phyla Arthropoda, Nematoda, Gastrotricha, Gnathostomulida, Platyhelminthes and Xenacoelomorpha. The mean number of OTUs per sample were 15 and 26 for respectively the extracellular and direct DNA extraction method.

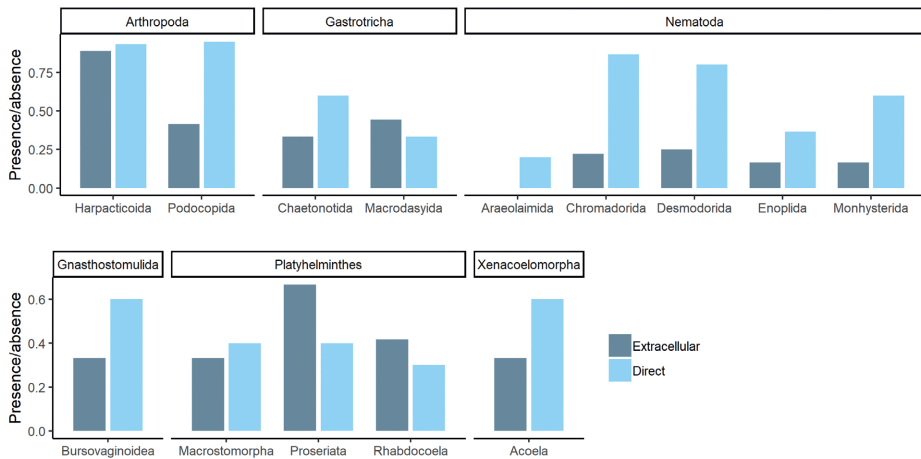


Figure 2.7 | Presence/absence ratios for meiofauna orders. Presence/absence ratios are reported for meiofaunal orders from the Arthropoda, Gastrotricha, Nematoda, Gnathostomulida, Platyhelminthes, Xenacoelomorpha phyla. Ratios are calculated as detection rate within the samples for either the extracellular or direct DNA extraction method.

DISCUSSION

Validation of methods

Assessing biodiversity from metabarcoding data is undergoing an exponential increase; in part due to the ability of these approaches to capture diversity in complex and diverse communities (i.a. Chariton et al, 2015; Lejzerowicz et al., 2015; Sinniger et al., 2016). Accordingly, metabarcoding-based assessments of biodiversity need standardization to allow comparison between studies. A mock sample was employed to assess the consistency of species identification from the metabarcoding sequences. The analysis of the mock sample exposed data gaps in the SILVA 18S rRNA reference database with respect to Wadden Sea fauna. The quality of taxonomic assignments is, to a large extent, depending on the completeness of the reference database. The sensitivity and accuracy of taxonomic assignment increases when more species are present in the reference database allowing OTU assignment to higher taxonomic levels (Carugati et al., 2015; Creer et al., 2016; Pompanon and Samadi, 2015; Richardson et al., 2017; Thomsen and Willerslev, 2015). In our study, only few reads were assigned to the arthropod species *Bathyporeia sarsi* and no reads were assigned to the species *Corophium arenarium* and *Urothoe poseidonis* or any other species within the class Malacostraca. This outcome persisted even after the addition of sequences from these species to the SILVA reference database. The overall absence of Malacostraca barcodes in the mock sample as well as in the environmental samples suggests methodological problems for certain arthropod species rather than misidentifications

during the bioinformatic process. The V4-V5 region of the 18S rRNA locus targeted in our study, has been reported to allow identification of OTUs across a wide taxonomic range (Hadziavdic et al., 2014; Hugerth et al., 2014). This specific region is also known to exhibit length polymorphism (Hadziavdic et al., 2014; Hugerth et al., 2014; Nickrent and Sargent, 1991). The amplicon size was in the range of 600bp to 650bs for the majority of our targeted species. However, the arthropod amplicons were longer, around ~1,000 bp. Longer amplicons may be underrepresented by PCR and Roche 454 sequencing. This may potentially explain the absence of arthropod OTUs (Berry et al., 2011; Engelbrektson et al., 2010; Herbold et al., 2015).

BLAST is typically the default method for taxonomic assignment in benthic metabarcoding studies (Coward et al., 2015; Dell'Anno et al., 2015; Lejzerowicz et al., 2015; Sinniger et al., 2016). However, this study, and in particular the analysis of the mock sample, revealed some incorrect assignments when using BLAST. Although all species that were included in the mock sample were recovered, five additional species were detected with BLAST. These five additional species were closely related to species in the mock sample but did not have identical barcodes for the subjected 18S rRNA region. This suggests that the BLAST taxonomic assignment in combination with our OTU clustering method was not strict enough, even at more stringent settings. The RDP classifier is not commonly used for marine benthic biodiversity studies but it performed well for the taxonomic assignment of metazoan genera in other studies (Chariton et al., 2015; Cole et al., 2009; Porter et al., 2014). During this study, the RDP classifier was able to recover the exact species present in the mock sample and caused no misidentifications. Although many more taxonomic assignment and aligning methods have been developed next to BLAST and the RDP classifier (i.a. Liu et al., 2008; Coissac, Riaz and Puillandre, 2012; Richardson, et al., 2017), it is beyond the scope of this study to compare all these methods. Our results indicate that the RDP classifier is an adequate tool for the taxonomic assignment of Wadden Sea benthic fauna studies.

Comparison of extraction methods

The recovery of DNA from marine benthic communities is a crucial first step in molecular-based assessments of biodiversity. This study presents one of the first comparative analysis of benthic assessment based on morphological and different molecular approaches with respect to different DNA extraction methods. Our results indicate that DNA extraction methods preceded by a lysis step are efficient in terms of recovering marine benthic macrofauna and meiofauna biodiversity. The utility of an additional sieving step prior to DNA extraction depends on which portion of biodiversity is of interest, in this study, sieving improved the detection of macrofaunal diversity.

All three DNA extraction methods recovered most of the macrofauna families that were detected with the traditional morphological identification method, as reported earlier (Guardiola et al., 2015; Lejzerowicz et al., 2015; Pearman et al., 2016). However, the sieved lysis method was

the only method from which presence/absence ratios of macrofaunal genera correlated to the ratios found in the morphological approach. Previous studies using a sieving, or an elutriation step, already showed good results for large metazoan species (Brannock and Halanych, 2015; Vanreusel et al., 2010; Yu et al., 2012). However, this is the first study that shows a one-to-one correlation with morphological approaches. The sieved lysis method in this study included organisms retained on a 1mm sieve and hence recovered only macrofaunal taxa. Mesh sizes used for separating benthic fauna from sediment substrates through sieving, or elution, can be adapted to broaden the size range of the sampled species and also include meiofaunal species (Brannock and Halanych, 2015; Creer et al., 2016). Presence/absence ratios of macrofauna genera based on the morphological approach did not correlate to the ratios based on the extracellular DNA extraction method and the direct DNA extraction method. Although some studies assumed that the extracellular DNA excreted by macrofauna species reflects the biodiversity present, this study could not support this assumption.

Meiofaunal diversity was represented best by the direct DNA extraction method compared to the extracellular DNA extraction method. In particular nematodes seemed underrepresented with the extracellular DNA extraction method. Nematodes are the most abundant and diverse meiofaunal group in the Wadden Sea (Blome, Schleier and Van Bernem, 1999; Heip et al., 1985; Witte and Zijlstra, 1984) and the extracellular method failed to capture this important group. The low nematode diversity detected with the extracellular DNA extraction method has been reported earlier and is possibly characteristic for this particular method (Guardiola et al., 2016, 2015).

The methods employed here were selected from methods currently employed in metabarcoding studies. The methods did not only differ in DNA extraction strategy, but also in the sample volume. The sample volume and the sieving procedure for the sieved lysis method was similar to the morphological method and the high correlation as found in this study between the results of these methods was expected. The sample volume of the extracellular DNA extraction method was also similar to the morphological method; however, this method was infeasible to reflect the macrofauna diversity and numbers of metazoan OTUs were relatively low. The direct DNA extraction method showed the highest OTU diversity, even though this method processed only 1/90 of the sample volume of the other two methods.

The numbers of metazoan OTUs detected were lowest with the extracellular DNA extraction method. The low recovery rates may be due to the relatively long DNA fragment (650bp used) targeted in this study, since the length of the amplicon affects the recovery rate of partly degraded extracellular DNA (Coissac et al., 2012; Corinaldesi et al., 2008; Sinniger et al., 2016; Taberlet et al., 2012b). OTU detection based on extracellular DNA is biased by species specific differences in DNA release and the fate of extracellular DNA. Many factors influence

environmental DNA release. The annelid *Lanice* sp. produces relatively high amounts of slime and is well represented in the extracellular DNA pool whereas the gastropod *Peringia* sp. is enclosed by a shell and may therefore be less prominent in assessments based on extracellular DNA (Barnes and Turner, 2016).

The suitability of different DNA extraction methods depends on the specific research objective. The sieved lysis method appears best suited to characterise marine macrofaunal biodiversity. However, the direct DNA extraction method provided a more complete characterization of the marine benthic diversity. Unsieved sediment samples included intracellular DNA of species present in the small sediment core as well as environmental DNA from surrounding species which makes this specific method versatile (Barnes and Turner, 2016; Delmont et al., 2011). Targeting shorter DNA fragments, as now done for Illumina sequencing, might increase the diversity found with the extracellular DNA extraction method. However, taxonomic resolution will decrease inherently (Elbrecht & Leese, 2015). Although 454 sequencing, as used in the study, is no longer operational, the results of these studies will still be relevant. Nanopore technologies are quickly emerging and have the ability to sequence the longer amplicons as used in this study.

This study demonstrated the feasibility of metabarcoding as a means to assess marine benthic biodiversity in the Dutch intertidal Wadden Sea. Metabarcoding allowed for a rapid, replicable and nearly complete approach for the study of benthic communities. However, the outcome of the classic morphological approach and the outcome of metabarcoding studies are not necessarily identical. A more comprehensive discussion about the interpretation of metabarcoding studies can be found in Cowart et al., (2015) and Lejzerowicz et al., (2015). Besides macrofauna, also meiofauna key indicators for ecosystem health (Balsamo et al., 2012; Carugati et al., 2015; Spilmont, 2013) can now easily be included in marine benthic studies. Still, caution is needed when designing and interpreting metabarcoding studies. This study shows that results, i.e. the biodiversity recovered, may vary with the DNA extraction method and the combination of amplicon and reference database used. Studies need to clearly describe the methods and the reference databases used in order to enable comparisons with other studies. The need for incorporating a mock sample to test for optimal bioinformatics methods is shown here.

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