# Detection and identification of North Sea macrobenthos

Thesis


Marcel Kistemaker
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Inholland University of Applied Sciences
Life Sciences & Chemistry, De Boelelaan 1109, Amsterdam
F. Spaapen

Naturalis Biodiversity Center
Biodiversity discovery, Sylviusweg 72, Leiden
B. van der Hoorn

## Samenvatting

Macrobenthos is een verzamelnaam voor alle dieren die groter zijn dan 1 millimeter en in of op het sediment van een waterlichaam leven. Zij worden vaak gebruikt als indicator voor het inschatten van de waterkwaliteit van de zee en om het gevolg van antropogene activiteiten zoals boren naar olie en aquafarmen in kaart te brengen. Voor het beoordelen van het milieu zijn zowel de aanwezigheid en kwantiteit van bepaalde macrobenthos soorten van belang. Rijkswaterstaat heeft de Bentische Indicator Soorten Index (BISI) overgenomen als leidende metriek voor het evalueren van 160 Noordzee bemonsteringsstations. De BISI geeft aan welke macrobenthos soorten en hoeveel er moeten zijn op bepaalde locaties als indicatie voor waterkwaliteit. Het probleem hierbij, is dat de soorten geïdentificeerd moeten worden. Voor het identificeren van macrobenthos is een hoog niveau taxonomische kennis nodig. Deze expertise is aan het verdwijnen in Nederland, maar er is een nieuwe methode voor identificatie voorgesteld op basis van DNA. Deze identificatie komt tot stand door een deel van het mitochondriaal DNA te sequencen. Dit deel is het cytochroom c oxidase I subunit (CO1). Er bestaan een grote hoeveelheid primers die het CO1 gen amplificeren, maar er is een groot verschil in de hoeveelheid soorten die deze primers detecteren.

Wij hebben meerdere CO1 primers *in silico* getest door te kijken of er binding plaats vindt tussen de Noordzee macrobenthos CO1 sequenties en de primers. Uit deze resultaten zijn er twee forward en twee reverse primers gekozen die samen vier combinaties maken. Wij hebben de verschillende primer combinaties getest op een mock community van 50 soorten waarvan de meeste BISI soorten waren. The mock communities zijn geamplificeerd met die verschillende primer combinaties via PCR waarna het uiteindelijk werd gesequenced. De data die hieruit voortkwam is gebruikt om te achterhalen hoeveel mock community soorten er daadwerkelijk zijn geamplificeerd door de verschillende primer combinaties.

De resultaten geven aan dat de forward primer mlCOIintF en de reverse primer Fol-Degen-Rev het hoogste aantal mock community soorten amplificeerde en detecteerde. Hierbij hebben wij ook gevonden dat er tien PCR replicaten nodig zijn om alle soorten aan te tonen en dat een verhoging van de hoeveelheid template DNA de hoeveelheid soorten die worden gedetecteerd verhoogd.

## Summary

Macrobenthos are animals larger than one millimeter that live at the bottom of a body of water. They are commonly used as indicator to assess the ecological quality of marine environments and the impact of anthropogenic actions (oil drilling, aquafarming). For environmental assessments, both the presence and the quantity of specific macrobenthos species are studied. Rijkswaterstaat has adopted the Benthische Indicator Soorten Index (BISI) as leading metric for the evaluation of 160 North Sea sampling stations. The BISI contains a detailed list that states how many and which benthic species are supposed to be present at these stations. However, identification of macrobenthos species based on morphology requires a high level of taxonomic expertise. This expertise is rare and even diminishing in the Netherlands. As such, a new method for DNA-based identification has been proposed. This identification is achieved by sequencing a part of the mitochondrial DNA named cytochrome c oxidase I (CO1). A multitude of primers is available for amplification of CO1, but their success in species detection varies strongly.

We compared several CO1 primers *in silico* in their ability to bind to the North Sea macrobenthos CO1 sequences and chose two forward and two reverse primers for a total of four combinations. We tested the primer combinations on a mock community consisting of 50 species, most of which were BISI species. The mock communities were amplified with the different primers through PCR and were eventually sequenced. The resulting data was used to determine how many mock community species were amplified by the different primer combinations.

We found that the forward primer mlCOIintF and reverse primer Fol-Degen-Rev combination amplified the highest number of mock community species. That ten PCR replicates were needed to detect all amplified mock community species and that an increase in the amount of template DNA increases the number of species detected in the sample.

# List of abbreviations

| | |
|---|---|
| BISI | Benthic indicator species index |
| BOLD | Barcode of Life Data Systems |
| bp | basepair |
| CO1 | Cytochrome *c* oxidase subunit I |
| DNA | Deoxyribonucleic acid |
| HTS | High-throughput sequencing |
| NGS | Next generation sequencing |

# Table of contents

# 1. Introduction

## 1.1 Introduction

Macrobenthos are animals larger than one millimeter that live at the bottom of a body of water. They are commonly used as indicator to assess the ecological quality of marine environments and the impact of anthropogenic actions (oil drilling, aquafarming). For environmental assessments, both the presence and the quantity of specific macrobenthos species are studied. Rijkswaterstaat has adopted the Benthische Indicator Soorten Index (BISI) as leading metric for the evaluation of 160 North Sea sampling stations. The BISI contains a detailed list that states how many and which benthic species are supposed to be present at these stations. However, identification of macrobenthos species based on morphology requires a high level of taxonomic expertise. This expertise is rare and even diminishing in the Netherlands. As such, a new method for DNA-based identification has been proposed. This identification is achieved by sequencing a part of the mitochondrial DNA named cytochrome c oxidase I (CO1). A multitude of primers is available for amplification of CO1, but their success in species detection varies strongly.

## 1.2 Justification

Previous research has shown that many of the species living in the benthic zone are not easily identified or even missed entirely during the sequencing process. Whether this stems from primer bias or sequencing depth (template DNA, PCR replicates) is not yet clear. This research aims to determine the best publicly available CO1 primers to amplify North Sea macrobenthos species. It also aims to determine the number of PCR replicates needed to detect the maximum amount of species, and to determine how the amount of template DNA influences the recovery of the species in a sample.

## 1.3 Research question

How can primer choice, the amount of template DNA and PCR replicates improve DNA metabarcoding protocols for the detection of Dutch macrobenthos species?

# 2. Theoretical framework

## 2.1 Benthos and their uses

The benthic zone of an aquatic environment is the lower most level which includes the sediment and a few centimeters of sub-surface sediment. Zoöbenthos is the collective name for all the species of (in)fauna that inhabit the benthic layer of body water. These benthic fauna are divided into 3 groups based on size. These groups are microbenthos, meiobenthos and macrobenthos. Microbenthos are smaller than 32 µm and the group is mainly composed of bacteria and Protista. [1] The second group are meiobenthos which range from 32 µm to 1 mm in length according to Herman *et al.* [1] but according to McIntyre [2] the lower limit lies somewhere between 0,04 mm and 0,1 mm, depending on the sieve used. *Ostracoda, Copepoda, Oligochaeta* and low density *Nematoda* are but a few of the animals which make up the group of meiobenthos. The animals larger than 1 mm are considered macrobenthos and this group is made up of *crustaceans, Mollusca, Polycheata, Echinodermata* among many others.

Due to the diversity of species the microbenthic infauna play several different and key roles inside of the aquatic ecosystem. Some benthic species such as Bivalva and Polycheata burrow into the sediment or move it around in a process known as bioturbation. [3] Through this activity the decomposition of organic matter is enhanced. [4, 5,] The creation of burrows allows for ventilation of lower sediment layers and results in higher exchange of nutrients between the water column and sediment by stimulating microbial denitrification. [6, 7, 8] Ieno *et al*. [7] noted that the function of certain species and their ability to carry out this function to a better degree when compared to ecologically equivalent species might have a higher importance than species diversity.

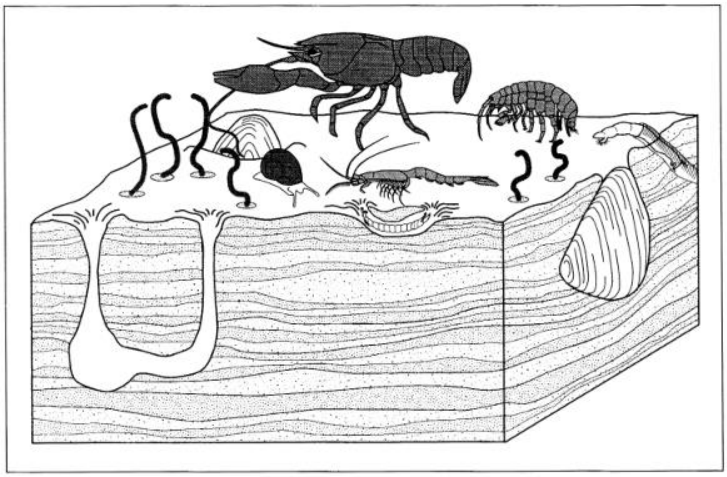

Figure 1: Illustration of several benthic species and the way in which they have an impact on nutrient generation and detritus decomposition by moving horizontally and vertically through the sediment. Aller (1982) [4]

The Benthic Indicator Species Index (BISI) [9] is an index in which benthic species have been given a score for their ability as an indicator. The aspects for being an indicator species are frequency, presence over several years, susceptibility to soil disturbance, importance to the food web, importance to soil structure/processes and if the species is potentially indicative for recovery. This index was developed to allow for the evaluation of habitat quality, soil integrity and ecological functioning of benthic fauna on different substrates in the North Sea. [10] A particular emphasis was placed on benthic species with a high longevity and increased susceptibility to soil disturbance as result of bottom trawling. [11] The effect of bottom trawling on the composition and structure of benthic communities has been observed by many. [12, 13, 14] A rapport of the Centraal Bureau voor de Statistiek (CBS) from the 31st of October 2017[1] noted that the Living Planet Index (LPI) of the North Sea has decreased by 30% between 1990 and 2015. 57 of the 140 total species observed declined, whilst 35 of the species showed an increase in numbers and the rest stayed the same. The decline was mostly attributed to the dwindling number of benthic species. On the other hand, the LPI

of the Oosterschelde and Westerschelde showed an increase whilst the North Sea coastal region and the Wadden Sea showed no change. As noted above, some of the indicator species have a potential indication for recovery. As such with time and careful monitoring, recovery of the numbers and biodiversity of benthic fauna in the North Sea might well be possible.[1]

## 2.2 DNA metabarcoding

DNA metabarcoding is a relatively new technique which holds the premise to allow for the identification of species and biodiversity without the need for taxonomic expertise. [15] DNA metabarcoding is the combination of high-throughput sequencing (HTS) together with DNA barcoding. HTS is an evolution of the standard DNA sequencing such as Sanger sequencing. The HTS platforms can generate millions of reads per sequencing run by sequencing fragments in a parallel fashion while Sanger sequencing sequences only one fragment at any given time. [16] DNA barcoding allows for discrimination between species based on the sequence variation in a specific gene region, also known as DNA barcodes. [17] CO1, 16S and 18S are examples of genes that are used for the identification of animals. There are several primers which amplify a part of these DNA barcodes and these primers are also known as barcoding primers/assays. The DNA barcodes gained from sequencing are then compared to a reference database for identification.

## 2.3 BOLD versus GenBank as reference database

In order to identify any species reliably a reference database is needed. This database needs to be complete with sequences of different barcoding markers and crucially it needs to be reliable. Misidentifications in the reference database has a profound impact on identification as might be expected. Currently two of these databases are publicly available, which are GenBank [18] and the Barcode of Life Data System (BOLD). [19] BOLD has, amongst other modules, a data collection and analysis workbench which allows for validation of DNA barcodes and sequences. GenBank is a comprehensive database with more nucleotide sequences when compared to BOLD which comes at the cost reliability. GenBank accepts all DNA sequences from projects with little to none criteria, BOLD on the other hand has strict criteria which need to be observed in order to be incorporated into the database to maintain quality assurance. These criteria include the availability of a voucher, a minimum sequence length of 500 base pairs and a taxonomic name. Either choice as reference database comes down to quality versus quantity. GenBank has more sequences which comes at the cost of quality and identification reliability. Although BOLD is not impervious to misidentification. This is due to not identifying the specimen morphologically but basing identification on barcoded sequences available in BOLD. This has given rise to private reference databases by research institutes to set their own criteria for the DNA sequences.

## 2.4 Primers make the difference

The choice for a marker (DNA metabarcode) and primer combination greatly influences the final number of detected species by metabarcoding. The most widely used marker to identify animals is the cytochrome c oxidase subunit 1 gene, in short CO1, located at the mitochondria. This was the original marker proposed at the very beginning of DNA barcoding activities. [17] There have been doubts on the accuracy and applicability of this marker for DNA metabarcoding studies with the years. [20] Still, some authors strongly advocate the use of CO1 as the community DNA metabarcode

[1] https://www.cbs.nl/nl-nl/nieuws/2017/44/biodiversiteit-noordzee-achteruit

for animals because, among others, the unprecedented coverage of reference sequence databases for this gene. [21] They argue that the right primers for DNA metabarcoding CO1 have not yet been developed.

Focusing on macrobenthos species, the primers used for DNA barcoding (determining a DNA barcode from a single voucher specimen) and building up a DNA reference library were developed by Folmer *et al*. (1994) [22]. However, these primers amplify the total length of the CO1 marker of 658 base pairs. DNA in the environment is mostly degraded which makes reads of these length less likely to be encountered, so the Folmer primers are less suitable as DNA metabarcodes. Currently, the most widely used general primers for metazoa are the Leray primers [23] with a length of 313 base pairs, recently improved to Leray-XT primers. [24] The improvement consists of the addition of some degenerated base pairs, base pairs that match with every of the four complementary base pairs, with the result that these primers are less specific and amplify the DNA metabarcodes of more macrobenthos species than the original Leray primers did. [24]

However, these primers still don't amplify all species groups equally well. Molluscs are notoriously difficult to detect in metabarcoding studies. [25, 26] And CO1 might not always be the right marker of choice, as has been demonstrated for Hydrozoans [27] and Tunicates. [28] Alternative 16S markers for dietary studies were developed for marine invertebrates such as cephalopods [29] and crustacean. [30] Studies focussing on eukaryotes in general use different regions of 18S as DNA barcode or DNA metabarcode. [28, 31, 32, 33] Although 18S seems to provide less accurate diversity estimates than CO1, a study on macrobenthos found species that were not detected by CO1 because reference barcodes of this gene were lacking. [25]

One universal primer to detect all macrobenthos species at once will be unlikely to be discovered. It is more likely that using primer cocktails will yield the best results. Development and testing new and existing primers for specific species groups has the potential to increase metabarcoding efficiency for North Sea macrobenthos species.

# 3. Materials and methods

## 3.1 *In silico* marker and primer test

Available CO1 sequences of North Sea macrobenthos species at Naturalis were loaded into Geneious (Geneious Prime 2019.2.1.) and split into families. These were complemented with full mitochondrial sequences from GenBank. CO1 sequences were mapped against the mitochondrial sequences to ensure the orientation for all sequences was from the 5' to 3'.

Universal CO1 primers were selected from literature. The best performing primers from previous studies appeared to be BF1, BF2, BF3, mlCOIintF and mlCOIintF-XT (table I). All primers were tested *in silico* on the macrobenthos sequences using the "Test with saved primer" function in Geneious, with the maximum number of accepted mismatches set to four. When a primer bound to a sequence, the theoretical amplicon (the marker) was then excised and put into a separate folder.

A phylogenetic tree was calculated for the markers for each primer pair, to determine whether the markers had sufficient resolving power to identify all macrobenthos to the species level. The phylogenetic trees were calculated using PHYML. Two or more species that were closely grouped might be impossible to identify separately.

Table I: List of all primers and their sequences that were tested *in silico* in during this study

| **Primer** | **Strand** | **Primer sequence (from 5' to 3')** | **Citation** |
|---|---|---|---|
| mlCOIintF | Forward | GGWACWGGWTGAACWGTWTAYCCYCC | Leray *et al*. 2013 [23] |
| mlCOIintF-XT | Forward | GGWACWRGWTGRACWITITAYCCYCC | Wangensteen *et al.* 2018 [24] |
| BF1 | Forward | ACWGGWTGRACWGTNTAYCC | Elbrecht & Leese 2017 [34] |
| BF2 | Forward | GCHCCHGAYATRGCHTTYCC | Elbrecht & Leese 2017 [34] |
| BF3 | Forward | CCHGAYATRGCHTTYCCHCG | Elbrecht *et al*. 2019 [35] |

## 3.2 Mock communities

Four mock communities were created for each of the four primer combinations. Only species that were part of the DNA collection of Naturalis could be used. In order to increase the relevance of the study, available species used for the BISI indicator were selected (33), supplemented with species (17) to cover a broad taxonomic range (appendix 1). The DNA concentration used per species was 5 ng/µl (mock 1). The second mock community (mock 2) was identical to the first except for 8 species (*Lutraria lutraria, Cerianthus llloydii, Nephtys hombergii, Pagurus bernhardus, Goneplax rhomboides, Aporrhais pespecelani, Astropecten irregularis* and *Echinocardium flavescens*) the amount of DNA was diluted a 1000 times before being added.

## 3.3 Environmental samples

During an expedition in June 2018, 36 boxcore samples were collected from the seafloor between the Dutch Wadden Sea island Terschelling and the UK Shetland islands. The contents were rinsed over a 1mm sieve and stored on 96% ethanol in a -20 freezer until further processing. The samples were blendered and stored on ethanol in a -20 freezer, again for future studies. Four blender samples with a high number of species were selected for this study to test the effectivity of the primer pairs on real environmental samples.

## 3.4 Experimental setup

All primer combinations were assessed with the following samples (table II): 12 replicates of 1 µl template DNA of each mock community to assess the number of PCR replicates to detect all amplified species. A 12 µl template DNA sample of each mock community to assess whether more template DNA would yield more species. Four environmental samples to compare the performance of the primer combinations on real samples. Two negative controls, one for 1 µl template DNA and one for 12 µl template DNA. A positive control was also added (*Linaria cannabina)*.

Table II: The number of replicates for the mock communities and environmental samples per primer combination used during this research.

| **Primer combinations** | **Mock 1** | | **Mock 2** | | **Environmental samples** | | | |
|---|---|---|---|---|---|---|---|---|
| | **1 µL DNA** | **12 µL DNA** | **1 µL DNA** | **12 µL DNA** | **LSI0448** | **LSI0460** | **LSI487** | **LSI502** |
| **mlCOIintF + jgHCO2198** | 12 | 1 | 12 | 1 | 1 | 1 | 1 | 1 |
| **mlCOIintF + Fol-Degen-Rev** | 12 | 1 | 12 | 1 | 1 | 1 | 1 | 1 |
| **mlCOIintF-XT + jgHCO2198** | 12 | 1 | 12 | 1 | 1 | 1 | 1 | 1 |
| **mlCOIintF-XT + Fol-Degen-Rev** | 12 | 1 | 12 | 1 | 1 | 1 | 1 | 1 |

## 3.5 DNA amplification and sequencing

A 313 bp fragment of the CO1 barcode region was amplified using the selected forward and reverse primers in four different combinations. (table III)

Table III: Selected primers and their sequences. The nucleotides in red indicate the Nextera adapter of the primer

| **Primer** | **Strand** | **Primer sequence (from 5' to 3')** | **Citation** |
|---|---|---|---|
| mlCOIintF | Forward | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC | Leray *et al*. 2013 [23] |
| mlCOIintF-XT | Forward | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWRGWTGRACWITITAYCCYCC | Wangensteen *et al.* 2018 [24] |
| jgHCO2198 | Reverse | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAIACYTCIGGRTGICCRAARAAYCA | Geller et al. 2013 [36] |
| Fol-Degen-Rev | Reverse | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTANACYTCNGGRTGNCCRAARAAYCA | Yu et al. 2012 [37] |

A dual indexed MiSeq amplicon library was prepared using two rounds of PCR amplifications. The PCR's of the first round were done with the different primer combinations with Nextera-tailed primers. All PCR's in the first round contained 20 µl reactions with 10 µl 2x TaqMan Environmental Master Mix 2.0 (Thermo Fisher, Waltham, MA, USA), 1 µl of template, 7 µl MiliQ and 1 µl (10 pMol) of both a forward and a reverse primer depending on the primer combination. The 12 µl template DNA reactions in the first round contained 10 µl 2x TaqMan Environmental Master Mix 2.0 (Thermo Fisher, Waltham, MA, USA), 12 µl of template DNA and 1 µl (10 pMol) of each primer of a combination. Initial denaturation was done at 96 °C for 10 min, followed by 30 cycles of at 96 °C for 30 s, 50 °C for 30 s, 72 °C for 20 s and followed by a final elongation 72 °C for 7 min. Each of the 96-well plates contained a blank containing no template for the 1 µl and 12 µl reactions as well as two positive controls for detection of cross-contamination. To ensure the PCR was successful the product was checked on an E-Gel 96 pre-cast agarose gel (Thermo Fisher, Waltham, MA, USA). Afterwards the PCR products were cleaned with NucleoMag NGS-Beads (Machery0Nagel, Düren, Germany) using a 1:0,9 ratio.

The second round of PCR's was done in 20 µl reactions using 10 µl 2x TaqMan Environmental Master Mix 2.0 (Thermo Fisher, Waltham, MA, USA),1 µl of MiSeq Nextera XT labels, 4 µl PCR product and 4 µl MiliQ. The initial denaturation was performed at 96 °C for 10 min, followed by 8 cycles at 96 °C for 30 s, 55 °C for 60 s, 72 °C for 30 s and followed by a final elongation at 72 °C for 7 min. Second round PCR products were cleaned with NucleoMag NGS-Beads using a ratio of 1:0,9 and then quantified on the QIAxcel (Qiagen, Venlo, the Netherlands) and pooled equimolarly with the QIAgility (Qiagen, Venlo, the Netherlands). The pools of the two plates were then pooled equimolarly. The equimolar

pool was quantified on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) with the DNA High Sensitivity Kit. The pool was then run on the Illumina MiSeq at BaseClear (Leiden, the Netherlands)

## 3.6 Quality filtering

Filtering and clustering of the raw data gained from the Illumina MiSeq was performed in the bioinformatics pipeline of Naturalis Biodiversity Center through a Galaxy instance. [38] The raw sequences were merged using FLASH [39] and all non-merged reads were discarded. The reads were split into the four different primer combinations and, the primers trimmed from the merged reads using Cutadapt. [40] All reads with primers not present or not anchored were discarded. Sequences with a length below 310 bp and above 316 bp were discarded using PRINSEQ. [41] The reads were split into the mock communities and environmental samples. Reads were clustered into OTU's with UNOISE2 [42] with an alpha value of 0.5. A 0.02% threshold was used to omit spurious reads from the OUT tables.

## 3.7 Taxonomic assignment and data analysis

All primer combinations amplify the same 313 bp DNA marker of the CO1 gene. The sequence of this DNA marker was extracted for each macrobenthos species in Geneious, using the DNA barcode collection of Naturalis, and added to a custom reference library. This collection was used because it contained the sequences from the DNA samples used to create the mock communities. A phylogenetic tree was created to check whether all species in the reference were individually identifiable with the chosen DNA marker. If the sequences of species were equal and clustered together, they were checked against BOLD to determine whether the original identifications of the species might be wrong. A total of eleven spurious sequences were removed from the reference database because their identification did not match the identification in BOLD. For three species (*Buccinum undatum, Alcyonium digitatum* and *Aequipecten opercularis*) the sequences available at Naturalis were of insufficient length or were not actually CO1 genes. For these species a sequence from the BOLD database was added to the custom reference database. For species with more than three sequences in the reference database, sequences with bad coverage and identity were removed. Finally, the mock community samples were identified using BLASTn on this custom reference database.

The environmental samples were identified using BLASTn on the BOLD reference database [19] version 17-04-2019.

The vegan package [43] in R was used to calculate and plot accumulation curves based on replicates for the undiluted mock community. They were calculated using 100 permutations in the "random" method.

# 4. Results

## 4.1 *In silico* primer test

To determine which forward and reverse primers were the most efficient in species detection, an *in silico* test was performed in Geneious. Several primer combinations were tested in their ability to amplify part of the CO1. All the available macrobenthos sequences at Naturalis were loaded into Geneious and were aligned for an orientation from 5' to 3'. The maximum amount of mismatches was set to four, as it is assumed a primer does not bind properly to a sequence when it has five mismatches.

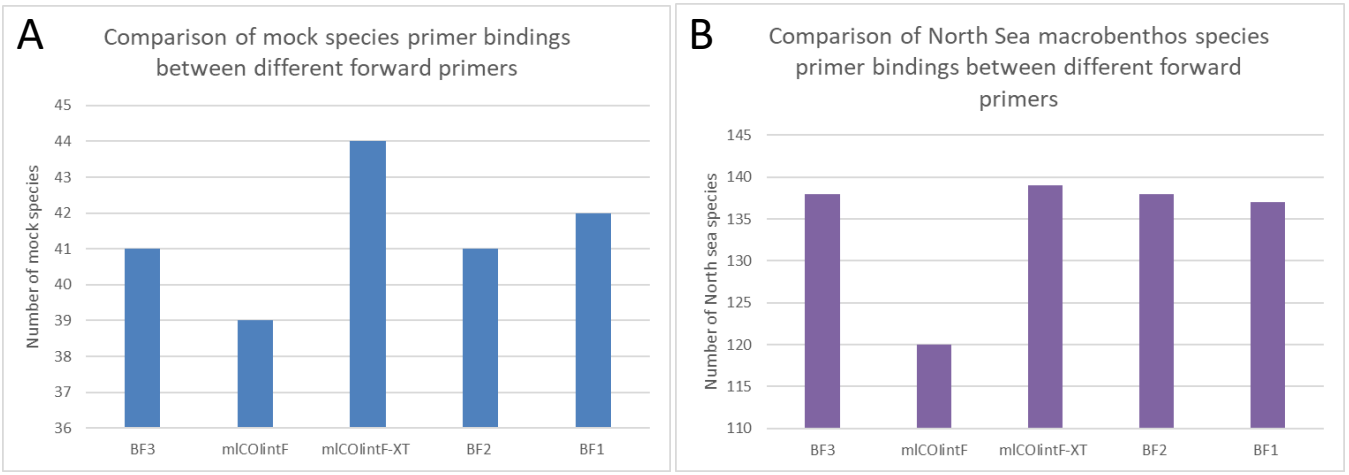


Figure 2: Different forward primers and their ability to bind to the mock species sequences (A)(N = 50) and to all North Sea macrobenthos species including all mock species (B)(N = 178).

The forward primer mlCOIintF-XT bound to the highest amount of the 50 mock species (figure 2A) and the 178 North Sea macrobenthos species (figure 2B). The BF primers performed quite similarly to one another and all bound roughly to the same amount of mock and North Sea macrobenthos species. mlCOIintF bound to noticeably less mock and North Sea macrobenthos species.

## 4.2 Mock community primer test

The four chosen primer combinations were tested on mock communities to determine the combination that amplified the highest number of species. A comparison between the primer combinations was made based on the number of species detected in all mock community replicates.

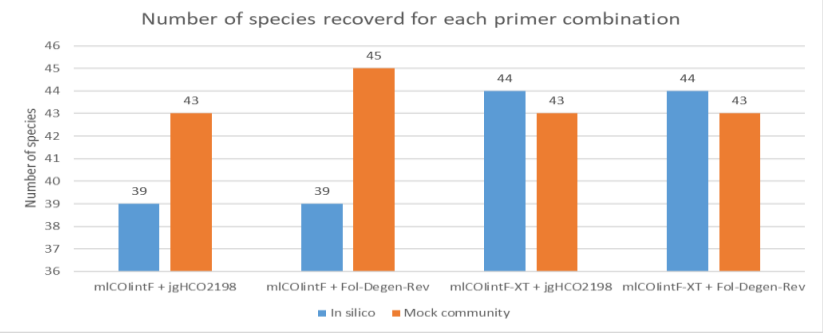


Figure 3: Bar graph showing different primer combinations and the number of mock community species recovered for the *in silico* test and the mock community samples.

The number of species detected in the mock communities with the primer combinations where mlCOIintF was the forward primer exceeded the *in silico* tests in the number of mock community species detected (figure 3). The opposite was found for the mlCOIintF-XT primer combinations. No primer combination detected all mock community species (table IV). All combinations failed to detect the species: *Arctica islandica, Cerianthus lloydii, Ensis siliqua* and *Ophiura albida* in contradiction to the *in silico* test. The combinations with Fol-Degen-Rev as reverse primer detected the species, *Alcyonium digitatum* and *Aphrodite aculeata,* which were undetected by the other primer combinations. *Aequipecten opercularis* was only detected by the mlCOIintF-XT + Fol-Degen-Rev primer combination even though it was not supposed to be amplified according to the *in silico* tests. A complete list of the fifty mock community species with *in silico* and mock community results is added in appendix 2.

Table IV: Undetected species in the mock communities for each primer combination. Green denotes the presence of the species and red the absence of the species. The "+" and "- "denotes whether the primer combination would or would not bind.

| Species | mlCOIintF + jgHCO2198 | mlCOIintF + Fol-Degen-Rev | mlCOIintF-XT + jgHCO2198 | mlCOIintF-XT + Fol-Degen-Rev |
|---|---|---|---|---|
| *Acanthocardia echinata* | + | + | + | + |
| *Aequipecten opercularis* | - | - | - | - |
| *Alcyonium digitatum* | + | + | + | + |
| *Aphrodite aculeata* | + | + | + | + |
| *Aporrhais pespelecani* | + | + | + | + |
| *Arctica islandica* | - | - | - | - |
| *Cerianthus lloydii* | + | + | + | + |
| *Ensis siliqua* | - | - | + | + |
| *Hyperia galba* | + | + | + | + |
| *Ophiura albida* | + | + | + | + |

The species *Spatangus purpureus, Ophelina acuminata* and *myxine glutinosa* made up 64%, 15% and 10% (figure 4) of the total number of DNA reads for each PCR replicate of the mlCOIintF + Fol-Degen-Rev primer combination. Despite the highly varying number of total reads per PCR replicate (figure 4a), the standardised species composition of each replicate is almost the same (figure 4b). Even the PCR with 12 µL template DNA that yielded considerably more DNA reads (>300.000) contained roughly the same standardised species composition (figure 4b). An identical situation can be seen in the composition based on the class-level taxonomic assignment (figure 5) of the species found in all environmental samples combined. The most prevalent classes for each of the primer combinations were Polycheata, Malacostraca, Ophiuroidea and Echinoidea. Primer combination mlCOIintF + jgHCO2198 was the only combination able to amplify a species belonging to the Asteroidea class. The same was true for mlCOIintF-XT + Fol-Degen-Rev primer combination only it was able to amplify a species belonging to the Scyphozoa class. The environmental samples for the combination mlCOIintF + Fol-Degen had less than 9000 reads with all samples combined while the other combinations had a minimum of 45.000 total reads. Species numbers were only slightly higher in PCR replicates which yielded a high amount of DNA reads. A scatterplot (figure 6) demonstrates this correlation.

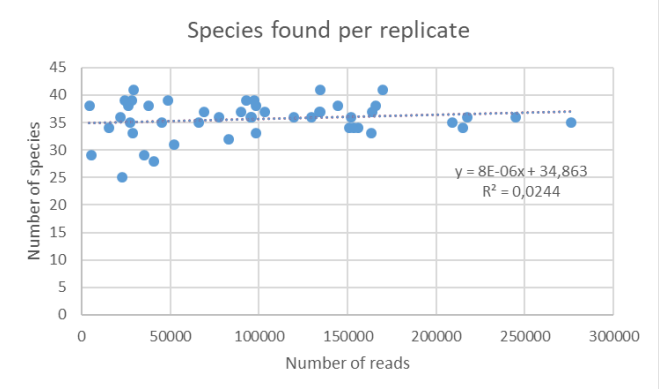


Figure 6: Scatterplot in which the amount of reads is compared to the number of species found.

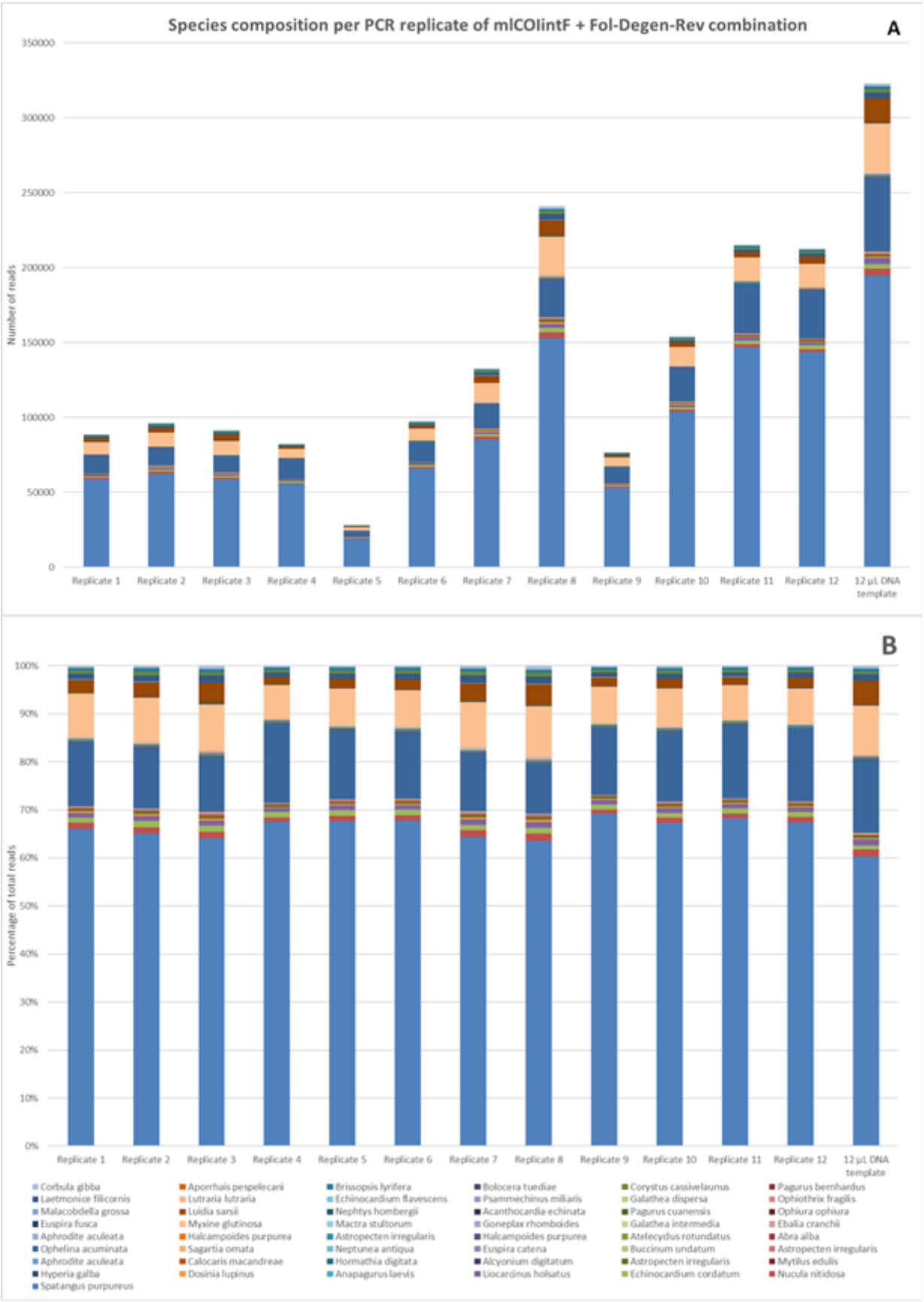


Figure 4: Species composition of PCR replicates of the undiluted mock community for the mlCOIintF + Fol-Degen-Rev combination. (A) Absolute number of DNA reads per species per PCR replicate. (B) Standardises the number of DNA reads per species per PCR replicate. Replicates 1 through 12 contained 1 µL of template DNA as input for the PCR process added during the PCR process. The 12 µL DNA template replicate contained 12 µL of template DNA.

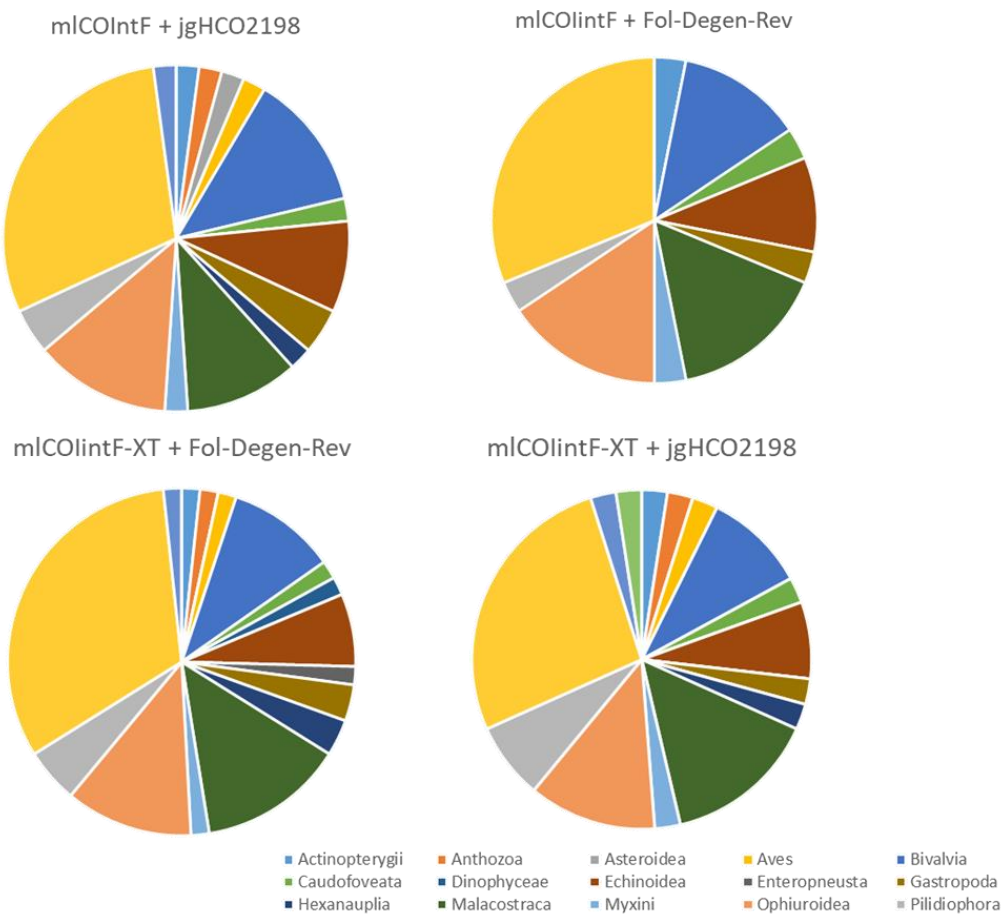


Figure 5: Class-level species composition found in all environmental samples combined per primer combination.

## 4.3 Required number of PCR replicates

In order to assess the number PCR replicates needed to detect all species in a sample, a statistical analysis has been performed in R based on the sequencing data. A species accumulation curve (figure 7) for each primer combination has been calculated in R based on the replicates of the undiluted mock community (mock community 1).

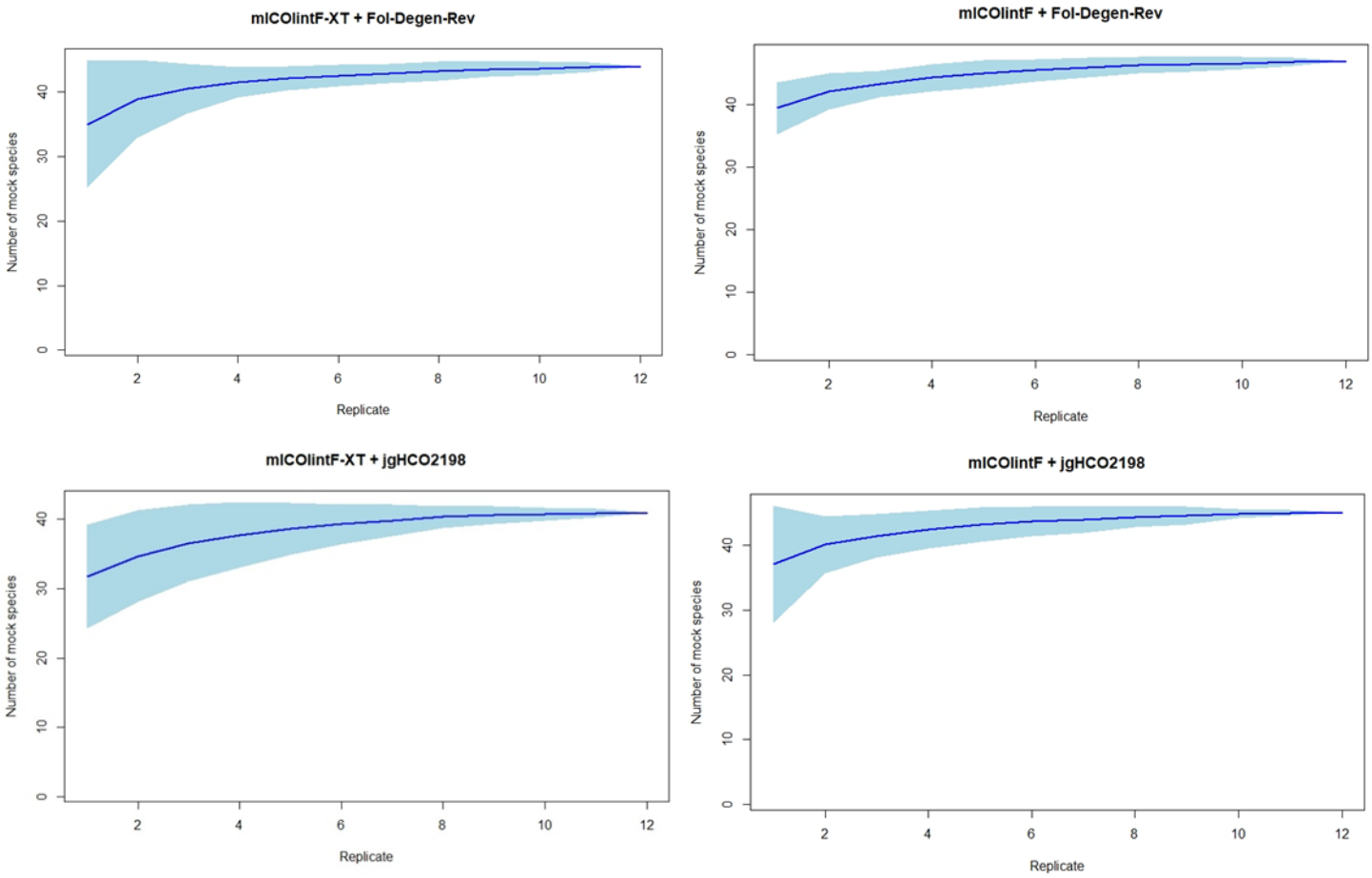


Figure 7: Species accumulation curves and their confidence intervals (p = 0,005) for all primer combinations based on the PCR replicates of the undiluted mock community. Accumulation curves were calculated in R using randomization with 100 permutations. on the randomised method permutated 100 times.

The accumulation curves of the undiluted mock communities demonstrate that both the mlCOIintF + Fol-Degen-Rev and mlCOIintF + jgHCO2198 primer combinations have a relatively small spread in the number of mock community species found in the first three replicates when compared to the other two primer combinations. The variation in species number found in the first three replicates is less for the mlCOIintF combinations. The curves start to flatten out after three replicates, and at six replicates only two to four mock community species were still not detected. The number of PCR replicates needed to detect all the amplified species present in the mock communities is ten.

Figure 8 shows several boxplots comparing the number of reads detected for each species which were diluted a 1000 times in mock 2 as compared to mock 1. *Lutraria lutraria, Pagurus bernhardus* and *Aporrhais pespecelani* have all been detected in the undiluted samples but were not detected in the diluted mock samples, despite the 12 PCR replicates. The number of reads for *Astropecten*

*irregularis* and *Echinocardium flavescens* show that a 1000 times dilution of a species does not equal a 1000 times less reads.

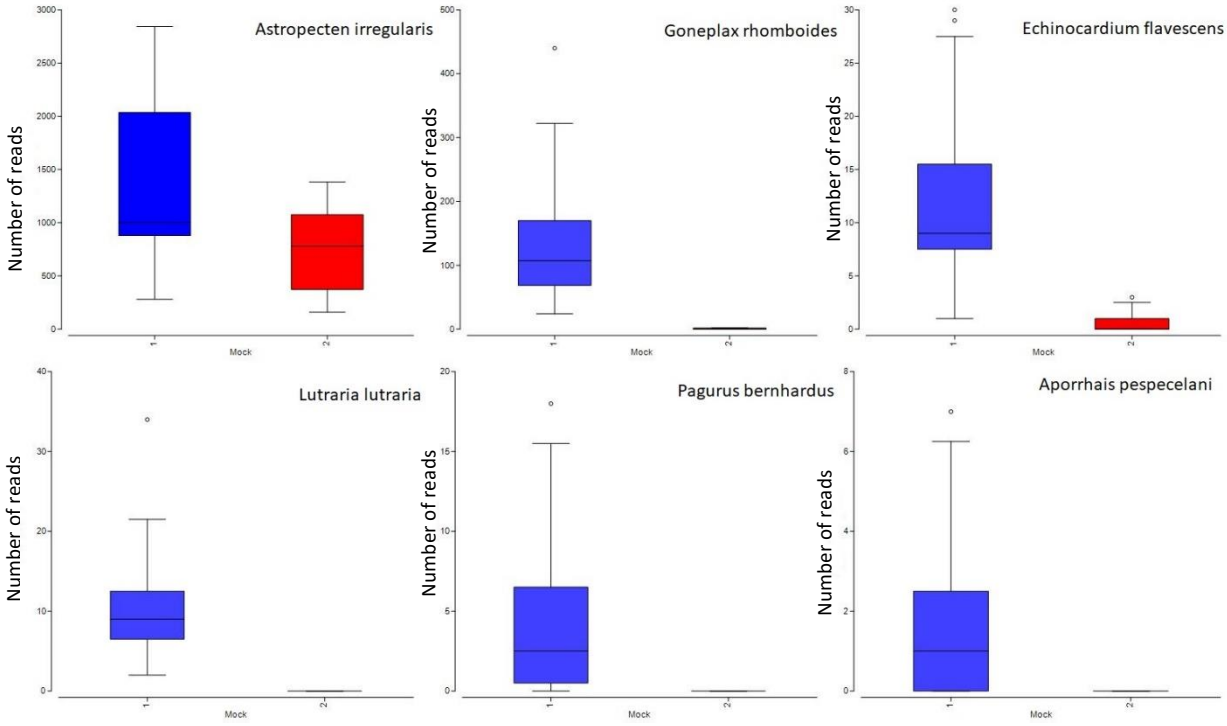


Figure 8: Boxplots for six species with normal (5 ng/µL) DNA concentrations in mock sample 1 and a 1000 times diluted DNA concentration in mock sample 2.

## 4.4 Variation in DNA concentration

To determine whether the amount of template DNA used for the PCR reaction had any effect on the number of detected species in the mock community, one PCR replicate with 12 µL of template DNA was added per primer combination. The results were compared to the 12 replicates with 1 µL template DNA.

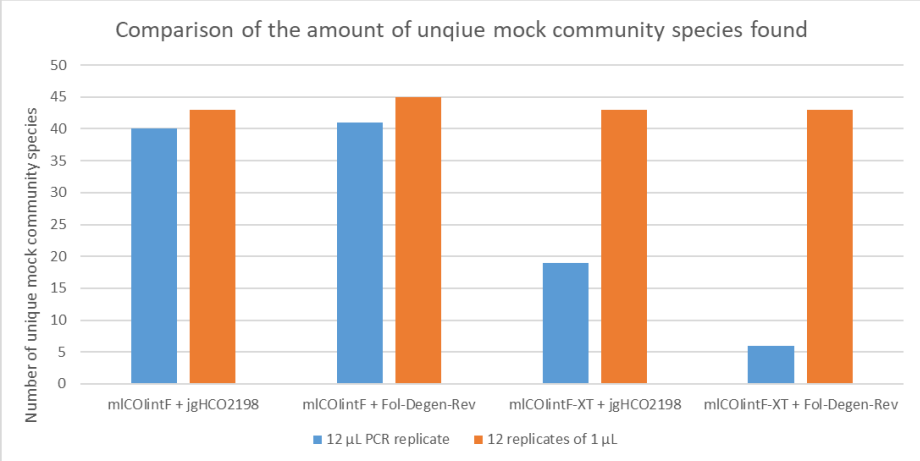


Figure 9: Detected mock community species with a 12 µL PCR replicate and with 12 1 µL PCR replicates per primer combination.

The PCR replicate with 12 µL template DNA in the mlCOIintF + Fol-Degen-Rev primer combination yielded more DNA reads (figure 4a) with a roughly similar species composition as the other PCR replicates (figure 4b). However, the 12 µL replicate detected more species than every individual PCR replicates with 1 µL template DNA, but three species fewer (figure 9) when all the individual PCR with 1 µL replicates were added up. The amount of reads for the 12 µL DNA template samples for different primer combinations was highly variable. The mlCOIintF-XT + Fol-Degen-Rev combination had only 40 reads while the other mlCOIintF-XT combination had 4500 reads. The other two combinations: mlCOIintF + Fol-Degen-Rev and mlCOIintF + jgHCO2198 had 140.000 and 327.000 reads respectively.

# 5. Discussion

## 5.1 Primers

Our results show a disparity between the *in silico* test of a primer combination and the actual amplification of species in a mock community. Several species of the mock community were not supposed to be amplified according to the *in silico* test, but they were. *Euspira fusca*, *Laetmonice filicornis* and *Buccinum undatum* are examples. Unfortunately, the reverse also holds true as *Ophiura albida* and *Cerianthus IIoydii* should have been amplified but were not. It has been noted [44] that mismatches within the last five nucleotides at the 3'-end of a primer are detrimental to the annealing of the primers, especially G/G and G/A mismatches. [45] A detailed check of primer binding for mlCOIintF on *Ophiura albida* demonstrated two mismatches. A G/T mismatch at the 9$^{th}$ nucleotide from the 5'-end and one T/C mismatch at the 5$^{th}$ nucleotide from the 3' -end. *Ophiotrix fragilis* has the exact same mismatches with mlCOIintF as *Ophiura albida* but did get amplified during the PCR process. Whilst the *in silico* primer tests can give a good indication of primer binding it has to be supported with tests of mock communities.

Forward primer mlCOIintF was tested *in silico* on freshwater invertebrates in a previous study [34] and it was found less efficient for freshwater *Polycheata* and *Bivalva.* Our results are in line with their findings even though we used marine species. The primer combination mlCOIintF + Fol-Degen-Rev detected 45 species from a total of 50. Three of the five undetected species were *Bivalva*. Elbrecht & Leese also tested jgHCO2198 as a reverse primer on the same freshwater species and found it to be potentially suitable. Our results show that the Fol-Degen-Rev reverse primer amplifies two species more than jgHCO2198. This could be due to the removal and replacement of the inosines with regular nucleotides, since inosine has a preferential binding with cytidine and tends to have the properties of guanine [46] and could facilitate mismatches.

Even though all the species in the mock community had the same concentration of 5 ng/µl DNA the results show that several species have a vastly increased amount of reads compared to other species. This disparity in amplification efficiency of the COI gene is known as primer bias and can greatly skew results. [47] In our results *Spatangus purpureus, Ophelina acuminata* and *myxine glutinosa* have 1.9 million, 400.000 and 260.000 reads respectively in the mlCOIintF + Fol-Degen-Rev combination. The other primer combinations had a similar distribution of reads where a few species had a drastically higher amount of reads compared to other species. The presence of these select few species with a high amount of reads show that each of the primers used suffered from primer bias. To what extend this primer bias had an effect on the results is unknown and cannot be traced back.

We were unable to test any other reverse primers than those used in this study. All the macrobenthos species at Naturalis were amplified and identified with Sanger sequencing using the jgLCO1409 forward primer and the jgHCO2198 reverse primer. When the sequences were processed the primers were trimmed off leaving a 658 bp sequence. Most of the reverse primers used for barcoding bind approximately to the same region as jgHCO2198. Since this part of the sequence was trimmed off we were unable to test the reverse primers and their binding ability. It may well be possible that other reverse primers will recover a higher number of macrobenthos species compared to the reverse primers we tested. We tested Fol-Degen-Rev as a reverse primer, as it is identical to jgHCO2198 in length and only the inosines are replaced with a degenerate nucleotide.

## 5.2 PCR replicates

The accumulation curves all follow the same pattern. They start to flatten out with three replicates and beyond six replicates only a few new OTU's were found and after ten replicates no new species were found. After six or seven replicates only a few of the rare species were detected. Four PCR replicates as a standard has been adopted for the Earth Microbiome Project [48], but no such standard exists for biomonitoring studies. Some studies use three replicates [49] while others use ten replicates. [50] Another study [51] suggest that sequencing depth and not PCR replicates allows for the detection of more OTU's and hence more species. Our results show that adding more template DNA during the PCR process increases the amount of mock community species found compared to a single replicate with a lower amount of template DNA, though not all of the rare species have been detected. We also show that the diluted species were hardly detected in the mock community and that a 1000 times less concentration does not equal to a 1000 times less reads. This result combined with knowledge of the primer bias support the conclusion of Elbrecht & Leese [50] that the number of reads is not a reliable indication for the number of species in an ecosystem.

Suggestions for future research based on the results of this study would be to look further into several other reverse primers to see if other primer combinations result in a higher amount of species detected. The BR1 and BR2 [34] primers are suggested as suitable for amplification of freshwater invertebrates. Whether these primers work equally well on marine macrobenthos is worth looking into. Another suggestion could be the development of family or genus specific primers to target a specific group of species that are known to be harder to amplify (e.g. Bivalva) with the standard barcoding primers. Then making a primer "cocktail" by mixing these with the standard barcoding primers and sequencing the results to see if the group specific primers worked. Although this would slow down the bioinformatics part of the research.
Further suggestions would be to investigate the impact of sequencing depth versus PCR replicates on the amount of OTU's detected. This would entail that during the equimolar pooling of e.g. six PCR replicates, one of these replicates would have a five times higher concentration than the other five replicates. With an increased sequencing depth some of the rare species could possibly be detected in one or two samples instead of having to use multiple PCR replicates. The same setup can be used in order to determine if diluted or rare species are lost during the PCR process or during sequencing.

# 6. Conclusion

Our findings show discrepancies between *in* silico primer tests and mock community tests exist. In our study, the mlCOIintF appeared the worst performing primer *in silico,* yet it amplified the highest amount of macrobenthos species in our mock community. Even though the different primer combinations amplified a varying amount of mock community species, ten PCR replicates seems sufficient to detecting all species present. Other studies will have to determine whether they need to detect all the macrobenthos species in a sample and go for many PCR replicates or suffice with a lower percentage of species and use fewer replicates. Increasing the amount of template DNA could also contribute to increasing species detection. Even with equal DNA concentrations, the DNA amplification efficiency differs greatly between species probably due to primer bias. Bivalves for example appear to be amplified less efficiently. We advise the development of group specific primers to increase species detection and minimize primer bias.

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# Appendix 1: List of all species in the mock communities

| *Soort* | taxon |
|---|---|
| *Echinocardium flavescens* | zeeegel |
| *Corbula gibba* | schelp |
| *Ophiothrix fragilis* | zeester |
| *Abra alba* | schelp |
| *Echinocardium cordatum* | zeeegel |
| *Luidia sarsii* | zeester |
| *Brissopsis lyrifera* | zeeegel |
| *Nucula nitidosa* | schelp |
| *Ophiura albida* | zeester |
| *Ophiura ophiura* | zeester |
| *Astropecten irregularis* | zeester |
| *Aporrhais pespelecani* | zeeslak |
| *Arctica islandica* | schelp |
| *Dosinia lupinus* | schelp |
| *Acanthocardia echinata* | schelp |
| *Neptunea antiqua* | zeeslak |
| *Aequipecten opercularis* | schelp |
| *Ensis siliqua* | schelp |
| *Mactra stultorum* | schelp |
| *Buccinum undatum* | zeeslak |
| *Goneplax rhomboides* | krab |
| *Corystus cassivelaunus* | krab |
| *Liocarcinus holsatus* | krab |
| *Aphrodite aculeata* | zeemuis |
| *Pagurus bernhardus* | kreeft |
| *Nephtys hombergii* | zeeworm |
| *Alcyonium digitatum* | zacht koraal |
| *Cerianthus lloydii* | anemoon |
| *Lutraria lutraria* | schelp |
| *Malacobdella grossa* | zeeworm |
| *Myxine glutinosa* | zeeworm |
| *Spatangus purpureus* | zeeegel |
| *Laetmonice filicornis* | zeeworm |
| *Euspira catena* | zeeslak |
| *Euspira fusca* | zeeslak |
| *Hyperia galba* | kreeft |
| *Anapagurus laevis* | kreeft |
| *Galathea dispersa* | kreeft |
| *Calocaris macandreae* | kreeft |
| *Ebalia cranchii* | krab |
| *Atelecyclus rotundatus* | krab |
| *Hormathia digitata* | anemoon |
| *Sagartia ornata* | anemoon |
| *Halcampoides purpurea* | anemoon |
| *Bolocera tuediae* | anemoon |
| *Ophelina acuminata* | zeeworm |

# Appendix 2: Macrobenthos species *in silico* test results plus mock community test results.

| Mock community species | mlCOIintF + jgHCO2198 | mlCOIintF + Fol-Degen-Rev | mlCOIintF-XT + jgHCO2198 | mlCOIintF-XT + Fol-Degen-Rev |
|---|---|---|---|---|
| *Abra alba* | + | + | + | + |
| *Acanthocardia echinata* | + | + | + | + |
| *Aequipecten opercularis* | - | - | - | - |
| *Alcyonium digitatum* | + | + | + | + |
| *Anapagurus laevis* | + | + | + | + |
| *Aphrodite aculeata* | + | + | + | + |
| *Aporrhais pespelecani* | + | + | + | + |
| *Arctica islandica* | - | - | - | - |
| *Astropecten irregularis* | - | - | + | + |
| *Atelecyclus rotundatus* | + | + | + | + |
| *Bolocera tuediae* | + | + | + | + |
| *Brissopsis lyrifera* | + | + | + | + |
| *Buccinum undatum* | - | - | - | - |
| *Calocaris macandreae* | + | + | + | + |
| *Cerianthus lloydii* | + | + | + | + |
| *Corbula gibba* | + | + | + | + |
| *Corystus cassivelaunus* | + | + | + | + |
| *Dosinia lupinus* | + | + | + | + |
| *Ebalia cranchii* | + | + | + | + |
| *Echinocardium cordatum* | + | + | + | + |
| *Echinocardium flavescens* | + | + | + | + |
| *Ensis siliqua* | - | - | + | + |
| *Euspira catena* | + | + | + | + |
| *Euspira fusca* | - | - | - | - |
| *Galathea dispersa* | + | + | + | + |
| *Galathea intermedia* | + | + | + | + |
| *Goneplax rhomboides* | + | + | + | + |
| *Halcampoides purpurea* | - | - | + | + |
| *Hormathia digitata* | + | + | + | + |
| *Hyperia galba* | + | + | + | + |
| *Laetmonice filicornis* | - | - | - | - |
| *Liocarcinus holsatus* | - | - | + | + |
| *Luidia sarsii* | + | + | + | + |
| *Lutraria lutraria* | - | - | + | + |
| *Mactra stultorum* | + | + | + | + |
| *Malacobdella grossa* | + | + | + | + |
| *Mytilus edulis* | + | + | + | + |
| *Myxine glutinosa* | + | + | + | + |
| *Nephtys hombergii* | + | + | + | + |
| *Neptunea antiqua* | - | - | - | - |
| *Nucula nitidosa* | + | + | + | + |
| *Ophelina acuminata* | + | + | + | + |
| *Ophiothrix fragilis* | + | + | + | + |
| *Ophiura albida* | + | + | + | + |
| *Ophiura ophiura* | + | + | + | + |
| *Pagurus bernhardus* | + | + | + | + |
| *Pagurus cuanensis* | + | + | + | + |
| *Psammechinus miliaris* | + | + | + | + |
| *Sagartia ornata* | + | + | + | + |
| *Spatangus purpureus* | + | + | + | + |