NGS-based biodiversity and community structure analysis of meiofaunal eukaryotes in shell sand from Hållö island, Smögen, and soft mud from Gullmarn Fjord, Sweden

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Abstract

Aim: The aim of this study was to assess the biodiversity and community structure of Swedish meiofaunal eukaryotes using metabarcoding. To validate the reliability of the metabarcoding approach, we compare the taxonomic resolution obtained using the mitochondrial cytochrome oxidase 1 (COI) ‘mini-barcode’ and nuclear 18S small ribosomal subunit (18S) V1-V2 region, with traditional morphology-based identification of Xenacoelomorpha and Nematoda.

Location: 30 samples were analysed from two ecologically distinct locations along the west coast of Sweden. 18 replicate samples of coarse shell sand were collected along the north-eastern side of Hållö island near Smögen, while 12 replicate samples of soft mud were collected in the Gullmarn Fjord near Lyseki.
Methods: Meiofauna was extracted using flotation and siphoning methods. Both COI and 18S regions were amplified from total DNA samples using Metazoan specific primers and subsequently sequenced using Illumina MiSeq, producing in total 24,132,875 paired-end reads of 300 bp in length, of which 15,883,274 COI reads and 8,249,601 18S reads. These were quality filtered resulting in 7,954,017 COI sequences and 890,370 18S sequences, clustered into 2805 and 1472 representative OTUs respectively, yielding 190 metazoan OTUs for COI and 121 metazoan OTUs for 18S using a 97% sequence similarity threshold.

Results: The Metazoan fraction represents 7% of the total dataset for COI (190 OTUs) and 8% of sequences for 18S (121 OTUs). Annelida (30% of COI metazoan OTUs and 23.97% of 18S metazoan OTUs) and Arthropoda (27.37% of COI metazoan OTUs and 11.57% of 18S metazoan OTUs), were the most OTU rich phyla identified in all samples combined. As well as Annelida and Arthropoda, other OTU rich phyla represented in our samples include Mollusca, Platyhelminthes and Nematoda. In total, 213 COI OTUs and 243 18S OTUs were identified to species using a 97% sequence similarity threshold, revealing some non-native species and highlighting the potential of metabarcoding for biological recording. Taxonomic community composition shows as expected clear differentiation between the two habitat types (soft mud versus coarse shell sand), and diversity observed varies according to choice of meiofaunal sampling method and primer pair used.

Keywords

Meiofaunal biodiversity, community structure, Illumina Mi-Seq, Metabarcoding, COI, 18S

Introduction

Microscopic interstitial marine organisms, also termed ‘meiofauna’, are often defined as animals that pass a 1 mm mesh but are retained on a 45 µm sieve (Higgins 1988). Meiofauna are an important component of sedimentary and benthic habitats due to their small size, abundance and rapid turnover rates. Moreover, meiofaunal surveys represent a useful tool for environmental impact assessments, underlying the urgent need for reliable, reproducible and rapid analytical methods. The breadth of taxonomic groups present in marine sediments makes meiofauna an ideal tool for detecting the effects of ecological impacts on marine biodiversity (Moreno et al. 2008). However, traditional morphology based taxonomy assignment methods are labour intensive and time consuming, leading us to explore recently developed metabarcoding methods for whole community analysis. Metabarcoding has previously been used to characterize plankton assemblages (Lindeque et al. 2013, de Vargas et al. 2015), marine benthic meiofaunal assemblages (Creer et al. 2010, Fonseca et al. 2014, Fonseca et al. 2010, Brannock and Halanych 2015, Cowart et al. 2015), meiofaunal communities colonizing autonomous reef monitoring structures (Leray and Knowlton 2015) or fish gut contents (Leray et al. 2013). The vast majority of studies have employed Roche 454 due to its long read lengths compared to other technologies (Table 1; Shokralla et al. 2012), but Illumina MiSeq is now able to provide
similarly long reads using paired-end sequencing (2x300 base pairs). As summarized in Table 1, there is no standardized method for metabarcoding of marine fauna, and a variety of sample extraction methods, sequencing platforms, molecular markers, bioinformatics pipelines and OTU clustering thresholds have been used to date, making these studies difficult to compare (Table 1).

Table 1.
Methodological comparison of benthic and pelagic metabarcoding studies of marine fauna published to date

| Authors                  | Sample type                  | Sample extraction method                  | Sequencing platform | Marker | Marker size (bp) | Chimera screening | OTU clustering method and threshold | Database                  |
|--------------------------|------------------------------|-------------------------------------------|---------------------|--------|------------------|-------------------|------------------------------------|---------------------------|
| Leray et al. 2013        | Coral reef fish gut contents | Dissection of fish gut                    | Roche 454 GS FLX   | COI    | 313              | UCHIME            | CROP 92-94%                        | Moorea Biocode Database, GenBank |
| Leray and Knowlton 2015  | Autonomous reef monitoring structures | 4 fractions (Sessile, 2mm, 500μm, 106μm) | Ion Torrent        | COI    | 313              |                   |                                    | BOLD, GenBank               |
| Lindeque et al. 2013     | Zooplankton from 50m to the surface | 200μm mesh WP2 plankton net               | Roche 454 GS FLX   | 18S    | (V1-V2 regions)  | ChimeraSlayer (QIIME 1.3.0) | UCLUST 97% (QIIME 1.3.0)        | Silva 108, GenBank          |
| de Vargas et al. 2015    | Plankton                     | 3 fractions (5-20μm, 20-180μm, 180-2000μm) | Paired-end Illumina Genome Analyser IIX system | 18S    | (V9 region)      | USEARCH           | V9_PR2, V9 rDNA, Protistan Ribosomal Reference Database |
| Fonseca et al. 2010      | Marine benthic meiofauna     | Decanting 45μm sieve Ludox                | Roche 454 GS FLX   | 18S    | (V1-V2 regions)  | OCTOPUS           | OCTOPUS 96%                        | GenBank                    |
| Fonseca et al. 2014      | Marine benthic meiofauna     | Decanting 45μm sieve Ludox                | Roche 454 GS FLX   | 18S    | (V1-V2 regions)  | Amplicon-Noise    | Amplicon-Noise 99% and 96%         | GenBank                    |
| Brannock and Halanych 2015 | Marine benthic meiofauna | Directly from sediment, elutriated on 45μm sieve | Paired-end 100 bp reads Illumina HiSeq | 18S    | (V9 region)      | USEARCH 6.1. (QIIME 1.8) | UPARSE 97% UCLUST and USEARCH (QIIME 1.8) | Silva 111                 |
In this study we used samples from muddy and sandy marine sediments to examine how results of metabarcoding based surveys of meiofaunal communities are impacted by three different meiofaunal extraction methods and three different primer pairs for COI and 18S. In order to validate the reliability of the metabarcoding approach, we compare the results obtained with traditional morphology-based taxonomic assignment for two test groups, Xenacoelomorpha and Nematoda, the latter previously shown to be the dominant taxon in meiofaunal communities in terms of number of OTUs (Fonseca et al. 2010).

### Materials and Methods

#### Sampling

Samples were collected in two ecologically distinct locations along the west coast of Sweden in August 2014.

**Hållö island samples**: Coarse shell sand was sampled by dredging at 7-8m depth along the north-eastern side of Hållö island near Smögen, Sotenäs municipality, Västra Götalands county (N 58° 20.32-20.38', E 11° 12.73-12.68').

**Gullmarn Fjord samples**: Soft mud was collected using a Waren dredge at 53 m depth in the Gullmarn Fjord near Lysekil, Lysekil municipality, Västra Götalands county (N 58° 15.73', E 11°26.10').

#### Meiofaunal extraction

**Hållö island**. Hållö island samples were extracted in the lab using two different variations of the flotation (decanting and sieving) technique.

**Flotation (freshwater)**: Freshwater was used to induce an osmotic shock in meiofaunal organisms and force them to detach from heavy sediment particles. 200 mL of sediment were placed in a large volume of fresh water and thoroughly mixed to suspend meiofauna.

| Cowart et al. 2015 | Benthic meiofauna from seagrass meadows | 2mm sieve, 1mm sieve, 0.5mm sieve | Roche 454 GS FLX | COI 18S | USEARCH 6.1 (QIIME 1.7) | UCLUST de novo (QIIME 1.7) | GenBank Silva 115 |
|-------------------|--------------------------------------|----------------------------------|-----------------|--------|----------------------|----------------------|-----------------|
| This study        | Meiofauna from coarse shell sand and muddy benthic sediment | Siphoning 125µm, flotation (MgCl2) 125µm, flotation (H2O) 45µm/70µm | Paired-end Illumina MiSeq | COI 18S (V1-V2 regions) | UCHIME (part of USEARCH 6.1) (QIIME 1.9.1) | CROP COI: 92-94% 18S: 95-97% | BOLD, SweBol and own databases for Nemertea, Acoela, Oligochaeta, Genbank Silva 111 |

**Table:** Adult meiofauna were extracted from two different seagrass meadow environments, using two different sieve combinations (2mm, 1mm, and 0.5mm). The extraction methodology included flotation using freshwater and saline media (MgCl2) and both sieving (H2O) and decanting techniques. The samples were then analyzed using Illumina MiSeq for COI and 18S rDNA. Bioinformatic tools such as USEARCH and UCLUST de novo were used for OTU picking, while GenBank Silva 115 provided a comprehensive database for taxonomic assignment.
and lighter sediment particles. The supernatant was sieved through a 1000 µm sieve to separate the macrofaunal fraction, which was then discarded. The filtered sample was sieved again through a 45 µm sieve to collect meiofauna and discard fine organic particles. This procedure was repeated three times. Meiofauna was then rinsed with seawater from the sieve into large falcon tubes. Twelve sediment samples were processed, ten of them were fixed immediately in 96% ethanol for molecular analysis and stored at -20°C. The other two samples were first screened for live representatives of Xenacoelomorpha, and later preserved in 4% formaldehyde for morphology-based identification of nematodes.

**Flotation (MgCl2 solution):** A 7.2% solution of MgCl2 was used to anesthetize meiofauna. As above, twelve samples were processed in total, ten of them were decanted through 125 µm sieve and fixed immediately in 96% ethanol for molecular analysis and stored at -20°C, while two samples were decanted through a 125 µm sieve which was subsequently placed in a petri dish with seawater. After 30 minutes, the petri dish as well as the inside of the sieve were searched for Xenacoelomorpha using a stereo microscope. Afterwards they were preserved in 4% formaldehyde for morphology-based identification of nematodes.

**Gullmarn Fjord.** Meiofauna was extracted from the Gullmarn Fjord samples using two different methods: flotation and siphoning.

**Flotation (freshwater):** Freshwater was used to induce an osmotic shock in meiofaunal organisms. 2.4 L of sediment were placed in a large volume of freshwater, thoroughly mixed to suspend meiofauna and lighter sediment particles. The supernatant was sieved through a 1000 µm sieve in order to separate macrofauna, which was then discarded. The filtered sample was then sieved three times through a 70µm sieve to collect meiofauna and discard fine organic particles. Meiofauna was then rinsed with seawater from the sieve into a large container and equally divided between 12 falcon tubes. Six samples were fixed in 96% ethanol for molecular analysis and stored at -20°C. Six samples were screened for live representatives of Xenacoelomorpha, and preserved in 4% formaldehyde for morphology-based identification of nematodes.

**Siphoning:** A total volume of 12 L of sediment was processed as follows: an approximately 5 cm thick layer of mud was placed in a container and covered with 20 cm of seawater. The sediment was allowed to settle for 20 hours. Half of the sediment area was then siphoned through a 125 µm sieve, the residue in the sieve was immediately fixed in 96% ethanol, large macrofauna was manually removed, and the entire volume was split equally into six samples and placed at -20°C for subsequent molecular analysis. The remaining half of the area was similarly siphoned through a 125 µm sieve, the sieve contents were stored in sea water, large macrofauna manually removed, the entire volume split into six samples, which were screened for live representatives of Xenacoelomorpha, and preserved in 4% formaldehyde for morphology-based identification of nematodes.

**Morphology-based identification**

*Xenacoelomorpha.* Four samples from Hållö and 12 samples from Gullmarn Fjord were used for morphology-based assessment of the diversity of Xenacoelomorpha. All samples
were stored in seawater and searched for Xenacoelomorpha with a stereo microscope. All specimens found were immediately identified to the lowest taxonomic rank possible using a compound microscope equipped with DIC.

**Nematoda.** Two samples from each location/extraction method were used to assess nematode diversity using morphology-based identification. Samples from Hållö (flotation with fresh water and MgCl2) and Gullmarn Fjord (siphoning) were processed whole and samples from Gullmarn Fjord extracted using flotation with fresh water were subsampled by taking 1/10 of the entire sample. Formaldehyde–preserved samples were transferred to glycerin using Seinhorst’s rapid method as modified by De Grisse (1969). Permanent nematode mounts on glass slides were prepared using the paraffin wax ring method. It is common practice to estimate the diversity of marine nematodes by counting a predetermined number (usually 100 or 200) of randomly picked nematodes per sample (Vincx 1996), which may not provide sufficiently detailed results for samples with high diversity. Therefore, all nematode specimens were counted and identified for each analyzed sample. All nematode specimens were identified to genus, and, when possible, to species level.

**DNA extraction, library preparation and sequencing**

**DNA extraction.** 30 samples were processed for total DNA extraction, twelve from the Gullmarn Fjord and eighteen from Hållö island, using 10g of sediment and the PowerMax® Soil DNA Isolation Kit (MO BIO Laboratories), according to manufacturer’s instructions.

**Primer design.** Illumina MiSeq reagent v3. produces paired-end reads of 300bp in length, allowing a maximum marker length of 500bp when taking into account a 50 bp overlap. Universal COI primers available for the Metazoa amplify a 658bp region (Folmer et al. 1994), which is too long for most NGS applications. Accordingly, primers amplifying a 313 bp fragment of the mitochondrial cytochrome oxidase 1 (COI) gene were used, as described in Bourlat et al. 2016. The primers used for COI are modified from Leray et al.’s ‘mini-barcode’ COI primers (mlCOlintF-dgHCO2198; Leray et al. 2013) by adding the Illumina MiSeq overhang adapter sequences. The Leray et al. ‘mini-barcode’ primers have been shown to amplify up to 91% of metazoan diversity in a sample (Leray et al. 2013). In combination with Leray et al.’s mini barcode forward primer (mlCOlintF), we used Folmer et al.’s COI reverse primer (dgHCO2198; Folmer et al. 1994) as well as a reverse primer developed by Lobo et al., shown to enhance amplification of the COI region in a wide range of invertebrates (Lobo et al. 2013).

For the 18S region, Illumina overhang adapter sequences were appended to the primers from Fonseca et al. (SSU_FO4-SSU_R22; Fonseca et al. 2010), yielding a 364 bp fragment. These primers target a homologous region of the gene and flank a region that is highly divergent, corresponding to the V1-V2 region of the 18S gene (Lindeque et al. 2013, Fonseca et al. 2010).
Sequence overlap in the paired-end reads was calculated in Geneious Kearse et al. 2012. COI shows a sequence overlap of 230 bp and 18S shows an overlap of 190 bp.

All primer sequences used are shown in Table 2.

| Marker | Primer name | Illumina adapter overhang (regular font), with primer sequence (in bold) |
|--------|-------------|------------------------------------------------------------------------|
| COI    | Leray       | 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC-3' |
|        | dgHCO2198   | 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAAACTTCAGGGGTGACCAAAARAYCA-3' |
| COI    | Lobo        | 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC-3' |
|        | LoboR1      | 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTAAACTTCAGGGGTGACCCRAAARAYCA-3' |
| 18S    | SSU_FO4     | 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTTGCTCTCAAAGATTAAGCC-3' |
|        | SSU_R22     | 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTCCTGCTGGCTCTTCTTT GGA-3' |

**Illumina MiSeq library preparation using fusion primers.** For Illumina MiSeq library preparation, we used a dual PCR amplification method as described in Bourlat et al. (2016). The first PCR, the amplicon PCR, uses amplicon specific primers including the Illumina adapter overhang, as described above. The second PCR, the index PCR, allows the incorporation of Illumina index adapters using a limited number of cycles (Bourlat et al. 2016).

**Amplicon PCR.** PCR amplifications of the COI and 18S regions were set up as follows. For a 50µl reaction volume, we used 5µl Pfu polymerase buffer (10x), 1µl dNTP mix (final concentration of each dNTP 200µM), 0.5 µl of each primer at 50 pm/µl, 2 µl DNA template (~10 ng), 0.5µl Pfu DNA polymerase (Promega) and 40.5µl of nuclease free water. Each DNA sample was amplified with the 3 primer pairs described above (COI Leray, COI Lobo and 18S). PCR cycling conditions were 2 min at 95°C (1 cycle); 1 min at 95°C, 45 s at 57°C, 2 min at 72°C (35 cycles); 10 min at 72°C (1 cycle). The PCR was checked on a 2% agarose gel. 20µl of each PCR reaction were then purified with Agencourt® AMPure® XP paramagnetic beads (Beckman Coulter), allowing size selection of PCR fragments by using different PCR product to bead ratios (Bourlat et al. 2016).

**Index PCR.** For dual indexing we used the Nextera XT index kit (96 indices, 384 samples, Illumina) according manufacturers’ instructions. Dual indexing allows an increase in the multiplex level of sequencing per lane, so that more samples can be sequenced on the same flow cell (Fadrosh et al. 2014). It also eliminates cross-contamination between samples and the occurrence of mixed clusters on the flow cell (Kircher et al. 2012). The index PCR was set up as 50µl reactions using 5µl of cleaned up PCR amplicons, 5µl of
Nextera XT Index Primer i5, 5µl of Nextera XT Index Primer i7, 25µl of 2x KAPA HiFi HotStart ready mix (Kapa Biosystems) and 10µl of nuclease free water. PCR cycling conditions were: 3 min at 95°C (1 cycle); 30 s at 95°C, 30 s at 55°C, 30 s at 72°C (8 cycles); 5 min at 72°C (1 cycle). A bead purification was carried out after the index PCR with Agencourt® AMPure® XP magnetic beads (Beckman Coulter) using a ratio of 0.8, allowing the selection of fragments larger than 200 bp. DNA was quantified before sequencing using a Qubit Fluoremeter (Invitrogen) and average fragment size was verified using Tapestation (Agilent Technologies). Further library normalization and pooling steps are described in Bourlat et al. (2016).

**Sequencing.** The pooled libraries were sequenced three times independently using Illumina MiSeq Reagent Kit v3, producing in total 24 132 875 paired-end reads of 300 bp in length, of which 15 883 274 COI reads and 8 249 601 18S reads (Table 3).

| Marker / Sequencing run | 1      | 2      | 3      | Total   |
|-------------------------|--------|--------|--------|---------|
| COI                     | 5 859 454 | 5 075 735 | 4 948 085 | 15 883 274 |
| 18S                     | 2 803 391 | 3 135 331 | 2 310 879 | 8 249 601  |
| **Total**               | 8 662 845 | 8 211 066 | 7 258 964 | 24 132 875 |

**Bioinformatic data processing and analysis**

Most analytical steps were performed using Qiime (Quantitative Insight Into Microbial Ecology) version 1.9.1 (Caporaso et al. 2010) and custom python scripts (Fig. 1).
Paired-end joining

Demultiplexed MiSeq paired-end reads were joined using the Qiime script `multiple_join_paired_ends.py` using the fastq-join tool (https://code.google.com/p/ea-utils/wiki/FastqJoin). Data from three sequencing runs were merged producing a total of 24,132,875 raw paired-end reads, 15,883,274 reads for the COI dataset and 8,249,601 reads for the 18S dataset (Table 3). The number of reads remaining after various bioinformatic data processing steps is presented in Table 4. After paired-end joining, 48% of sequences were lost leading to a total of 12,543,198 reads, due to an observed decrease in sequence quality at the end of the reads, resulting in a bad overlap between the paired-ends. This loss is much more important for the longer 18S region (2,131,102 reads after joining, corresponding to a 74% loss) than for the COI region (10,412,096 reads after joining, corresponding to a 34.5% loss).

| Marker / Step | Raw data | Paired-end joining | Primer trimming | Quality filtering | Chimera removal |
|---------------|----------|--------------------|-----------------|------------------|----------------|
| COI           | 15,883,274 | 10,412,096         | 8,099,507       | 7,976,649        | 7,954,017      |
| 18S           | 8,249,601  | 2,131,102          | 1,071,871       | 1,015,874        | 890,370        |
| Total         | 24,132,875 | 12,543,198         | 9,171,378       | 8,992,523        | 8,844,387      |

Primer trimming and quality filtering

Dual indexes and Illumina overhangs were removed by the sequencing platform. COI and 18S primer sequences were removed using a custom python script designed for this study (https://github.com/Quiterie90/Primer_Removal). The script retains and trims reads that have the exact sequence of the forward and reverse primers at the beginning and at the end of the reads respectively, while other reads not meeting these criteria are discarded. The script takes into account the presence of ambiguous bases in the primer sequence (such as W, R, S, Y, M, K, H, D, B and V). In the case that an unassigned base (N) is found in the primer sequence, the read is also discarded. The primer-trimming step resulted in 9,171,378 reads remaining corresponding to a 27% loss. As the script is quite stringent, it quality filters reads by removing incomplete reads or chimeras. At this step 1,071,871 reads remained after trimming for the 18S dataset corresponding to a 50% loss and 8,099,507 reads remained after trimming for the COI dataset corresponding to a 22% loss. A quality filtering step was then carried out using the Qiime script `multiple_split_libraries_fastq.py` to remove reads with a Q Score inferior to 30 (corresponding to a base call accuracy of at least 99.9%). A total of 2% of sequences were lost after the quality-filtering step leading to 8,992,523 reads remaining. 5% of the reads were lost in the 18S dataset corresponding to a final 1,015,874 reads and 1.5% of the reads were lost in the COI dataset corresponding to a final 7,976,649 reads.
Chimera removal and OTU clustering

Chimeric reads were removed with UCHIME (Edgar et al. 2011) using the Qiime scripts `identify_chimeric_seqs.py` followed by `filter_fasta.py` based on the Usearch61 software. After chimera removal, 7,954,017 sequences remained in the COI dataset (0.3% loss) and 890,370 sequences remained in the 18S dataset (12% loss).

For clustering sequences into Operational Taxonomic Units (OTUs) we used CROP, a Bayesian clustering algorithm that delineates OTUs based on the natural distribution of the data, using a Gaussian mixture model (Hao et al. 2011). The program allows the user to define a lower and upper bound variance to cluster the sequences, instead of a fixed sequence similarity value. According to a benchmarking study by Leray et al. based on the Moorea Biocode barcode library (http://mooreabiocode.org/; Leray et al. 2013), the best lower and upper bound values to cluster metazoan COI sequences are 3 and 4, corresponding to sequence dissimilarities between 6% and 8%. According to an 18S benchmarking experiment with a set of 41 known nematode species carried out by Porazinska et al., a 96% threshold most accurately reflects taxonomic richness, yielding 37 OCTUs, whereas a 97% threshold yielded 51 OCTUs (Porazinska et al. 2009). According to this benchmark, a range of sequence dissimilarities between 3% and 5% were used in CROP (1.5 and 2.5 respectively for the lower and upper values, corresponding to 95-97% similarity).

Parameters used in CROP for the analysis were as follows:

```
CROP -i   -b 160 000 -z 470 -l 3 -u 4 -o
CROP -i   -b 18 000 -z 470 -l 1.5-u 2.5 -o
```

The 7,954,017 COI sequences and the 890,370 18S sequences were clustered into 2805 and 1472 representative OTUs respectively, 213 of which were identified to species for COI and 243 of which were identified to species for 18S, using a 97% sequence similarity threshold (Table 5 Fig. 2).

| Phylum           | COI | 18S |
|------------------|-----|-----|
|                  | OTUs| Percentage | OTUs | Percentage |
| Annelida         | 57  | 30.00 | 29   | 23.97      |
| Arthropoda       | 52  | 27.37 | 14   | 11.57      |
| Bryozoa          | 5   | 2.63  | 3    | 2.48       |
| Cephalorhyncha   | 0   | 0.00  | 1    | 0.83       |
| Chaeetognatha    | 1   | 0.53  | 0    | 0.00       |
| Chordata         | 12  | 6.32  | 7    | 5.79       |

Table 5.
Number of OTUs and percentage per phylum for COI and 18S for the metazoan fraction. Based on a 97% similarity threshold.
## Taxonomic Composition Overview at Species Level

| Taxonomic Group | OTUs | Percentages | Counts | Percentages |
|-----------------|------|-------------|--------|-------------|
| Cnidaria        | 8    | 4.21        | 4      | 3.31        |
| Echinodermata   | 13   | 6.84        | 5      | 4.13        |
| Gastrotricha    | 1    | 0.53        | 9      | 7.44        |
| Gnathostomulida | 1    | 0.53        | 0      | 0.00        |
| Mollusca        | 26   | 13.68       | 6      | 4.96        |
| Nematoda        | 0    | 0.00        | 10     | 8.26        |
| Nemertea        | 3    | 1.58        | 6      | 4.96        |
| Platyhelminthes | 0    | 0.00        | 13     | 10.74       |
| Phoronida       | 1    | 0.53        | 0      | 0.00        |
| Porifera        | 2    | 1.05        | 3      | 2.48        |
| Priapulida      | 1    | 0.53        | 0      | 0.00        |
| Rotifera        | 2    | 1.05        | 0      | 0.00        |
| Sipuncula       | 1    | 0.53        | 1      | 0.83        |
| Tardigrada      | 0    | 0.00        | 1      | 0.83        |
| Xenacoelomorpha | 4    | 2.11        | 9      | 7.44        |
| **Total OTUs Metazoa** | **190** | **100** | **121** | **100** |

**Figure 2.**

Taxonomic composition overview at species level based on a 97% sequence similarity threshold. A) Percentages and counts of OTUs for the COI gene with unassigned OTUs. B) Percentages and counts of OTUs for the COI gene without unassigned OTUs. C) Percentages and counts of OTUs for the 18S gene with unassigned OTUs. D) Percentages and counts of OTUs for the 18S gene without unassigned OTUs.
Taxonomic assignment

As Qiime is normally used for metagenomic analyses of prokaryotes, default databases are not suited for taxonomic assignment of Metazoa. Custom databases consisting in a taxonomy file associated with a reference sequence file can be created, or alternatively, a preformatted database such as the Silva database (http://www.arb-silva.de/no_cache/download/archive/qiime/) can be used. For the COI region, a custom database of 1,947,954 sequences was created consisting of the BOLD database (http://www.boldsystems.org/downloaded on October 8 2015), combined with own reference databases of Nemertea, Xenacoelomorpha and Oligochaeta and barcodes of Swedish Echinodermata, Mollusca, Cnidaria and Arthropoda from the Swedish Barcode of Life database (SweBol). For the 18S rRNA region, a custom database of 732,419 reference sequences was created using the Silva database release 111 (http://www.arb-silva.de/no_cache/download/archive/qiime/) and own barcodes for Acoela and Oligochaeta. Corresponding tab-delimited taxonomy files were created including a sequence ID and taxonomic lineage information (Phylum, Class, Order, Family, Genus and Species) derived from BOLD, Swebo, Silva and WoRMS (http://www.marinespecies.org/).

Taxonomic assignments were carried out using both 80% and 97% sequence similarity thresholds, to obtain identifications at phylum and species levels respectively (Giongo et al.
yielding 690 metazoan OTUs for COI and 793 metazoan OTUs for 18S at 80% threshold and 190 metazoan OTUs for COI and 121 metazoan OTUs for 18S at 97% threshold. For COI, taxonomic assignment was done with the Qiime script `assign_taxonomy.py` using the Uclust software (Edgar 2010). With Uclust, a query sequence matches a database sequence if the identity is high enough. The identity is calculated from a global alignment, which differs from BLAST and most other database search programs, which search for local matches. By default, Uclust stops searching when it finds a match, but also stops searching if it fails to find a match after eight failed attempts. Within Qiime, Uclust is the default algorithm for the `assign_taxonomy.py` script and two parameters are associated to the algorithm. The minimum fraction of database hits that must have a specific taxonomic assignment to assign that taxonomy to a query that was fixed at 0.51 and the number of database hits to consider when making an assignment that was fixed at 3, corresponding to the default values. To obtain matches for non-Metazoan taxa, a Megablast search with 70% minimum coverage was done against the Genbank nt (nucleotide) database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/ downloaded on June 27 2015) using Geneious (Kearse et al. 2012). For taxonomic assignment of the 18S dataset, the Qiime script `assign_taxonomy.py` was used with Uclust (Edgar 2010) default settings against the Silva database. Some taxonomic errors were detected for Nematodes in the Silva database.

Note on the taxonomic assignement of Nematodes: The output from the Qiime analysis included 145 18S OTUs assigned to the phylum Nematoda. Three of them (HE1.SSU866120, HE6.SSU382930 and HF6.SSU331569) Suppl. material 1 were incorrectly placed among the nematodes due to errors in the reference database they derived from – they group among Arthropod taxa by the Megablast search and were excluded for that reason. Another OTU (TS6.SSU559982) is placed among Phoronida by the Megablast search and was also excluded. Two more sequences that were assigned to Nematoda appear to have long insertions within conserved regions (HE6.SSU358113 and TF5.SSU411806). Both of them were found only in one sample each, further supporting the idea that they are derived from erroneous amplification product, and were removed from any further analysis.

Invasive Alien Species (IAS) were detected in our samples by comparing our species list (Suppl. material 1) to the Helcom-Ospar list (http://www.helcom.fi/about-us/partners/ospar) and the Swedish Främmande Arter invasive species lists (http://www.frammandearter.se/).

Taxonomic composition bar plots (Fig. 4) were created using OTU tables (Suppl. materials 2, 3) and the Qiime scripts `make_otu_table.py`, `split_otu_table_by_taxonomy`, `merge_otu_table.py` and `summarize_taxa_through_plots.py`. The bar plots created for Fig. 4 take into account the relative abundance or number of reads for each OTU, whereas Table 5 and Fig. 3 do not take relative abundances of each OTU into account. Fig. 3 showing community composition per phylum and marker was created using PhyloT (http://phylot.biobyte.de/) and Evolview tools (http://www.evolgenius.info/evolview.html; (Zhang et al. 2012).
Diversity analyses

Alpha and beta diversity analyses were carried out with and without unassigned OTUs for both COI and 18S datasets. Unassigned OTUs were removed using the Qiime script `filter_otus_from_otu_table.py`. Alpha diversity (species richness) was calculated using the nonparametric Chao1 index using rarefied datasets to correct bias in species number due to unequal sample size. One of the samples in the COI dataset was removed prior to rarefaction analysis due to low sequence number (1122 sequences including unassigned OTUs and 280 sequences excluding unassigned OTUs at 97% sequence similarity) using the Qiime script `filter_sample_from_otu_table.py`. Rarefaction, alpha diversity calculation and generation of plots were performed using the Qiime scripts i) `multiple_rarefactions.py`, ii) `alpha_diversity.py`, iii) `collate_alpha.py` and iv) `make_rarefaction_plots.py`. Rarefaction was done to a depth corresponding to the total number of sequences in the smallest dataset (20405 sequences including unassigned OTUs and 5442 sequences excluding unassigned OTUs at 97% sequence similarity for COI, and 7561 sequences including unassigned OTUs and 5399 sequences excluding unassigned OTUs at 97% sequence similarity for 18S). Alpha diversities were compared between locations and extraction methods.
methods for both datasets and COI primer sets using the Qiime script `compare_alpha_diversity.py`. The script performs Monte-Carlo permutations to determine p-values.

Beta diversity was calculated using the abundance-based Bray-Curtis index for both COI and 18S datasets. The Qiime script `beta_diversity_through_plots.py` was used to compute beta diversity distance matrices from the rarefied samples and generate Principal Coordinate Analysis (PCoA) plots. Beta diversity was compared according to location, extraction method and primer pair both with and without the unassigned OTUs using the Qiime script `compare_categories.py`. The script uses R and the vegan and ape libraries to compute statistical tests. We performed ANOSIM (ANalysis Of SIMilarity) tests, which are nonparametric, through 999 permutations. This method tests whether two or more groups of samples are significantly different by taking as null hypothesis that there is no difference between the two or more groups studied.

Alpha and beta diversities were calculated including and excluding the unassigned OTUs and results obtained were similar. Here we present plots including the unassigned OTUs (Figs 5, 6).

**Figure 5.**

Alpha diversity rarefaction plots for COI and 18S datasets including unassigned OTUs. According to location for COI (A) 18S (B). Hållö Island (HI) in red, Gullmarn Fjord (GF) in blue. According to extraction method for COI (C) 18S (D). HI flotation in red, HI MgCl2 in blue, GF flotation in yellow, GF siphoning in green. According to primer pair for COI (E). CO1 Leray primer in red, COI Lobo primer in blue.
Data resources

The data underpinning the analysis reported in this paper are deposited at the GenBank SRA under project number PRJNA388326 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA388326).

Results and discussion

Phylum-level community composition of meiofaunal samples from the Swedish west coast

Illumina MiSeq produced at total of 24,132,875 raw reads, of which 15,883,274 COI reads and 8,249,601 18S reads. These were quality filtered (see methods section for details) resulting in 7,954,017 COI sequences and 890,370 18S sequences. These were clustered into 2805 and 1472 representative OTUs respectively, yielding 190 metazoan OTUs for COI and 121 metazoan OTUs for 18S at 97% sequence similarity (see methods, Table 5 & Fig. 2).

Taxonomic assignment of OTUs at a 97% similarity threshold shows community composition of the samples at the phylum level (Fig. 2). Of 2805 COI OTUs, 190 (7%) were assigned to the Metazoa, 22 (1%) to plants and algae, 1 (0%) to Fungi. 2592 OTUs remained unassigned, corresponding to 92% of COI OTUs.
For the 18S dataset, 121 of 1472 OTUs (8%) were assigned to Metazoa, 104 (7%) to plants and algae, 10 (1%) to Fungi, and 8 (1%) to Protozoa. 1229 OTUs remained unassigned, corresponding to 83% of all 18S OTUs.

The large numbers of unassigned OTUs reflect the incompleteness of the databases used for COI and 18S. When unassigned OTUs are disregarded, differences between the taxonomic coverage of the markers can be observed (Fig. 2, B and D). COI is the ‘standard’ animal barcode and is thus mostly useful for diversity surveys within the Metazoa (Hebert et al. 2003). 18S has on the other hand much larger taxonomic coverage and can be used for biodiversity profiles of whole eukaryotic communities, at higher taxonomic scales.

Of all OTUs classified as Metazoa, a detailed breakdown per phylum is presented in Table 5 and Fig. 3. Annelida (30% of COI metazoan OTUs and 23.97% of 18S metazoan OTUs) and Arthropoda (27.37% of COI metazoan OTUs and 11.57% of 18S metazoan OTUs), were the most OTU rich phyla identified in all samples combined, a similar pattern as observed in a recent study on coastal seagrass meadows in Brittany, France (Cowart et al. 2015).

As well as Annelida and Arthropoda, other phyla represented by a high number of OTUs in our samples include Mollusca (13.68% of COI metazoan OTUs and 4.96% of 18S metazoan OTUs), Platyhelminthes (10.74% of 18S metazoan OTUs and 0% of COI metazoan OTUs) and Nematoda (8.26% of 18S metazoan OTUs and 0% of COI metazoan OTUs) (Table 5 & Fig. 3). Other benthic metabarcoding studies based on the 18S V1-V2 region, found Nematoda and Platyhelminthes as the most OTU rich phyla represented (Fonseca et al. 2014, Fonseca et al. 2010), or Nematoda and Annelida (Bik et al. 2012b), alternatively Nematoda and Arthropoda (Bik et al. 2012a, Lallias et al. 2015).

**Meiofaunal community composition differs according to location**

Taxonomic community composition at both locations surveyed is illustrated in Fig. 4. The bar plots in Fig. 4 take into account the read counts for each OTU, whereas Table 5 and Fig. 3 do not take these into account.

In Fig. 4, clear differentiation in biodiversity between the two habitat types (soft mud versus coarse shell sand) can be observed, as expected. Echinodermata (such as Ophiurida, Echinoidea and Asteroidea), Mollusca (Bivalvia, Gastropoda), Annelida and Arthropoda are represented by higher numbers of reads in samples from the muddy sediments in the Gullmarn fjord samples (grain size 100 μm approx.).

In coarse shell sand in shallow areas, such as in the Hållö island samples, Annelida and Arthropoda are represented by higher numbers of reads, followed by Chordata (cephalohordata such as Branchiostoma sp., ascidians and various fish species such as Gobius sp., Ctenolabrus rupestris, Solea solea) with in addition a larger diversity of small taxa such as Bryozoa, Gnathostomulida, Gastrotircha, Tardigrada, Rotifera, Sipuncula and Phoronida, reflecting the high diversity of interstitial taxa found in sandy sediments.
Sample diversity and composition analyses

A greater number of phyla were uncovered in the Hållö Island samples than in the Gullmarn Fjord samples (Fig. 4A and 4B) and this observation was corroborated by the alpha diversity rarefaction plots showing that Hållö Island samples (in red) present a higher diversity than the Gullmarn Fjord samples (in blue) (p-value = 0.001) regardless of the marker used (Fig. 5A and 5B). Within the same location, choice of extraction method does not have a significant impact on sample diversity (p-value ~ 1) (Fig. 5C and 5D, Table 6). However, for the 18S dataset, the flotation method seems to be more effective for extraction of nematodes than the siphoning method in the Gullmarn Fjord samples (Fig. 4A and 4B). Moreover, the beta diversity PCoA results highlight the fact that sample composition is influenced by the choice of extraction method for both COI and 18S datasets (p-value = 0.001) leading to four different clusters (Fig. 6A and 6B, Table 6). For the COI dataset, in addition to extraction method as a factor of divergence, choice of primer (COI Leray or COI Lobo) also influences the grouping of the samples (p-value = 0.003 excluding unassigned OTUs and 0.001 including unassigned OTUs), in particular for the Hållö Island samples (Fig. 6C). Moreover, the COI Lobo primer seems to uncover a higher diversity of taxa than the COI Leray primer (Fig. 5E) even if the results are considered to be non significant (p-value = 0.585 excluding unassigned OTUs and 0.111 including unassigned OTUs) (Table 6Table 7).

| Table 6. |
| Nonparametric t-test results with 999 Monte-Carlo permutations for both datasets with and without unassigned OTUs (97% taxonomic assignment) |

|                  | COI dataset                     | 18S dataset                      |
|------------------|---------------------------------|----------------------------------|
|                  | Excluding unassigned OTUs       | Including Unassigned OTUs        | Excluding unassigned OTUs       | Including Unassigned OTUs        |
|                  | Test value | P-value  | Test value | P-value  | Test value | P-value  | Test value | P-value  |
| **Location**     |            |          |            |          |            |          |            |          |
| HI vs. GF        | -14.453    | 0.001    | -21.455    | 0.001    | -6.929     | 0.001    | -7.170     | 0.001    |
| **Method**       |            |          |            |          |            |          |            |          |
| HI H2O vs. HI MgCl2 | -0.437    | 1.0      | -0.691     | 1.0      | -0.906     | 1.0      | -0.174     | 1.0      |
| GF flotation vs. GF siphoning | 1.567     | 0.792    | 1.546      | 0.99     | -1.427     | 1.0      | -0.744     | 1.0      |
| **Primer**       |            |          |            |          |            |          |            |          |
| COI Leray vs. COI Lobo | -0.508    | 0.596    | -1.614     | 0.111    | -         | -        | -         | -        |
Molecular identifications to species level

Using a sequence similarity search at 97% similarity allowed us to identify 213 COI OTUs and 243 18S OTUs to species level (Table 8 and Suppl. material 1). For the COI dataset, 81 species (of which 70 metazoans) were found in both locations, 36 (of which 35 metazoans) were found in the Gullmarn fjord only and 96 (of which 85 metazoans) were found in Hållö island only. For the 18S dataset, 108 species (of which 48 metazoans) were found in both locations, 44 (of which 21 metazoans) were found in the Gullmarn fjord only and 91 (of which 52 metazoans) were found in Hållö Island only (Suppl. material 1). These species observations from metabarcoding represent ‘molecular occurrence records’ that could be used in monitoring and other types of biodiversity surveys, in the same way as physical observations, such as for mapping species distributions (Bohmann et al. 2014, Lawson Handley 2015).

Table 7.
ANOSIM test results (999 permutations) for both COI and 18S datasets with and without unassigned OTUs (97% taxonomic assignment)

|                      | COI dataset Excluding unassigned OTUs | COI dataset Including unassigned OTUs | 18S dataset Excluding unassigned OTUs | 18S dataset Including unassigned OTUs |
|----------------------|--------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
|                      | R-value | P-value | R-value | P-value | R-value | P-value | R-value | P-value |
| Location             | 0.976   | 0.001   | 1.0     | 0.001   | 0.935   | 0.001   | 0.929   | 0.001   |
| Method               | 0.660   | 0.001   | 0.738   | 0.001   | 0.889   | 0.001   | 0.895   | 0.001   |
| Primer               | 0.200   | 0.003   | 0.218   | 0.001   | -       | -       | -       | -       |

Table 8.
Metazoa identified to species level using 97% sequence similarity (HI: Hållö island, GF: Gullmarn Fjord)

| COI  | OTU ID           | Nb of reads | Phylum    | Class    | Order    | Species                        | HI | GF |
|------|------------------|-------------|-----------|----------|----------|--------------------------------|----|----|
|      | HE6.Lobo_7972794| 3           | Annelida  | Clitellata| Haplotaxida| Adelodrilus pusillus          | +  | -  |
|      | HE1.Lobo_933012 | 14954       | Annelida  | Clitellata| Haplotaxida| Grania postclitellochaeta      | +  | +  |
|      | HF8.Lobo_5239705| 241         | Annelida  | Clitellata| Haplotaxida| Grania variochaeta            | +  | +  |
|      | HF4.Lobo_97092  | 29391       | Annelida  | Clitellata| Haplotaxida| Tubificoides benedii           | +  | +  |
| Code          | Taxonomy                  | Genus       | Species                | Family | Order      | Genus       | Species                | Family | Order      | Genus       | Species                | Family | Order      | Genus       | Species                |
|--------------|---------------------------|-------------|------------------------|--------|------------|-------------|------------------------|--------|------------|-------------|------------------------|--------|------------|-------------|------------------------|
| HF5.Lobo_3297996 | Annelida Clitellata      | Haplotaxida | *Tubificoides kozloffii* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TS1.Leray_545620 | Annelida Polychaeta      | Amphinomida | *Paramphinome jeffreysi* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HF1.Lobo_4996219 | Annelida Polychaeta      | Canali palpata | *Polygordius appendiculatus* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TF6.Lobo_5247622 | Annelida Polychaeta      | Capitellida |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TF1.Lobo_4669404 | Annelida Polychaeta      | Capitellida |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TF5.Lobo_6394093 | Annelida Polychaeta      | Capitellida |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TS3.Leray_6813257 | Annelida Polychaeta      | Eunicida    |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HF5.Leray_4035802 | Annelida Polychaeta      | Eunicida    | *Ophryotrocha maculata* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TS2.Leray_4445240 | Annelida Polychaeta      | Eunicida    | *Parougie eliasoni* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TF3.Leray_6645504 | Annelida Polychaeta      | Ophelliida  |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TS5.Lobo_6031643 | Annelida Polychaeta      | Ophelliida  |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HF9.Lobo_7587930 | Annelida Polychaeta      | Ophelliida  |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HE8.Leray_7284535 | Annelida Polychaeta      | Phyllodocida |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TS5.Leray_1557252 | Annelida Polychaeta      | Phyllodocida |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TS3.Leray_6744085 | Annelida Polychaeta      | Phyllodocida |                      |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TS3.Leray_6805306 | Annelida Polychaeta      | Phyllodocida | *Aphrodita aculeata* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TS3.Lobo_1308935 | Annelida Polychaeta      | Phyllodocida | *Eumida ockelmanni* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HE6.Leray_2958692 | Annelida Polychaeta      | Phyllodocida | *Glycera alba* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HF7.Leray_1672792 | Annelida Polychaeta      | Phyllodocida | *Glycinde nordmanni* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| TF5.Leray_2872180 | Annelida Polychaeta      | Phyllodocida | *Gyptis mackiei* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HF1.Lobo_5059232 | Annelida Polychaeta      | Phyllodocida | *Gyptis propinqua* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HF9.Lobo_7695035 | Annelida Polychaeta      | Phyllodocida | *Lepidonotus squamatus* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HE6.Lobo_7972042 | Annelida Polychaeta      | Phyllodocida | *Myrianida edwarsi* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HF9.Lobo_7688887 | Annelida Polychaeta      | Phyllodocida | *Nereimyra punctata* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| HF2.Lobo_2136301 | Annelida Polychaeta      | Phyllodocida | *Pisione remota* |        |            |              |                        |        |            |              |                        |        |            |              |                        |
| Sample Code       | Count | Phylum       | Class       | Order       | Family       | Species          | + | - |
|-------------------|-------|--------------|-------------|-------------|--------------|------------------|---|---|
| HE3.Leray_364663  | 59407 | Annelida     | Polychaeta  | Phyllodocida|              | Platynereis dumerilli |  |   |
| TS4.Leray_7471107 | 1     | Annelida     | Polychaeta  | Phyllodocida|              | Sige fusigera     | - | + |
| HE5.Lobo_493462   | 571790| Annelida     | Polychaeta  |             |              |                  |  |   |
| TS2.Lobo_6962270  | 4595  | Annelida     | Polychaeta  | Sabellida   | Galathowenia  | oculata           |  |   |
| TS2.Leray_4491798 | 316559| Annelida     | Polychaeta  | Spionida    |              |                  |  |   |
| TS4.Lobo_1502925  | 195999| Annelida     | Polychaeta  | Spionida    |              |                  |  |   |
| HF9.Lobo_7588557  | 891   | Annelida     | Polychaeta  | Spionida    |              |                  |  | - |
| TS6.Leray_5665274 | 936   | Annelida     | Polychaeta  | Spionida    |              |                  | - |  |
| TF1.Lobo_2668551  | 874   | Annelida     | Polychaeta  | Spionida    |              |                  | - |  |
| HE4.Leray_3067470 | 3     | Annelida     | Polychaeta  | Spionida    | Chaetopterus  | sarsi            |  | - |
| HF1.Lobo_4965916  | 1     | Annelida     | Polychaeta  | Spionida    | Malacoceros  | fuliginosus       |  | - |
| HF9.Leray_4404528 | 1     | Annelida     | Polychaeta  | Spionida    | Polydrada    | cornuta           |  | + |
| HF5.Lobo_3178882  | 2824  | Annelida     | Polychaeta  | Spionida    | Spiophanes    | bombyx            |  | + |
| TF1.Leray_2314881 | 29235 | Annelida     | Polychaeta  | Terebellida |              |                  |  |   |
| TF1.Lobo_2832834  | 9348  | Annelida     | Polychaeta  | Terebellida |              |                  |  |   |
| TS1.Leray_614419  | 788   | Annelida     | Polychaeta  | Terebellida |              |                  |  |   |
| HE8.Lobo_858951   | 1     | Annelida     | Polychaeta  | Terebellida |              |                  |  | - |
| TS2.Lobo_6889557  | 184   | Annelida     | Polychaeta  | Terebellida |              |                  |  | + |
| TS6.Lobo_255019   | 3     | Annelida     | Polychaeta  | Terebellida |              |                  | - |  |
| TS2.Lobo_6860909  | 1     | Annelida     | Polychaeta  | Terebellida |              |                  |  | + |
| TS5.Leray_1638640 | 1     | Annelida     | Polychaeta  | Terebellida |              |                  |  | + |
| TF1.Lobo_2848745  | 1305  | Annelida     | Polychaeta  | Terebellida | Amphictene    | auricoma          |  |   |
| TS3.Leray_6729893 | 1     | Annelida     | Polychaeta  | Terebellida | Brada villosa |                  | - |  |
| HF4.Lobo_96799    | 102   | Annelida     | Polychaeta  | Terebellida | Cirratulus cirratus |               | + |  |
| HF2.Lobo_2052205  | 285   | Annelida     | Polychaeta  | Terebellida | Dodecaceria concharum |            | + |  |
| TS5.Leray_1638834 | 102   | Annelida     | Polychaeta  | Terebellida | Lagis koreni  |                  |  | + |
|     |     |     |     |     |
|-----|-----|-----|-----|-----|
| HE9.Lobo_2191024 | 8   | Annelida | Polychaeta | Terebellida | Macrochaeta clavicornis | +  |
| TF1.Leray_2475372 | 6353 | Annelida | Polychaeta | Terebellida | Sosane wahrbergi | +  |
| HE1.Lobo_982378  | 38  | Arthropoda | Branchiopoda | Diplostraca | Evadne nordmanni | -  |
| TF5.Lobo_6391642  | 10097 | Arthropoda | Branchiopoda | Diplostraca | Penilia avirostris | +  |
| HF9.Lobo_7623741  | 1   | Arthropoda | Branchiopoda | Diplostraca | Pleopsis polyphemoides | +  |
| TS4.Leray_7402581  | 10  | Arthropoda | Insecta | Diptera | Chironomus aprilinus | +  |
| TS3.Lobo_1162454  | 2   | Arthropoda | Insecta | Diptera | Procladius sp. | +  |
| HF4.Lobo_5006     | 1   | Arthropoda | Insecta | Diptera | Cryptochironomus supplicans | -  |
| TF5.Leray_2910679  | 6   | Arthropoda | Insecta | Diptera | Psectrocladius yunoquartus | +  |
| HF9.Lobo_7599310  | 3   | Arthropoda | Insecta | Diptera | Tanytarsus usmaensis | +  |
| HE5.Lobo_479906   | 152 | Arthropoda | Insecta | Diptera | Atylus vedlomensis | +  |
| HE2.Lobo_2023271  | 21589 | Arthropoda | Malacostraca | Amphipoda | Ampelisca brevicornis | +  |
| HF1.Leray_2493444  | 3911 | Arthropoda | Malacostraca | Amphipoda | Monocorophium insidiosum | +  |
| HE8.Lobo_860608   | 1   | Arthropoda | Malacostraca | Amphipoda | Corophium volutator | +  |
| HE3.Lobo_4900763  | 1   | Arthropoda | Malacostraca | Amphipoda | Leptocheirus hirsutimanus | +  |
| HF4.Leray_6193380  | 66039 | Arthropoda | Malacostraca | Amphipoda | Athanas nitescens | +  |
| HE8.Leray_7216397  | 1   | Arthropoda | Malacostraca | Amphipoda | Monoculodes packardi | -  |
| HE6.Lobo_7849183  | 1   | Arthropoda | Malacostraca | Amphipoda | Monoculodes packardi | -  |
| HF9.Leray_4291607  | 1372 | Arthropoda | Malacostraca | Decapoda | Athanas nitescens | +  |

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| Code       | Species ID | Taxonomy        | Phylum               | Kingdom           | Class            | Order             | Family   | Genus       | Species     | Annotation     | Status1 | Status2 |
|------------|------------|-----------------|----------------------|-------------------|------------------|-------------------|----------|-------------|-------------|----------------|---------|---------|
| HF8.Leray_5586003 | 2864       | Arthropoda      | Malacostraca        | Decapoda          | Eualus cranchii  | +                  | +        |
| HF8.Leray_5612792 | 37         | Arthropoda      | Malacostraca        | Decapoda          | Eualus cranchii  | +                  | -        |
| HE1.Lobo_952576   | 3739       | Arthropoda      | Malacostraca        | Decapoda          | Liocarcinus      | navigator         | +        |
| TF5.Lobo_6459477  | 1279       | Arthropoda      | Malacostraca        | Decapoda          | Philocheras      | bispinosus        | +        |
| HE4.Lobo_4138563  | 42         | Arthropoda      | Malacostraca        | Decapoda          | Plisidia         | longicornis       | +        |
| HE8.Leray_7306131 | 2          | Arthropoda      | Malacostraca        | Decapoda          | Processa         | modica            | +        |
| TS3.Lobo_1213146  | 17         | Arthropoda      | Malacostraca        | Isopoda           | Asellus          | aquaticus         | +        |
| TF5.Leray_2897128 | 3          | Arthropoda      | Maxillopoda         | Calanoida         | Acartia          | bifilosa          | -        |
| HF3.Leray_7129076 | 22         | Arthropoda      | Maxillopoda         | Calanoida         | Acartia          | clausi            | +        |
| TF6.Leray_5332240 | 7399       | Arthropoda      | Maxillopoda         | Calanoida         | Acartia          | tonsa             | +        |
| HF7.Leray_1683272 | 927        | Arthropoda      | Maxillopoda         | Calanoida         | Acartia          | tonsa             | +        |
| HE2.Lobo_2010882  | 1          | Arthropoda      | Maxillopoda         | Calanoida         | Anomalocera      | patersoni         | +        |
| TS2.Leray_4478240 | 2          | Arthropoda      | Maxillopoda         | Calanoida         | Calanus          | euxinus            | -        |
| HF7.Lobo_5810493  | 41         | Arthropoda      | Maxillopoda         | Calanoida         | Centropages      | hamatus            | +        |
| HF8.Lobo_5106754  | 82         | Arthropoda      | Maxillopoda         | Calanoida         | Centropages      | typicus            | +        |
| HE8.Leray_7251655 | 1          | Arthropoda      | Maxillopoda         | Calanoida         | Eurytemora       | affinis            | +        |
| HE7.Leray_3803390 | 5325       | Arthropoda      | Maxillopoda         | Calanoida         | Paracalanus      | parvus             | +        |
| HF9.Leray_4411242 | 1          | Arthropoda      | Maxillopoda         | Calanoida         | Pseudocalanus    | elongatus          | +        |
| TS4.Leray_7515925 | 2          | Arthropoda      | Maxillopoda         | Calanoida         | Pseudocalanus    | elongatus          | -        |
| TS3.Lobo_1208165  | 1          | Arthropoda      | Maxillopoda         | Calanoida         | Scolecithricella | minor              | -        |
| TF5.Lobo_6373065  | 809        | Arthropoda      | Maxillopoda         | Calanoida         | Temora           | longicornis       | +        |
| TF1.Leray_2453024 | 1          | Arthropoda      | Maxillopoda         | Calanoida         | Temora           | longicornis       | -        |
| HF4.Leray_6242499 | 45         | Arthropoda      | Maxillopoda         | Cyclopoida        | -                  | +        |
| Code    | Taxonomy                                      | Species                        | Reference  |
|---------|----------------------------------------------|--------------------------------|------------|
| HF4.Leray_6206299 | Arthropoda Maxillopoda Harpactoida | + - | 24 Haenel Q et al |
| HE8.Lobo_823478 | Arthropoda Maxillopoda Harpactoida | Harpacticoida sp. + - | 24 Haenel Q et al |
| TS3.Lobo_1208905 | Arthropoda Maxillopoda Harpactoida | Harpacticus flexus + + | 24 Haenel Q et al |
| HE1.Lobo_995710 | Arthropoda Maxillopoda Harpactoida | Tachidius discipes + - | 24 Haenel Q et al |
| HF4.Leray_6092514 | Arthropoda Maxillopoda Poecilostomatoida | + - | 24 Haenel Q et al |
| HF9.Leray_4391714 | Arthropoda Maxillopoda Sessilia | Balanus balanus + + | 24 Haenel Q et al |
| HF4.Leray_6295260 | Arthropoda Maxillopoda Sessilia | Balanus balanus + + | 24 Haenel Q et al |
| HF7.Leray_1785147 | Arthropoda Maxillopoda Sessilia | Verruca stroemia + - | 24 Haenel Q et al |
| HE1.Leray_1117391 | Arthropoda Pycnogonida Pantopoda | Endeis spinosa + - | 24 Haenel Q et al |
| HE9.Lobo_2173983 | Bryozoa Gymnolaemata Cheilostomatida | Escharella immersa + - | 24 Haenel Q et al |
| HF7.Leray_1838377 | Bryozoa Gymnolaemata Cheilostomatida | Membranipora membranacea + - | 24 Haenel Q et al |
| HE3.Lobo_4881810 | Bryozoa Gymnolaemata Cheilostomatida | Scrupocellaria scruposa + - | 24 Haenel Q et al |
| HF6.Lobo_2617384 | Bryozoa Gymnolaemata Ctenostomata | Amathia gracilis + - | 24 Haenel Q et al |
| HF5.Lobo_3158598 | Bryozoa Stenolaemata Cyclostomatida | Crisia eburnea + - | 24 Haenel Q et al |
| HE6.Leray_2983148 | Chaetognatha Sagittoidea Aphyrognomina | + - | 24 Haenel Q et al |
| TS1.Leray_646185 | Chordata Actinopterygii Gasterosteiformes | Gasterosteus aculeatus + + | 24 Haenel Q et al |
| HF4.Lobo_208606 | Chordata Actinopterygii Perciformes | Ammodtes marinus + - | 24 Haenel Q et al |
| HF1.Leray_2487062 | Chordata Actinopterygii Perciformes | Ctenolabrus rupestris + - | 24 Haenel Q et al |
| HF3.Lobo_3538759 | Chordata Actinopterygii Perciformes | Gobius niger + - | 24 Haenel Q et al |
| TF1.Lobo_2807051 | Chordata Actinopterygii Perciformes | Lesueurigobius friesii + + | 24 Haenel Q et al |
| HF9.Lobo_7596943 | Chordata Actinopterygii Perciformes | Mullus surmuletus + - | 24 Haenel Q et al |
| HF5.Lobo_3273051 | Chordata Actinopterygii Perciformes | Trachinus draco + - | 24 Haenel Q et al |
| HE2.Lobo_1914646 | Chordata Actinopterygii Pleuronectiformes | Limanda limanda + - | 24 Haenel Q et al |
| HE8.Lobo_879846 | Chordata Actinopterygii Pleuronectiformes | Solea solea + - | 24 Haenel Q et al |
| HE8.Lobo_756051 | Chordata Actinopterygii Salmoniformes | Salmo trutta + - | 24 Haenel Q et al |
| HF3.Lobo_3595218 | Chordata Ascidiacea Phlebobranchia | Phallusia ingeria + - | 24 Haenel Q et al |
| Reference | Code      | Group    | Subgroup  | Species               | Count | Group Description | Subgroup Description | Species Description |
|-----------|-----------|----------|-----------|-----------------------|-------|-------------------|----------------------|---------------------|
| HE8.Lobo  | 873511    | Chordata | Leptocardii | Branchiostoma lanceolatum | 131011 | +                 | +                    |                     |
| TF3.Leray | 6588680   | Cnidaria | Anthozoa   | Funiculina sp.          | 3869  | +                 | +                    |                     |
| TF6.Lobo  | 5251371   | Cnidaria | Hydrozoa   | Corynmorpha nutans      | 1    | +                 | +                    |                     |
| HE9.Lobo  | 2164485   | Cnidaria | Hydrozoa   | Littorina littorea      | 2    | +                 | -                    |                     |
| TF6.Leray | 5512978   | Cnidaria | Hydrozoa   | Eutima gracilis         | 1481  | +                 | +                    |                     |
| HF5.Lobo  | 3253786   | Cnidaria | Scyphozoa  | Aurelia aurita          | 232   | +                 | +                    |                     |
| HE3.Leray | 361248    | Cnidaria | Scyphozoa  | Cyanea capillata        | 2    | +                 | -                    |                     |
| HE2.Leray | 6553538   | Cnidaria | Staurozoa  | Craterolophus convolvulus | 1    | +                 | -                    |                     |
| HE7.Leray | 3802459   | Echinodermata | Asteroidea | Asterias rubens        | 570   | +                 | -                    |                     |
| HE3.Leray | 388102    | Echinodermata | Asteroidea | Marthasterias glacialis | 85    | +                 | -                    |                     |
| HF4.Leray | 6293728   | Echinodermata | Echinoidea | Echinocystis pusillus   | 71    | +                 | +                    |                     |
| HE8.Leray | 7326980   | Echinodermata | Echinoidea | Psammechinus miliaris   | 315   | +                 | -                    |                     |
| HE6.Lobo  | 7886165   | Echinodermata | Echinoidea | Spatangoidea            | 1     | +                 | -                    |                     |
| TF3.Leray | 6591339   | Echinodermata | Echinoidea | Brissopsis lyrifera     | 2079  | +                 | +                    |                     |
| HF7.Leray | 1843674   | Echinodermata | Echinoidea | Echinocardium cordatum  | 94    | +                 | -                    |                     |
| TS5.Lobo  | 6025603   | Echinodermata | Holothuroidea | Thyone fusus            | 11    | +                 | +                    |                     |
| TS3.Leray | 6733304   | Echinodermata | Ophiuroidea | Ophiurida               | 1027065 | +                 | +                    |                     |
| TS1.Leray | 663710    | Echinodermata | Ophiuroidea | Ophiurida               | 3     | -                 | +                    |                     |
| TF1.Lobo  | 2726978   | Echinodermata | Ophiuroidea | Ophiurida               | 298   | -                 | +                    |                     |
| TF1.Leray | 2426830   | Echinodermata | Ophiuroidea | Ophiurida               | 16603 | +                 | +                    |                     |
| TF5.Leray | 2879711   | Echinodermata | Ophiuroidea | Ophiurida               | 1     | -                 | +                    |                     |
| HF3.Leray | 7012508   | Echinodermata | Ophiuroidea | Ophiurida               | 44    | +                 | +                    |                     |
| HE1.Lobo  | 948618    | Mollusca | Bivalvia   | Mytilus galloprovincialis | 14   | +                 | -                    |                     |
| TS2.Leray | 4506244   | Mollusca | Bivalvia   | Mytilus edulis          | 1     | -                 | +                    |                     |

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| Reference | Species Name       | Class   | Order   | Family      | Genus      | ± ± | + + | - - | + + | + - | + + | ± ± |
|-----------|-------------------|---------|---------|-------------|------------|-----|-----|-----|-----|-----|-----|-----|
| HF3.Leray_7058438 | 371 Mollusca Bivalvia Myoida Corbula gibba |          |         |             |            |     |     |     |     |     |     |     |
| HE1.Lobo_894587 | 22 Mollusca Bivalvia Mytilioida Mytilus edulis |          |         |             |            |     |     |     |     |     | + - |     |
| TS1.Lobo_4571224 | 4 Mollusca Bivalvia Nuculida Nucula nucleus |          |         |             |            |     |     | - + |     |     |     |     |
| TS3.Leray_6727248 | 56213 Mollusca Bivalvia Veneroida Abra nitida |          |         |             |            |     |     | + + |     |     |     |     |
| HE4.Lobo_4121128 | 25 Mollusca Bivalvia Veneroida Dosinia lupinus |          |         |             |            |     |     | + + |     |     |     |     |
| TF5.Leray_2915847 | 1911 Mollusca Bivalvia Veneroida Kurtiella bidentata |          |         |             |            |     |     | + + |     |     |     |     |
| TS6.Leray_5683559 | 2 Mollusca Bivalvia Veneroida Lucinoma borealis |          |         |             |            |     |     | - + |     |     |     |     |
| HF1.Leray_2592679 | 33 Mollusca Bivalvia Veneroida Spisula subtruncata |          |         |             |            |     |     | + - |     |     |     |     |
| HE7.Leray_3779267 | 14392 Mollusca Bivalvia Veneroida Tellimya ferruginosa |          |         |             |            |     |     | + + |     |     |     |     |
| HF5.Lobo_3246886 | 1 Mollusca Cephalopoda Sepiida Sepietta neglecta |          |         |             |            |     |     | + - |     |     |     |     |
| TS1.Lobo_4750257 | 2 Mollusca Gastropoda Cephalaspidea |          |         |             |            |     |     | - + |     |     |     |     |
| TS1.Lobo_4792606 | 2 Mollusca Gastropoda Cephalaspidea |          |         |             |            |     |     | - + |     |     |     |     |
| HF8.Lobo_5143779 | 2 Mollusca Gastropoda Littorinimorpha Euspira nitida |          |         |             |            |     |     | + - |     |     |     |     |
| HE3.Lobo_4838288 | 34 Mollusca Gastropoda Neogastropoda Mangelia attenuata |          |         |             |            |     |     | + + |     |     |     |     |
| HF6.Lobo_2622544 | 37 Mollusca Gastropoda Neogastropoda Nassarius nitidus |          |         |             |            |     |     | + - |     |     |     |     |
| HE2.Lobo_1993552 | 50 Mollusca Gastropoda Nudibranchia |          |         |             |            |     |     | + - |     |     |     |     |
| HE6.Leray_2935130 | 2 Mollusca Gastropoda Nudibranchia |          |         |             |            |     |     | + - |     |     |     |     |
| HF1.Leray_2520121 | 559 Mollusca Gastropoda Nudibranchia Favorinus branchialis |          |         |             |            |     |     | + - |     |     |     |     |
| HE2.Lobo_1978270 | 5 Mollusca Gastropoda Nudibranchia Onchidoris muricata |          |         |             |            |     |     | + - |     |     |     |     |
| HE2.Lobo_1939813 | 155 Mollusca Gastropoda Nudibranchia Polycera quadrilineata |          |         |             |            |     |     | + - |     |     |     |     |
| HE2.Lobo_1938412 | 10 Mollusca Gastropoda Nudibranchia Polycera quadrilineata |          |         |             |            |     |     | + - |     |     |     |     |
| HF5.Leray_3991765 | 847 Mollusca Gastropoda Pulmonata Microhedyle glandulifera |          |         |             |            |     |     | + - |     |     |     |     |
| HF4.Leray_6299554 | 2965 Mollusca Gastropoda Sacoglossa Elysia viridis |          |         |             |            |     |     | + + |     |     |     |     |
| HF5.Lobo_3167773 | 166 Mollusca Gastropoda Sorbeoconcha Onoba semicostata |          |         |             |            |     |     | + - |     |     |     |     |
| OTU ID       | Nb of reads | Phylum     | Class | Order           | Species                     | HI | GF |
|--------------|-------------|------------|-------|-----------------|-----------------------------|----|----|
| TF5.SSU_460284 | 121639      | Annelida   | _     | _               | _                           | +  | +  |
| TS3.SSU_470635 | 59          | Annelida   | _     | _               | _                           | -  | +  |
| HF9.SSU_7624   | 12          | Annelida   | Clitellata | Enchytraeida | Grania sp.                 | +  | -  |
| TF5.SSU_453927 | 2687        | Annelida   | Clitellata | Haplotaxida   | Tubificoides insularis     | +  | +  |
| HF3.SSU_985477 | 1090        | Annelida   | Polychaeta | _             | Aricia sp.                  | +  | +  |
| HF6.SSU_322303 | 10          | Annelida   | Polychaeta | _             | Protodriloides chaetifer   | +  | -  |
| Code     | Species Count | Class          | Subclass     | Order            | Family            | Genus       | Species       | Remarks          |
|----------|---------------|----------------|--------------|------------------|-------------------|-------------|---------------|------------------|
| HF4.SSU_622170 | 1             | Annelida       | Polychaeta   | _                | Scalibregma       | inflatum    | + -           |                  |
| HF9.SSU_25735  | 3753          | Annelida       | Polychaeta   | _                | Trilobodrilus     | heideri     | + -           |                  |
| TS3.SSU_480632 | 189           | Annelida       | Polychaeta   | Phyllodocida     | Aphrodita         | sp.         | - +           |                  |
| HE6.SSU_371492 | 49226         | Annelida       | Polychaeta   | Phyllodocida     | Brania            | sp.         | + +           |                  |
| HE4.SSU_913344 | 37252         | Annelida       | Polychaeta   | Phyllodocida     | Glycera           | sp.         | + +           |                  |
| HF5.SSU_997904 | 64            | Annelida       | Polychaeta   | Phyllodocida     | Glycinde          | armigera    | + +           |                  |
| TS5.SSU_870099 | 69            | Annelida       | Polychaeta   | Phyllodocida     | Goniada           | maculata    | - +           |                  |
| TF6.SSU_42415  | 2             | Annelida       | Polychaeta   | Phyllodocida     | Harmothoe         | imbricata   | - +           |                  |
| HE6.SSU_350003 | 5             | Annelida       | Polychaeta   | Phyllodocida     | Myrianida         | sp.         | + -           |                  |
| HF6.SSU_324605 | 2             | Annelida       | Polychaeta   | Phyllodocida     | Nereis            | pelagica    | + -           |                  |
| HE7.SSU_239005 | 67220         | Annelida       | Polychaeta   | Phyllodocida     | Pisione           | remota      | + +           |                  |
| HE2.SSU_637269 | 49            | Annelida       | Polychaeta   | Phyllodocida     | Platynereis       | dumerillii  | + -           |                  |
| HE8.SSU_832291 | 1             | Annelida       | Polychaeta   | Phyllodocida     | Progoniada        | regularis   | + -           |                  |
| HE8.SSU_834197 | 1             | Annelida       | Polychaeta   | Sabellida        | Fabriciola        | liguronis   | + -           |                  |
| HF2.SSU_202737 | 4             | Annelida       | Polychaeta   | Sabellida        | Laeospira         | corallinae  | + -           |                  |
| HE2.SSU_640060 | 3             | Annelida       | Polychaeta   | Sabellida        | Myriochele        | sp.         | + -           |                  |
| TS5.SSU_869292 | 123           | Annelida       | Polychaeta   | Spionida         | Apistobranchus    | sp.         | - +           |                  |
| TS3.SSU_517096 | 1407          | Annelida       | Polychaeta   | Spionida         | Laonice           | sp.         | - +           |                  |
| HE3.SSU_123438 | 1952          | Annelida       | Polychaeta   | Spionida         | Spio              | sp.         | + +           |                  |
| TS5.SSU_882766 | 60            | Annelida       | Polychaeta   | Terebellida      | Diplocirrus       | glaucus     | - +           |                  |
| HF2.SSU_193854 | 1             | Annelida       | Polychaeta   | Terebellida      | Flabelligera      | sp.         | + -           |                  |
| TF6.SSU_63146  | 669           | Annelida       | Polychaeta   | Terebellida      | Pectinaria        | sp.         | - +           |                  |
| TS5.SSU_883475 | 4155          | Annelida       | Polychaeta   | Terebellida      | Terebellides      | stroemii    | - +           |                  |
| TF4.SSU_139713 | 193           | Arthropoda     | Branchiopoda | _                |                   |             | - +           |                  |
| Taxonomy            | Sequence Number | Diversity | Rank   | Order             | Family          | Species            | Presence | Absence |
|---------------------|-----------------|-----------|--------|-------------------|-----------------|--------------------|----------|---------|
| Arthropoda Malacostraca | HE5.SSU_184679 | 149       | _      | _                | _               | _                  | _        | _       |
| Arthropoda Malacostraca | HE8.SSU_832214 | 1         | _      | _                | _               | _                  | _        | _       |
| Arthropoda Malacostraca | HF5.SSU_994971 | 7         | Decapoda | Nikoides sp.   | _               | _                  | _        | _       |
| Arthropoda Malacostraca | TF6.SSU_56595  | 65992     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Malacostraca | HF9.SSU_15855  | 31800     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Malacostraca | HF2.SSU_208480 | 21241     | Decapoda | Praebella longidactyla | _ | _                  | _        | _       |
| Arthropoda Malacostraca | TS2.SSU_812824 | 433       | Maxilipoda | _               | _               | _                  | _        | _       |
| Arthropoda Malacostraca | TF3.SSU_955499 | 185       | Maxilipoda | _               | _               | _                  | _        | _       |
| Arthropoda Malacostraca | TF5.SSU_470101 | 360       | _      | _                | _               | _                  | _        | _       |
| Arthropoda Ostracoda Podocopida | HE1.SSU_864375 | 1160     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Ostracoda Podocopida | HE7.SSU_253407 | 2584     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Ostracoda Podocopida | HE5.SSU_181011 | 1       | _      | _                | _               | _                  | _        | _       |
| Arthropoda Ostracoda Podocopida | TF6.SSU_56595  | 65992     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Ostracoda Podocopida | HF9.SSU_15855  | 31800     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Ostracoda Podocopida | HF2.SSU_208480 | 21241     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Pycnogonida Pantopoda | HE8.SSU_832214 | 1         | _      | _                | _               | _                  | _        | _       |
| Arthropoda Pycnogonida Pantopoda | TF5.SSU_470101 | 360       | _      | _                | _               | _                  | _        | _       |
| Arthropoda Pycnogonida Pantopoda | TF6.SSU_56595  | 65992     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Pycnogonida Pantopoda | HF9.SSU_15855  | 31800     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Pycnogonida Pantopoda | HF2.SSU_208480 | 21241     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Pycnogonida Pantopoda | TF6.SSU_56595  | 65992     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Pycnogonida Pantopoda | HF9.SSU_15855  | 31800     | _      | _                | _               | _                  | _        | _       |
| Arthropoda Pycnogonida Pantopoda | HF2.SSU_208480 | 21241     | _      | _                | _               | _                  | _        | _       |
| Bryozoa Stenolaemata Cyclostomatida | HE1.SSU_850917 | 4         | _      | _                | _               | _                  | _        | _       |
| Bryozoa Stenolaemata Cyclostomatida | TF5.SSU_412099 | 18       | Cephalorhyncha | Kinorhyncha | Homalorhagida | Pycnophyse kielensis | _        | _       |
| Bryozoa Stenolaemata Cyclostomatida | HE7.SSU_239963 | 45       | _      | _                | _               | _                  | _        | _       |
| Bryozoa Stenolaemata Cyclostomatida | HE3.SSU_123107 | 7        | Chordata Actinopteri | Perciformes | Hypseleotris sp. | _        | _       |
| Bryozoa Stenolaemata Cyclostomatida | HE7.SSU_239963 | 45       | _      | _                | _               | _                  | _        | _       |
| Chordata Stolidobranchia | HE6.SSU_373369 | 2         | Bryozoa | _                | _               | _                  | _        | _       |
| Chordata Stolidobranchia | HE1.SSU_850917 | 4         | Bryozoa | _                | _               | _                  | _        | _       |
| Cnidaria Hydrozoa | TF1.SSU_674740 | 2212      | _      | _                | _               | _                  | _        | _       |
| Cnidaria Hydrozoa | TS3.SSU_472524 | 2741      | _      | _                | _               | _                  | _        | _       |
| Reference | Code      | Species                | Domain   | Kingdom  | Phylum           | Class               | Order         | Family        | Genus            | Species         |
|-----------|-----------|------------------------|----------|----------|------------------|---------------------|---------------|--------------|------------------|-----------------|
| TS3.SSU_518760 | 7860 | Cnidaria Hydrozoa Anthoathecata | Euphysa sp. | + + |
| HE2.SSU_639670 | 1 | Cnidaria Hydrozoa Leptothecca Abietinaria filicula | + - |
| TF4.SSU_152912 | 61418 | Echinodermata _ _ _ _ | + + |
| HE5.SSU_186025 | 8038 | Echinodermata _ _ _ _ | + + |
| TF4.SSU_155631 | 5491 | Echinodermata _ _ _ _ | + + |
| TS5.SSU_881395 | 25 | Echinodermata _ _ _ _ | - + |
| HE4.SSU_914821 | 1 | Echinodermata Holothuroidea Apodida Leptosynapta sp. | + - |
| HF9.SSU_2577 | 1006 | Gastrotricha _ _ | Chaetonotida Chaetonotus sp. | + + |
| HE7.SSU_244283 | 249 | Gastrotricha _ _ | Macrodasyida Diplodasys melioriae | + - |
| HF5.SSU_996540 | 161 | Gastrotricha _ _ | Macrodasyida Lepidodasys sp. | + - |
| HF5.SSU_995416 | 636 | Gastrotricha _ _ | Macrodasyida Macrodasys sp. | + - |
| HF2.SSU_192734 | 479 | Gastrotricha _ _ | Macrodasyida Macrodasys sp. | + - |
| HF7.SSU_385728 | 6934 | Gastrotricha _ _ | Macrodasyida Mesodasys sp. | + + |
| HE7.SSU_242889 | 3013 | Gastrotricha _ _ | Macrodasyida Tetranchyroderma thysoniphorum | + - |
| HF1.SSU_770513 | 339 | Gastrotricha _ _ | Macrodasyida Thaumastoderma ramuliferum | + - |
| HF1.SSU_760431 | 5 | Gastrotricha _ _ | Macrodasyida Urodasys sp. | + - |
| TF6.SSU_44832 | 3816 | Mollusca Bivalvia _ | + + |
| HF2.SSU_208561 | 14 | Mollusca Bivalvia Anomalodesmata | + + |
| HF8.SSU_788507 | 1 | Mollusca Bivalvia Limoida Limaria hians | + - |
| TF3.SSU_924397 | 11725 | Mollusca Bivalvia Veneroida Abra sp. | + + |
| HE9.SSU_317977 | 1982 | Mollusca Bivalvia Verenoida Arcticoida islandica | + + |
| TF4.SSU_132537 | 1581 | Mollusca Gastropoda Neogastropoda Nassarius festivus | + + |
| HF1.SSU_779114 | 65 | Nematoda Chromoidea Araeolaimida Odontophora sp. | + + |
| TF6.SSU_48167 | 2940 | Nematoda Chromoidea Araeolaimida Sabatieria sp. | + + |
| TF1.SSU_710679 | 639 | Nematoda Chromoidea Chromadoroida | + + |
| HF2.SSU_192072 | 2 | Nematoda Chromoidea Chromadorida Chromadora nudicapitata | + - |
| HF1.SSU_759758 | 4 | Nematoda Chromoidea Plectida | + - |
| HF9.SSU_20251 | 636 | Nematoda Desmodorida Microlaimidae | + + |
| Identifier   | Sequence | phylum  | superclass | subclass | class | family | Species                      | + | - |
|--------------|----------|---------|------------|----------|-------|--------|-------------------------------|---|---|
| HE3.SSU_124287 | 13       | Nematoda | Enoplea    | Enoplida | Enoploides | sp.    | + -                          |   |   |
| HE3.SSU_110275 | 8        | Nematoda | Enoplea    | Enoplida | Enoplus | sp.    | + -                          |   |   |
| HE5.SSU_188855 | 27       | Nematoda | Enoplea    | Enoplida | Symphlocostoma | sp.    | + +                          |   |   |
| TS6.SSU_587229 | 493      | Nematoda | Enoplea    | Enoplida | Viscosia | viscosa | + +                          |   |   |
| TF3.SSU_938615 | 642      | Nemertea | __         | __       | Cerebratulus | marginatus | + +                          |   |   |
| TF6.SSU_49192  | 265      | Nemertea | Anopla     | __       | Lineus | bilineatus | + +                          |   |   |
| HE4.SSU_908113 | 877      | Nemertea | Anopla     | __       | Callinera | grandis | + -                          |   |   |
| HF9.SSU_3582   | 6        | Nemertea | Paleonemertea | __       | Cephalothrix | filiformis | + +                          |   |   |
| HE3.SSU_121696 | 12053    | Nemertea | Paleonemertea | __       | Hubrechtella | dubia | + +                          |   |   |
| TS2.SSU_818002 | 1        | Platyhelminthes | Rhabditophora | Cestoda | __         | -         | + |   |
| HE9.SSU_303121 | 1939     | Platyhelminthes | Rhabditophora | Haplopharyngida | Haplopharynx | rostratus | + -                          |   |   |
| HF1.SSU_773830 | 1        | Platyhelminthes | Rhabditophora | Prolecithophora | Aloostoma | neostiliferum | + -                          |   |   |
| HE2.SSU_650311 | 8        | Platyhelminthes | Rhabditophora | Prolecithophora | Cylindrostoma | sp.    | + -                          |   |   |
| HE5.SSU_177399 | 4        | Platyhelminthes | Rhabditophora | Prolecithophora | Euxinia | baltica | + -                          |   |   |
| HF9.SSU_23023  | 8367     | Platyhelminthes | Rhabditophora | Prolecithophora | Plagiostomum | cinctum | + +                          |   |   |
| TS2.SSU_822141 | 938      | Platyhelminthes | Rhabditophora | Prolecithophora | Plagiostomum | cuticulata | - +                          |   |   |
| TF6.SSU_52738  | 214      | Platyhelminthes | Rhabditophora | Prolecithophora | Plagiostomum | striatum | - +                          |   |   |
| TF5.SSU_433159 | 2        | Platyhelminthes | Rhabditophora | Prolecithophora | Ulianinia | mollissima | - +                          |   |   |
| HF9.SSU_24513  | 59       | Platyhelminthes | Rhabditophora | Proseriata | Monocelis | lineata | + +                          |   |   |
| HF2.SSU_201740 | 2        | Platyhelminthes | Rhabditophora | Rhabdocoela | Phonorchynchus | helgolandicus | + -                          |   |   |
| TS6.SSU_592673 | 245      | Platyhelminthes | Rhabditophora | Rhabdocoela | Proxenetes | sp.    | + +                          |   |   |
| HF4.SSU_616041 | 771      | Platyhelminthes | Rhabditophora | Seriata | __         | -         | + -                          |   |   |
| HE3.SSU_117223 | 181      | Porifera  | Calcarea   | __       | __         | +         | + +                          |   |   |
Invasive and alien species detected in the samples

Five alien species were detected in the sample, of which two are considered invasive (in bold; Table 9), and the other three are on alert lists. The two invasive species (Acartia tonsa, a copepod, and Alexandrium ostenfeldii, a dinoflagellate) could easily be overlooked in routine monitoring programs. Species within the genus Acartia are difficult to distinguish (Jensen 2010) and the invasive species can be confused with other native species. Also A. ostenfeldii is easily misidentified as other Alexandrium species; detailed thecal plate observation is often necessary for proper identification (Balech 1995). This shows the potential of molecular techniques for monitoring invasive species, and points to problems using traditional identification techniques. Many invasive species arrive in an area as spores, larvae or juveniles - all life stages that may be easily overlooked and problematic to identify to species level. Target barcoding of environmental DNA (eDNA) shows a great promise for detecting species without the need of costly sampling schemes. This would also allow for more random sampling in an area, increasing the probability of actually finding a species even when they occur in low numbers.
Comparison of metabarcoding versus morphology-based identification of Xenacoelomorpha

Comparison of morphology-based assessment of Xenacoelomorpha diversity with metabarcoding using taxonomic assignments to the phylum level (with 80% similarity threshold; Suppl. materials 2, 3), shows that extraction procedures have strong impact on the effectiveness of morphology-based identification (Tables 10, 11). Using freshwater for extraction of Xenacoelomorpha rendered most of them unrecognizable and unidentifiable, but left their DNA intact and suitable for metabarcoding. No identifiable Xenacoelomorpha were found in the Hållö samples extracted using flotation with fresh water, while all specimens found in Gullmarn Fjord were treated together as one taxon "Acoela sp." for the lack of better alternative. Metabarcoding, on the other hand, recovered between 6 and 15 taxa (OTUs) from the Hållö samples extracted using flotation with fresh water (Table 11), and up to 13 taxa (OTUs) from the same type of samples from the Gullmarn Fjord site (Table 11), depending on the barcoding region used. Just like for nematodes (see below), 18S barcodes always gave higher overall estimates of diversity (number of OTUs) compared to COI (Table 11). 18S also gave higher diversity estimates, compared to morphology-based identification for the Hållö samples extracted using flotation with MgCl2 (11 versus 7), but lower for the Gullmarn Fjord site samples extracted using siphoning (9 versus 15). COI Leray primers were less effective compared to the COI Lobo primers that recovered 2-6 OTUs more in all samples (Table 11). The most numerous of the morphologically identified species, Mecynostomum tenuissimum, was present with 120 specimens in the manually sorted samples, but was not detected at all in the 18S samples. Note that the 18S and COI sequences for all of the species identified in the visually sorted samples are present in the reference database. This raises the question of the efficacy of using the SSU_FO4-SSU_R22 18 S fragment for metabarcoding of acoelomorphs. A recent study found a number of unknown xenacoelomorph taxa while data mining metabarcoding sequences from surveys of pelagial and deep benthic habitats (Arroyo et al. 2016). Unknown xenacoelomorph species may exist also at the moderate sampling depths we sampled in the Gullmarn Fjord. Our siphoning technique relies on migration of specimens to the sediment surface in response to hypoxia. It is possible that

Table 9.
Invasive species (in bold) and species on alert lists (not bold) found in the samples. X indicates where the species were found.

| Species               | Phylum    | COI        | 18S        |
|-----------------------|-----------|------------|------------|
|                       |           | Hållö island | Gullmarn Fjord | Hållö island | Gullmarn Fjord |
| *Acartia tonsa*       | Arthropoda| x          | x          |
| *Alexandrium ostenfeldii* | Dinoflagellata | x          | x          |
| *Bonnemaisonia hamifera* | Rhodophyta    | x          | x          |
| *Penilia avirostris*  | Arthropoda | x          | x          |
| *Thalassiosira punctigera* | Bacillariophyta | x          |
there are xenacoelomorphs with high tolerance for hypoxia that are not captured by the siphoning method, and thus would not be found in the manually sorted samples, but could be detected by metabarcoding of unprocessed samples. It should be noted that the extraction method used on the Hållö samples does not rely on migration of specimens to the surface.

Table 10.
Taxonomic composition and relative abundance (% of the total number of specimens) of Xenacoelomorpha species in Gullmarn Fjord and Hållö sites.

| Taxon                      | Gullmarn Fjord | Hållö          |
|----------------------------|----------------|---------------|
|                            | Siphoning      | Flotation with fresh water | Flotation with MgCl2 solution | Flotation with fresh water |
| **Acoela**                 |                |               |                            |                            |
| 1 Haploposthia rubropunctata | 1.03           | 0             | 0                           | 0                           |
| 2 Childia brachyposthium    | 3.78           | 0             | 0                           | 0                           |
| 3 Childia submaculatum      | 1.03           | 0             | 0                           | 0                           |
| 4 Childia trianguliferum    | 2.06           | 0             | 0                           | 0                           |
| 5 Childia crassum           | 3.44           | 0             | 0                           | 0                           |
| 6 Childia sp.               | 25.09          | 0             | 0                           | 0                           |
| 7 Mecynostomum tenuissimum  | 43.99          | 0             | 0                           | 0                           |
| 8 Mecynostomum aurum        | 0.34           | 0             | 0                           | 0                           |
| 9 cf. Eumecynostomum altitudi | 4.81          | 0             | 0                           | 0                           |
| 10 Philactinoposthia sp.    | 0.34           | 0             | 0                           | 0                           |
| 11 Acoela sp.               | 2.06           | 100           | 88.71                       | 0                           |
| 12 Faerlea glomerata        | 3.09           | 0             | 0                           | 0                           |
| 13 Archaphanostoma sp.      | 0.34           | 0             | 0.81                        | 0                           |
| 14 Postmecynostomum glandulosum | 0             | 0             | 2.42                        | 0                           |
| 15 Paramecynostomum sp.     | 0             | 0             | 0.81                        | 0                           |
| 16 Eumecynostomum macrobursalum | 0             | 0             | 0.81                        | 0                           |
| 17 Isodiametra sp.          | 0             | 0             | 0.81                        | 0                           |
| 18 Haplogonaria viridis/Archocelis macrorhabditis | 0             | 0             | 5.65                        | 0                           |
| **Nemertodermatida**        |                |               |                             |                             |
| 19 Nemertoderma westbladi   | 8.25           | 0             | 0                           | 0                           |
| 20 Flagellophora apelti     | 0.34           | 0             | 0                           | 0                           |
Comparison of metabarcoding versus morphology-based identification of Nematoda

Both study sites are characterized by rich and diverse nematode fauna. The Hållö site had a total of 107 species of nematodes, belonging to 86 genera (Holovachov et al. 2017). Of these, 88 species belonging to 73 genera were found in samples extracted by flotation with a MgCl₂ solution, and 101 species belonging to 83 genera were found in samples extracted by flotation with fresh water. The Gullmarn fjord site had a total of 113 nematode species of nematodes, belonging to 77 genera (Holovachov et al. 2017). Of these, 81 species belonging to 62 genera were found in samples extracted by siphoning, and 102 species belonging to 70 genera were found in samples extracted by flotation with fresh water. A certain small number of nematode individuals in each sample were not identified to species/genus/family, either due to their developmental stage or quality of preservation.

The final list of nematode OTUs includes 139 18S sequences. Only two 18S OTUs were positively identified using QIIME to species level using 97% similarity threshold: *Viscosia viscosa* (TS6.SSU58722) and *Chromadora nudicapitata* (HF2.SSU192072), six more were assigned to reference sequences identified to genus level only (Suppl. material 1). Only 22 COI sequences were assigned to the phylum Nematoda, and none was identified to species level.

When comparing the results of morphology-based assessment of nematode diversity with metabarcoding using taxonomic assignments to the phylum level in this particular study (with 80% similarity threshold; Suppl. materials 2, 3), the detailed and extensive examination of samples and morphology-based species identification provided more comprehensive estimates of nematode diversity (107 species in Hållö and 113 species in Gullmarn Fjord) than metabarcoding using either one of the molecular markers, independently of the extraction technique or locality (Table 12). Moreover, COI barcodes were much harder to obtain for marine nematodes using either one of the primers (16 OTUs in Hållö and 9 OTUs in Gullmarn Fjord using Lobo primers; 17 OTUs in Hållö and 4 OTUs in Gullmarn Fjord using Leray primers), comparing to 18S (95 OTUs in Hållö and 78

| Site / extraction method           | morphology-based | 18S | COI (Lobo) | COI (Leray) |
|------------------------------------|------------------|-----|------------|-------------|
| Hållö, flotation with MgCl₂        | 7                | 11  | 8          | 6           |
| Hållö, flotation with fresh water  | 0                | 15  | 11         | 6           |
| Hållö, total                       | 7                | 16  | 12         | 7           |
| Gullmarn Fjord, siphoning          | 15               | 11  | 9          | 4           |
| Gullmarn Fjord, flotation with fresh water | 1       | 13  | 2          | 0           |
| Gullmarn Fjord, total              | 15               | 19  | 10         | 4           |

Table 11.
Total number of Xenacoelomorpha taxa or OTUs distinguished based on morphology (Table 10), 18S and COI from different sampling sites and extraction methods (placement of OTUs is based on 80% similarity threshold, Suppl. materials 2, 3)
OTUs in Gullmarn Fjord site; Table 12). Due to the very limited reference databases available for marine nematodes, very few nematode OTUs can be identified to species or genus level, making it difficult to use metabarcoding data in ecological studies.

| Site / extraction method                        | morphology-based | 18S | COI (Lobo) | COI (Leray) |
|------------------------------------------------|------------------|-----|------------|------------|
| Hålloo, flotation with MgCl2                    | 88               | 71  | 12         | 11         |
| Hålloo, flotation with fresh water              | 101              | 78  | 14         | 14         |
| Hålloo, total                                   | 107              | 95  | 16         | 17         |
| Gullmarn Fjord, siphoning                       | 81               | 47  | 8          | 4          |
| Gullmarn Fjord, flotation with fresh water      | 102              | 67  | 4          | 2          |
| Gullmarn Fjord, total                          | 113              | 78  | 9          | 4          |

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Supplementary materials

Suppl. material 1: OTUs identified to species level in the samples using 97% sequence similarity, all organism groups.

Authors: Quiterie Haenel, Oleksandr Holovachov, Ulf Jondelius, Per Sundberg and Sarah J. Bourlat

Data type: Occurrence records from Metabarcoding for Hållö island and Gullmarsfjord, Sweden.

Brief description: Sequence similarity search at 97% similarity allowed us to identify some OTUs to species level. 215 COI OTUs and 243 18S OTUs were identified to species from both sites (Hållö island and Gullmarsfjord).

Filename: TableS1.xlsx - Download file (85.61 kb)
Suppl. material 2: OTU table for 18S

**Authors:** Quiterie Haenel, Oleksandr Holovachov, Ulf Jondelius, Per Sundberg and Sarah J. Bourlat

**Data type:** Metagenomic, OTU table

**Brief description:** OTU table showing all 18S OTUs, their taxonomic assignment at 80% similarity and number of reads per sample (HE: Hållö Flotation, HF: Hållö Flotation MgCl2, TS: Gullmarn Fjord Siphoning, TF: Gullmarn Fjord Flotation)

**Filename:** 18S.otu_table.txt - Download file (318.45 kb)

Suppl. material 3: OTU table for COI

**Authors:** Quiterie Haenel, Oleksandr Holovachov, Ulf Jondelius, Per Sundberg and Sarah J. Bourlat

**Data type:** Metagenomic, OTU table

**Brief description:** OTU table showing all COI OTUs, their taxonomic assignment at 80% similarity and number of reads per sample (HE: Hållö Flotation, HF: Hållö Flotation MgCl2, TS: Gullmarn Fjord Siphoning, TF: Gullmarn Fjord Flotation)

**Filename:** COI.otu_table.txt - Download file (728.63 kb)