Laboratory protocol for DNA metabarcoding of soft sediment macrobenthos from the North Sea

Interreg North Sea Region GEANS
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Genetic tools for Ecosystem health Assessment in the North Sea region

Laboratory protocol for DNA metabarcoding of soft sediment macrobenthos from the North Sea

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1. Introduction

The Interreg North Sea Region funded project GEANS (Genetic tools for Ecosystem health Assessment in the North Sea region) strives to harmonise and implement DNA-based tools in routine monitoring programs in support of policy and decision making concerning ecosystem health. To ensure the applicability and implementation of the developed genetic tools in ecosystem health assessment, pilots are being conducted in the partner countries in close cooperation with relevant stakeholder groups. For all pilots, GEANS applies traditional morphological monitoring in tandem with DNA metabarcoding and focuses on macrobenthos from the North Sea. Read more about our project at https://northsearegion.eu/geans/

The described protocol uses organismal DNA and is based on results of two pilot tests conducted by ILVO with macrobenthos from field samples from the Belgian part of the North Sea that were first morphologically identified. Four well characterized macrobenthos communities have been sampled with three replicate Van Veen grabs (A, B and C). The sediment was sieved on a 1 mm sieve. All material (macrobenthos organisms + residue) that remained on the sieve was fixed in absolute ethanol, and stored at -20°C until further processing. Five different COI primer sets have been tested for their ability to recover macrobenthos species. A detailed description of the locations and results can be found in Derycke et al. (2021). The comparison between metabarcoding eDNA from the ethanol fixative and DNA from the bulk organisms showed more consistent results in species detection when using organismal DNA (Derycke et al., 2021). The same samples have been used to investigate DNA and PCR replication for reliable diversity measurements using DNA metabarcoding (Van den Bulcke et al., in preparation) and to evaluate repeatability and robustness of DNA metabarcoding in a proficiency test (GEANS consortium, in preparation).

2. Blending/grinding of macrobenthos organisms

Organisms preserved in ethanol were mixed into a homogeneous solution using a blender or, in case less than 100 ml of sample was available, grinded with mortar and pestle. The homogeneous "soup" was distributed in 2ml Eppendorf tubes and stored at -20°C until further processing. Between samples, the blender or mortar and pestle were rinsed with tap water until all visible material was removed, soaked with soap and thoroughly wiped with a sponge, again rinsed with tap water and then wiped with RNAse DNAway®. After 5 minutes, blender or mortar and pestle were dried with paper.

3. Contamination

DNA metabarcoding is a very sensitive technique, and special care should be taken to avoid contamination during the lab protocol by using sterile material at all times (see also step 5). DNA extraction and PCR amplification should be conducted in separated rooms. We recommend to include DNA extraction controls (eg using nuclease free water instead of homogenized organisms at the DNA extraction step or in between homogenising samples in the blender) and PCR controls (using nuclease free water instead of DNA extract during PCR preparation) to be able to correct for cross contamination in the lab during bioinformatic processing of the sequencing data. We recommend to include at least three DNA and three PCR controls for every batch of samples.

4. Material and reagents requirements

Equipment:

- Laminar Flow Cabinet Class II (Vertical): CleanAir
- Thermomixer: Bio-rad
- Centrifuge 5424R: Eppendorf
- Incubator: Bruswick
- Magnetic 96-well Plate: Agencount
- TruSeq Index Plate Fixture: Illumina
- Bioanalyzer or Qiaxcel
- Qubit or Quantus
- Gel electrophorese Mupid-One: Eurogentec
- P 0.1-2.5: Transferpette
- P1-100: Transferpette
- P100-1000: Transferpette

Consumables:

- DNeasy PowerSoil DNA Isolation kit, catno: 12888-100, Qiagen
- Proteinase K (20 mg/ml solution in nuclease-free water),catno: V3021 , Promega
- Wizard DNA Clean-up System, catno: A7280, Promega
- Absolute Ethanol 99.9%, catno: CL00.0505.5000, Chem-Lab
- 95% Absolute Ethanol (95% Absolute Ethanol 99.9% + 5% DNAse RNAse Free Distillated Water)
- 70% Absolute Ethanol (70% Absolute Ethanol 99.9% + 30% DNAse RNAse Free Distillated Water)
- 80% Isopropanol (80% Isopropanol, Chem-Lab catno: CL00.0906.2500 + 20% DNAse RNAse Free Distillated Water)
- RNAse DNAway®, catno: 732-2271, Molecular BioProducts
- DNAse RNAse Free Distillated Water, catno: 10977-049, Life Technologies
- Amplicon PCR forward and reverse primer: Sigma-Aldrich
- 2 x KAPA HiFi HotStart Readymix, catno: KK2602, Roche
- CleanPCR® Reagent 50 ml, catno: CPCR-0050, GC Biotech
- 1xTE-buffer 100ml, catno: 93283, Sigma-Aldrich
- Illumina Nextera XT Index Primers set A, catno: FC-131-2001, Illumina
- 1.5ml PCR Clean Eppendorf tubes catno: 211-2164, Eppendorf
- Sterile MµltiGuard filtertips 1-20µl, catno: A14954, Sorenson
- Sterile MµltiGuard filtertips 10-200µl, catno: A14995, Sorenson
- Sterile MµltiGuard filtertips 100-1000µl, catno: A14953, Sorenson
- Sterile Petri dish 90 mm, catno: A23632, Gosselin
- 12-well PCR strips, catno: B6601, BiozymTC
- 12-well PCR caps, catno: B56511, BioZymTC
- Forceps, catno: 232-2156, VWR
- Micropestle: Eppendorf
- Gloves EcoShield category III, Small catno: A18990, SHIELD Scientific
- TE buffer

5. Preparation of your equipment and work station

It is important to start the laboratory procedure with RNA and DNA decontaminated equipment and work station. Decontaminate the laminar flow cabinet and lab bench with RNAse DNAway® before each step. Be sure that you wear clean gloves and a separate clean lab coat for NGS work. Clean all pipettes with RNAse DNAway® between each step.

6. Laboratory protocol

***** DNA extraction*****

For each biological replicate, three subsamples of 2ml of homogenized organisms are extracted separately.

1/ Centrifuge the Eppendorf tubes containing 2ml of homogenized organisms for 5 minutes and 8000 rpm at room temperature.

2/ Remove supernatant (= ethanol) by pipetting.

3/ Leave the Eppendorf tubes open for one hour (or until dry) at 50°C so that remaining ethanol can evaporate.

4/ The pellet from each Eppendorf tube is transferred to a 2ml tube with beads from the DNeasy PowerSoil Kit (QIAGEN, catno: 12888-100). When the pellet is stuck to the wall of the Eppendorf, bring the content of the Powerbead tube in the Eppendorf.

5/ Add 10 µl Proteinase K (20 mg/ml) (Promega catno V3021) and vortex. Incubate overnight at 56°C and 1000 rpm.

6/ Follow the protocol of the DNeasy PowerSoil kit provided by the manufacturer.

7/ Pool 10 µl of the three subsamples from the same biological replicate into one tube, to obtain 30 µl of pooled DNA extract.

8/ Clean-up the pooled DNA extract with the Wizard DNA Clean-Up System (Promega catno: A7280) according to manufacturer's protocol. Elute in 50 µL heated TE-buffer (Sigma-Aldrich catno: 93283-100ML).

***** PCR 1*****

9/ Three PCR replicates are conducted on the pooled DNA extract (the PCR mix is prepared three times, and each mix undergoes a separate PCR cycling run). Best results (in terms of congruence with morphological data) have been achieved with the Leray primers (Leray et al. 2013) that amplify 313bp of the Folmer region (primer sequence includes the Illumina adaptors):

miCOIintF :

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGWACWGGWTGAACWGTWTAYCCYCC-3'

jgHCO2198:

5'- GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTANACYTCNGGRTGNCCRAARAAYCA-3'

Prepare the following PCR mix (for one reaction of 25µl):

Nuclease free water	8,5
2 x KAPA HiFi HotStart ReadyMix	12,5
MiCOIintF primer 10 µM	0,75
jgHCO2198 primer 10 µM	0,75

10/ add 2.5µl from the pooled DNA extract to 22.5µl of the PCR mix.

11/ Incubate the PCR mix in the cycling machine using the following PCR conditions:

95°C 3min, 35x (98°C 30s, 57°C 30s, 72°C 30s), 72°C 1min

12/ Pool the three PCR replicates into one well of a 96 well plate (75 µl). This can be stored at -20°C until further processing, by covering with a seal.

***** PCR Clean-up*****

13/ a. If plate was stored in freezer: centrifuge the 96 well plate at 1,000 × g at 20°C for 1 minute to collect condensation and carefully remove seal. Pipet 37.5 µl of the Amplicon PCR in a new 96-well plate.

b. if plate is immediately used for clean-up: pipet 37.5 µl of the Amplicon PCR in a new 96-well plate.

14/ Vortex the CleanNGS beads for 30 seconds to make sure that the beads are evenly dispersed.

15/ Using a multichannel pipette, add 30 µl of CleanNGS beads to each well of the Amplicon PCR

plate. Change tips between columns. Gently pipette entire volume up and down 10 times.

16/ Incubate at room temperature without shaking for 5 minutes.

17/ Place the plate on a magnetic stand for 2 minutes or until the supernatant has cleared

(can take 10 minutes).

18/ With the Amplicon PCR plate on the magnetic stand, use a multichannel pipette to remove and

discard the supernatant. Change tips between samples.

19/ With the Amplicon PCR plate on the magnetic stand, wash the beads with freshly prepared 80% ethanol as follows:

a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.

b. Incubate the plate on the magnetic stand for 30 seconds.

c. Carefully remove and discard the supernatant.

20/ With the Amplicon PCR plate on the magnetic stand, perform a second ethanol wash as follows:

a. Using a multichannel pipette, add 200 µl of freshly prepared 80% ethanol to each sample well.

b. Incubate the plate on the magnetic stand for 30 seconds.

c. Carefully remove and discard the supernatant.

d. Use a P20 multichannel pipette with fine pipette tips to remove excess ethanol.

21/ With the Amplicon PCR plate still on the magnetic stand, allow the beads to air-dry for 10 minutes.

22/ Remove the Amplicon PCR plate from the magnetic stand. Using a multichannel pipette, add 40 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.

23/ Gently pipet the mix up and down 10 times, changing tips after each column.

Make sure that beads are fully resuspended.

24/ Incubate at room temperature for 2 minutes.

25/ Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

26/ Using a multichannel pipette, carefully transfer 37.5 µl of the supernatant from the Adaptor PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

Store it at -15° to -25°C for up to a week.

***** INDEX PCR*****

27/ Perform the index PCR with Illumina Nextera XT Index kit v2 (catno: 15052163) using the following PCR mix:

KAPA Hifi Readymix	12.5
Nuclease free water	5
Nextera XT primer 1	2.5
Nextera XT primer 2	2.5
PCR1 product	2.5

28/ Incubate the index PCR using the following PCR cycling conditions:

95°C 3min, 8x (95°C 30s, 55°C 30s, 72°C 30s), 72°C 5min

29/ Centrifuge the Index PCR plate at 280 × g at 20°C for 1 minute to collect condensation.

30/ Vortex the CleanNGS beads for 30 seconds to make sure that the beads are evenly dispersed.

31/ Bring 25 µl of INDEX PCR in a new 96-well plate.

32/ Using a multichannel pipette, add 28 µl of CleanNGS beads to each well of the Index PCR plate.

Gently pipet the mix up and down 10 times.

33/ Repeat step 16 until step 21.

34/ Remove the INDEX PCR plate from the magnetic stand. Using a multichannel pipette,

add 25 µl of 10 mM Tris pH 8.5 to each well of the Amplicon PCR plate.

35/ Gently pipette mix up and down 10 times, changing tips after each column.

Make sure that beads are fully resuspended.

36/ Incubate at room temperature for 2 minutes.

37/ Place the plate on the magnetic stand for 2 minutes or until the supernatant has cleared.

38/ Using a multichannel pipette, carefully transfer 37.5 µl of the supernatant from the Adaptor PCR plate to a new 96-well PCR plate. Change tips between samples to avoid cross-contamination.

Store it at -15° to -25°C for up to a week.

39/ Load 5 samples from PCR1 and the indexPCR on the Bioanalyzer/Qiaxcel to confirm the index PCR was successful.

***** Pooling of indexed PCR products*****

Illumina recommends quantifying your libraries using a fluorometric quantification method that uses dsDNA binding dyes.

40/ Measure samples with the Qubit/Quantus twice. The average value is used to calculate the DNA concentration in ng/µl.

41/ Calculate the DNA concentration in nM, based on the average size of DNA amplicons (380 bp) and the DNA concentration in ng/µl:

$$\text{concentration in nM} = \frac{concentration\ in\ ng/\mu l}{660\ \frac{g}{mol} * average\ library\ size} * 10^6$$

42/ Dilute samples using 10 mM Tris pH8.5 to **10 nM** (dependent on provider! Before sending, ask for the required concentration. Normally, 10 nM is more than sufficient (Illumina recommends 4 nM).

43/ Take 5 µl of diluted DNA from each sample to obtain a pooled library. Depending on coverage needs, up to 192 libraries can be pooled for one MiSeq run. Aim to have at least 10 000 reads per sample.

44/ Ask for an addition of 20% PhiX to the sequencing run. This greatly improves the quality of sequencing reads for low diversity samples.

7. References

Derycke, S., Maes, S., Van Den Bulcke, L., Vanhollebeke, J., Wittoeck, J., Hillewaert, H., Ampe, B., Haegeman, A., Hostens, K., and De Backer, A. (2021). Detection of Macrobenthos Species With Metabarcoding Is Consistent in Bulk DNA but Dependent on Body Size and Sclerotization in eDNA From the Ethanol Preservative. *Frontiers in Marine Science* 8.

Leray, M., Yang, J.Y., Meyer, C.P., Mills, S.C., Agudelo, N., Ranwez, V., Boehm, J.T., and Machida, R.J. (2013). A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Frontiers in Zoology* 10.

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