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Marine technology — Marine environment impact assessment (MEIA) — General protocol for observation of meiofaunal community

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 8, *Ships and marine technology*, Subcommittee SC 13, *Marine technology*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

For environmental impact assessments (EIA) of plans for seabed mineral resource exploitation, objective, comprehensive and easy-to-apply analysis techniques are required (see ISO 23730). Traditionally, relatively large organisms have been used as indicators for environmental impact assessments, and labour-intensive surveys using morphological characteristics were mainstream. Compared to larger macrofauna and megafauna, meiofauna in the deep sea have high abundance and biomass and are an important component of deep-sea ecosystems[2]. In addition, meiofauna have a considerable influence on the nutrient cycling in the sediments and sediment stability. Therefore, meiofauna are important as biological indicators used to monitor natural or anthropogenic disturbances[3]. The International Seabed Authority (ISA) guidelines for contractors on the assessment of possible environmental impacts due to exploration activities (see Reference ISBA/25/LTC/6) mandate the reporting of the abundance and diversity of seafloor biotic communities, including meiofauna. Therefore, meiofauna, being ubiquitous as well as sensitive to environmental perturbations, have been chosen as indicator organisms for the analyses in this document. However, traditional methods for meiofaunal community analysis are extremely time-consuming, which is economically problematic due to the costs of conducting EIA as part of resource development. In addition, advanced expertise is required for the identification of meiofauna to the species, genus, or even family level, and the number of experts qualified to do this is limited. Also, if a technician does not have the training or knowledge to identify meiofauna, a dissemination of inaccurate data could result. For these reasons, accurate, efficient and objective analytical tools for the identification of meiofauna are needed.

Thus, the purpose of this document is to establish a convenient protocol for MEIA using meiofauna as biological indicators. The role of EIA is the determination of fluctuation or change in the community structure after environmental impacts. Data of species level composition and population size are essential information to assess the effect of impacts.

Therefore, a meiofaunal analysis, following two methods is proposed, including:

1) imaging flow cytometry;

2) environmental metagenomic analysis.

By this protocol, the population density (number of individuals per unit area) is obtained by analysis using an imaging flow cytometer, and the species composition is acquired by metagenomic analysis. These methods obtain data faster than traditional analysis methods that have been done so far. Further, it is possible to compensate for the disadvantages of both methods with each other. By using both methods complementarily, it becomes possible to grasp communities of meiofauna in the environment objectively, comprehensively, quickly and easily (it is a method aligned to the ISA recommendation, see ISBA/25/LTC/6, mandating to obtain data on population density, biomass and species composition for meiofauna).

Marine technology — Marine environment impact assessment (MEIA) — General protocol for observation of meiofaunal community

1 Scope

This document specifies a general protocol for the observation of the meiofaunal community in the deep seabed.

The standardized method can be used in any phase [baseline data acquisition, monitoring during and after mining (testing)] accompanying resource development, making it easier to compare data beyond differences in workers and waters.

This document is intended for marine environment impact assessments and other occasions where long-term image-based data are required.

2 Normative references

The following document is referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISA[1)] ISBA/25/LTC/6, *Recommendations for the guidance of contractors for the assessment of the possible environmental impacts arising from exploration for marine minerals in the Area*, 2013. Available at https://www.isa.org.jm

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISBA/25/LTC/6 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
meiofauna
animals of the benthic community that are intermediate in size between macrofauna and microfauna, operationally defined as > 32 µm and < 250 µm

[SOURCE: ISBA/25/LTC/6:2013, Annex II.]

3.2
PCR
polymerase chain reaction
DNA sequence synthesis reaction repeated to amplify DNA fragments of target regions of hundreds of thousands of times of genes using template DNA, and two types of short DNA fragments (primers), and DNA polymerases

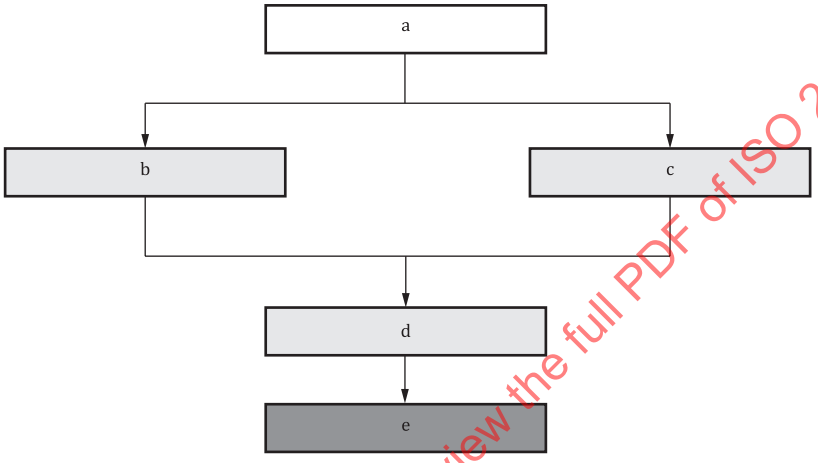
1) ISA: International seabed authority.

3.3
NGS
next generation sequencing/sequencer
<genome sequencing, transcriptome, and amplicon sequencing> device capable of reading nucleotide sequences of huge numbers of genes at high speed

Note 1 to entry: High-throughput or pyro-sequence.

4 Principle

A collected sediment sample is divided into two sub-samples and an analysis is performed by the two methods as illustrated in Figure 1. The results obtained by each method are integrated and analysed. Information on the meiofaunal community at the sampling point can be obtained.



Key
a Sediment samples.
b Morphological data [imaging flow cytometry].
c Molecular sequence data [next-generation sequencing].
d Integrated analysis.
e Result of meiofaunal community.

Figure 1 — Schematic overview of the meiofaunal community analysis procedure

5 Sampling

5.1 Method and equipment

Marine sediments should be quantitatively collected for the analysis of the meiofaunal density and community composition. Examples of equipment (corers) for quantitative sampling include box corers, grab corers, and multiple corers[4].

The corers are deployed from a research ship to the seafloor; thus, it is not suitable for surveys that require a selection of sampling points while observing seafloor conditions. In areas where pin-point sampling is required, e.g. hydrothermal fields where chimneys and mounds exist, sampling using push corers operated by manned or unmanned submersibles is recommended.

In addition, if samples from different deployments, different samplers or corers with different diameters are used, the community composition and vertical distribution of the meiofauna can be changed[5, 6, 7]. Thus, for the same EIA program, identical samplers should be employed, and when comparing results with other programs, differences in sampling gear shall also be considered.

5.2 Sub-sampling

Sediment samples are sub-sampled by cutting into several layers from the surface layer. ISA guidelines suggest the following cutting layers (depths) for meiofaunal investigation: 0 cm to 0,5 cm, 0,5 cm to 1,0 cm, 1 cm to 2 cm, 2 cm to 3 cm, 3 cm to 4 cm, 4 cm to 5 cm.

5.3 Sample preservation

5.3.1 Preservation method before analysis

The collected samples should be preserved by suitable ways to avoid affecting the analytical results.

— Morphological observation; fix by formaldehyde or suitable fixative reagents.

— DNA/RNA analysis; deep-freeze (−80 °C), or suitable buffers for the preservation of DNA/RNA.

5.3.2 Preservation method after analysis

The sample after analysis should be stored in a way that can maintain the same state.

— Morphological observation, samples are preserved by 5 % neutral formalin.

— Extracted DNA/RNA are preserved in the deep-freezer (−80 °C).

5.4 Recording sample information

Sample information shall be recorded, such as core colour, texture, a photograph of the core, sampling place (latitude, longitude, depth), date, sampling method, core number, layer, usage method, storage method, etc.

6 Procedures for imaging flow cytometry (morphological analysis)

6.1 Pre-processing of samples

Before any observation using an imaging flow cytometer, the sediment sample shall be sieved to extract a meiofaunal size fraction. Superfluous sediment particles should be removed as far as possible, because sediment particles obstruct to obtain clear images of meiofauna in most other cases. These qualifications can be realized with the method described by References [8], [9] and [10].

6.2 Observation using imaging flow cytometer

Observation shall be done with instruments suitable for imaging flow cytometry, such as given in Annex A. Examples of imaging flow cytometer are shown in Clause D1. Perform the following:

— operate an imaging flow cytometer in accordance with the manufacture's manual;

— use a colloidal silica solution as a sample flowing medium;

— put the sample and retrieve the image.

6.3 Image processing

The taxon should be identified based on the obtained images with reference to Reference [11] and other relevant data sources. Alternatively, the obtained images can be referred to the database created as necessary or open to the public.

7 Procedures for metagenomic analysis (molecular analysis)

7.1 General

For metagenomic analysis, DNA or RNA shall be targeted. When RNA is used, it can be easily matched with the results of imaging flow cytometry. RNA is subjected to reverse transcription reaction and treated as cDNA (complementary DNA) (see 7.4.2). Examples of metagenomic analysis are shown in Annex B.

7.2 Nucleic acids (DNA/RNA) extraction

A DNA/RNA extraction kit by using beads beating method for soil sample is recommended.

The same DNA/RNA extraction method for the sample collected at the same sampling point during time course observation should be used.

For RNA extraction, purification with DNase should be performed to digest all DNA fragments.

7.3 PCR primers

For metagenomic analysis by NGS, the target gene that is used for taxonomic identification is recommended [e.g. 18S rRNA, ITS (internal transcribed spacer), 28S rRNA (large subunit rRNA), and mitochondrial COI (cytochrome oxidase subunit I)]. The target gene should be chosen depending on the purpose. The primers for this step, the sequence should consist of specific sequence for target gene sequence and overhang adaptor sequence for NGS.

7.4 PCR protocol

7.4.1 DNA

In PCR (amplicon PCR), DNA fragments are amplified by repeated cycles (thermal cycling), which consists of 3 steps, (1) denaturation (denaturing a double stranded DNA into two single stranded DNA molecules by heat), (2) annealing (annealing of a primer with a single stranded complementary DNA), and (3) elongation (elongation of a single stranded DNA from the site where a primer annealed). PCR conditions (number of cycles and reaction temperature) shall be designated for appropriate amplification.

The amplifications should be done in 3 replicate PCR reactions for each sample, after electrophoresis to check the amplicon, combined triplicate PCR reactions of the same sample into a single volume.

7.4.2 RNA (cDNA library production)

A reverse transcription shall be performed for preparing a cDNA library using the extracted RNA. Usually, reverse primer is used to extend the target region. Only one reaction should be done for a single primer extension. The generated cDNA library is used as templates for amplicon PCR (see 7.4.1).

At the same time, to confirm extracted RNA quality (to check the DNA contamination), the same PCR should be performed by RNA before reverse transcription. If the amplified fragments are seen on the agarose gel as a band by RNA template PCR, the RNA may contain co-extracted DNA fragments. In that case, it should purify the RNA.

7.5 Check and purification of PCR amplicon

The amplicons should be checked by agarose gel electrophoresis. If the DNA fragment is amplified with appropriate PCR conditions, the expected length amplicon is seen as a band on the agarose gel.

After checking the amplicons, 3 replicate PCR reactions of the same sample should be combined into the single volume, and proceed to the purification step.

Amplicons shall be purified using magnetic beads to remove residual PCR primers and reagents, according to the supplier's manual. Magnetic beads can purify the large DNA fragments (such as amplicons) without excess primers, non-specific short fragments, salts, and enzymes by paramagnetic bead technology.

7.6 Index PCR

Performance of NGS depends on the type of a sequencer; however, it is known to read 10 million sequences (called reads) in one analysis. So, it is possible to analyse multiple samples simultaneously and be often used to sequence all of mixed multiple amplicons. Therefore, to distinguish the amplicon which derived from different source, tag sequence is added to each amplicon by index PCR. Primers for index PCR shall be comprised overhang adaptor sequence which is the same sequence of amplicon PCR, tag sequence (index sequence), and sequence adaptor for NGS.

7.7 Purification of amplicon by magnetic beads

A purification shall be performed for index PCR products after checking by agarose gel electrophoresis as described in 7.5.

7.8 Concentration measurement

In order to equalize the DNA concentration of each amplicon used in NGS, the concentration shall be measured. For this purpose, quantitative PCR or fluorometric quantitation can be used.

7.9 Quality check for amplicon by an automated electrophoresis system using microfluidic chip

If necessary, a quality check for created amplicons should be performed using an automated electrophoresis system.

7.10 Sequencing by a next generation sequencer

Follow the protocol of each manufacturer as to how to use the equipment.

The samples mixed to equal concentrations shall be denatured beforehand and applied for sequencing.

7.11 Data processing

The same program shall be used to compare the samples. Some analysing methods (programs) for NGS data are available as freeware softs. Examples of metagenomic analysis programs are shown in Clause D2. The parameters affect analysed data, especially taxon assignment. Analysing programs used, database, and other related information shall be recorded.

8 Data and sample management

DNA sequence and its metadata should be submitted to the DNA database (GenBank/DDBJ/ENA).

Appearance data of organisms should be described and registered in compliance with Darwin Core (DwC[12]) (also adopted in GIF and CoML) including image files.

9 Combination of morphological analysis and molecular analysis (integrated analysis)

The morphological data derived from imaging cytometry provide information on the taxonomical group and its population size (density) of the meiofaunal community. The nucleotide sequence data derived from metagenomic analysis provide also the community composition and diversity. By conducting these

two analyses for the same sample and by integrating the two data, the analytical method complement each other (see Annexes B and C).

Annex A
(informative)

Example procedure for the analysis of the meiofaunal community by imaging flow cytometry

A.1 General

Although few instruments are known for imaging flow cytometry, this Annex provides an example of the procedure by FlowCam[2)], see References [10] and [13].

A.2 Sampling and sub-sampling

Core sampling is the same method as described in Clause 5. The following procedure provides details for sub-sampling.

1. Make filtered seawater using a 0,22 µm filter and keep it in a wash bottle.
2. Place the collected sediment core onto an extruder.
3. Using a siphon tube, remove the seawater above the sediment to approximately 1 cm above the sediment surface.
4. Using a syringe, remove the remaining seawater 1 cm above the sediment and place in a plastic bag.
5. Using a spatula, slice the sediment cores into layers: 0 cm to 0,5 cm, 0,5 cm to 1 cm, 1 cm to 2 cm, 2 cm to 3 cm, 3 cm to 4 cm, and 4 cm to 5 cm from the surface. Place each layer into separate plastic bags.
6. Use filtered seawater to rinse off any sediments remaining on the spatula into the same plastic bag holding the layer.
7. Use pure water or clean tap water to completely wash the spatula and dry it thoroughly with paper wipes each time the spatula is used.

A.3 Fixation and storage of samples

Each sediment layer (sub-sampling of core sample) should be fixed and preserved separately. Add the neutral buffered formalin to the samples for a final concentration of about 5 %. Seal tightly and store in room temperature. Fixation with formalin is suitable for preservation of the morphology of meiofauna.

2) FlowCam is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

A.4 Analysis using flow cytometer

A.4.1 Pre-processing

Based on differences in density of meiofaunal specimens and sediment particles, a centrifugal separation method using colloidal silica solution has been devised[8, 9]. This method was modified to apply the imaging flow cytometry observation for meiofauna[10]. The procedures are as follows.

1. Add rose bengal staining solution into the sediment samples (final concentration, 0,05 g/l) and leave to stand for at least one night.
2. Sequentially pass the sediment samples through 1 mm, 250 µm and 63 µm mesh size sieves. Rinse away the seawater using pure water or clean tap water as much as possible.
3. Using pure water or clean tap water, transfer the fractions retained on the 1 mm and 250 µm mesh size sieves to 50 ml conical tubes. Add formalin for storage.
4. Use a spoon to transfer the fraction retained on the 63 µm mesh size sieve to a 50 ml conical tube. Keep the tube as dry as possible.
5. Rinse any sediments remaining on the sieve into a conical tube using a wash bottle containing Ludox HS-40[3)].
6. Centrifuge the conical tube containing sediment/Ludox HS-40 at 800 *g* for 10 min.
7. Transfer the supernatant to the 63 µm sieve.
8. Add more Ludox HS-40 to the pellet, resuspend, and centrifuge again; repeat once more for a total of three times.
9. Using Ludox HS-40, transfer particles retained on the sieve to a conical tube.

A.4.2 Observations using FlowCam

1. Set the focus using 50 µm size marker beads. If the auto-focus feature is installed, follow the instructions supplied with the flow cytometer. If not, set the focus manually.
2. Fill the flow line with colloidal silica (commercially available as Ludox-HS40).
3. Start the observation.
4. Introduce the sample using a Pasteur pipette while occasionally stirring the sample in the conical tube.
5. Conclude the observation when the entire sample has flowed through the flow line.
6. Rinse the flow line with water.
7. Transfer recaptured samples to a sieve, and use pure water or clean tap water to rinse the collected material into a new conical tube. Save and store the samples as appropriate.

A.4.3 Special remarks for observation and sample preservation after examination

Record information such as the magnification of the objective lens used and the created file name in a log.

Colloidal silica solution will still remain in the conical tubes with the recaptured samples. If colloidal silica solution is left to dry, it will form crystals centred on the meiofaunal specimens as nuclei.

3) Ludox HS-40 is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Therefore, the solution should be immediately replaced with pure water or clean tap water and the samples should be saved and stored as appropriate.

A mixture with 2:3 ratio as volume of colloidal silica solution and water can prevent drying and crystallization during the observation period.

A.5 Image analysis

A.5.1 Image analysis software

The analytical software VisualSpreadSheet[4)] is included with the FlowCam system and is introduced as an example of image analysis software.

Organisms are stained red (or pink) with rose bengal dye, which allows to pick the organisms using a sorting function by colour.

1. Start VisualSpreadSheet and open the acquired file.
2. Use the sort function to sort images according to the red/green ratio.
3. Go to the end of the sorted images and select images of organisms.
4. Save the selected images to a separate file.
5. Count the organisms by group at the taxonomic levels determined from the images.

Images obtained by the FlowCam are saved in TIFF format, which allows other image analysis software (for example, ZooImage[4)]) to be used.

4) VisualSpredSheet and ZooImage are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

Annex B
(informative)

Example procedure for analysis of meiofaunal community by NGS

B.1 General

This Annex provides an example of environmental DNA amplicon sequencing using MiSeq (Illumina)[5], see Reference [14].

B.2 Sampling and sub-sampling

The sampling and sub-sampling are performed according to the methods described in Annex A.

The obtained samples should be stored in a deep-freezer or appropriate buffer (including ethanol) for nucleic acid extraction. Formalin should not be used for this purpose.

B.3 DNA extraction

DNA extraction kits are available commercially, a kit that can perform cell disruption conducted by combined methods such as physical disruption with beads-beating and chemical disruption with surfactants and cell wall digesting enzymes should be selected.

B.4 Construction of amplicons

B.4.1 Primers

For example, V1-V2 region of 18S rRNA gene is used as the target gene to detect Eukaryotes (including meiofauna) in the sediment sample. The primers sequences are as follows[14, 15, 16]:

Forward primer (F04): 5’ > 3’ [Overhang adaptor sequence]-GCTTGTCTCAAAGATTAAGCC

Reverse primer (R22mod): 5’- > 3’ [Overhang adaptor sequence]-CCTGCTGCCTTCCTTRGA

NOTE Overhang adaptor sequence is specified for NGS amplicon analysis, and dependent on the sequencing kit or sequencer.

B.4.2 PCR enzyme and component of reaction mixture

Various enzymes for PCR are currently commercially available. It is required to include high fidelity, low attenuation (slow) even when the number of cycles increases, and resistance to amplified nucleotide sequences and inhibitors.

An example of PCR reaction mixture is as follows: total volume 20 µl: 2 µl of 10 × buffer, 1,6 µl of dNTP, 0,4 µl of 10 µM forward primer, 0,4 µl of 10 µM reverse primer, 0,1 µl of ExTaq (Takara)[6], 1 µl of template DNA (extracted DNA) (0,5 to 1 ng/µl), 14,5 µl of DNase/RNase free distilled water.

5) MiSeq (Illumina) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

6) ExTaq (Takara) is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

B.4.3 PCR cycle condition

An example of PCR cycle condition is as follows: 1 min of Taq DNA polymerase activation at 96 °C; next, 20 to 30 cycles, denaturation at 96 °C for 25 s; annealing at 55 °C for 45 s; extension at 72 °C for 1 min; then final extension at 72 °C for 7 min: by using thermal cycler.

B.4.4 Confirmation of PCR amplicon

PCR amplicons are confirmed by agarose gel electrophoresis. Commonly, high purity agarose (e.g. analytical grades) should be used, and made a 1% to 2% (weight per volume) solution by TAE (tris-acetate-ethylenediaminetetraacetic acid) or TBE (tris-borate-ethylenediaminetetraacetic acid) buffer. Moreover, a DNA ladder marker with an appropriate range should be electrophoresed simultaneously in order to confirm the length of amplified fragment. Several dyes are available to visualize PCR amplified fragments. Sensitivity of detection of fragments depends on dyes; therefore, it is recommended to use dyes with higher sensitivity in order to identify presence of nonspecific PCR products as much as possible. If PCR has finished successfully, amplified DNA fragments of the expected length are shown as bands on the gel.

B.4.5 Purification of PCR amplicon

After confirming the presence of an appropriate band by agarose gel electrophoresis, unnecessary primers and reagents are removed from the PCR reacted mixture for the next step. Purification of amplicon is performed by magnetic beads.

B.4.6 Index PCR

To add the index tag sequence, an index PCR is carried out by the primer sets which have specific sequences. For this step, kits are commercially available. After this step, PCR products are confirmed by agarose gel electrophoresis, and purified by magnetic beads.

B.4.7 Concentration measurement

For NGS analysis, concentrations of each amplicon should be adjusted so that all products are mixed at equal concentrations. For this purpose, quantitative PCR or fluorometric quantitation can be used. When MiSeq is used for an amplicon sequencing, for example, a specialized kit is commercially available for quantitative PCR. Or, when fluorometric quantitation is used, some kits and devices are supplied commercially.

B.4.8 Quality check of amplicon by an automated electrophoresis system using microfluidic chip

Fragment lengths of samples purified by magnetic beads and impurities contained in amplicons (including products with unpredicted fragment lengths) are examined by an automated electrophoresis system using microfluidic chip such as bioanalyzer, Agilent 2100 (Agilent)[7], MultiNA (Shimadzu)[7]. The quality of samples that are to be analysed by NGS is checked by this process.

B.5 Sequencing

Sequencing of samples where each sample is adjusted to have the same concentration are performed by a next generation sequencer. Sequencing methods are different depending on the manufactures and type of instruments.

7) Agilent 2100 (Agilent) and MultiNA (Shimazu) are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

B.6 Data analysis

Sequences generated by a NGS are sorted according to an identification tag attached using index PCR and nonspecific DNA fragments determined by length of nucleotides are removed (removal of low quality reads). The generated sequences are sorted into a classification unit called operational taxonomic unit (OTU) according to the similarity of the sequences, and the classification group and composition ratio are estimated in the sample using OTU as a unit. Software and database used for data analysis should be recorded.

Annex C
(informative)

Example procedure for quantitative analysis of meiofaunal community by NGS

C.1 General

This Annex provides an example procedure of quantitative amplicon sequence analysis by NGS using MiSeq, see References [14], [17] and [18]. This method allows to obtain the biomass information as numbers of copies of target gene as well as compositional information. This makes it easy to compare metagenomic data with image data, resulting in a more accurate estimation.

C.2 Sampling and sub-sampling

The sampling and sub-sampling are performed according to the methods described in Annex A.

The obtained samples should be stored in a deep-freezer or appropriate buffer (including ethanol) for nucleic acid extraction. Formalin fixation should not be used for this purpose.

C.3 DNA extraction

DNA extraction kits are available commercially, a kit that can perform cell disruption conducted by combined methods such as physical disruption with beads-beating and chemical disruption with surfactants and cell wall digesting enzymes should be selected.

C.4 Construction of amplicons for quantitative NGS

C.4.1 Primers

Three type primers are used for amplicon construction for quantitative NGS. One primer (first primer) including random-sequence barcodes with target sequence is used for single primer extension (SPE) reaction. A second primer and third primer are used for amplicon construction by PCR.

For example, V1-V2 region of 18S rRNA gene is used as the target gene to detect Eukaryotes (including meiofauna) in the sediment sample. Each primer sequence is shown as follows:

First primer (N8-F04): 5'- > 3' [Overhang adaptor sequence]-nnnnnnnn-GCTTGTCTCAAAGATTAAGCC

Second primer (N8-R22mod): 5'- > 3' [Overhang adaptor sequence]-nnnnnnnn-CCTGCTGCCTTCCTTRGA

Third primer (a part of overhang adaptor sequence of the first primer): example for Illumina sequencer: ACACTCTTTCCCTACACG.

NOTE 1 Overhang adaptor sequence is specified for NGS amplicon analysis, and dependent on sequencing kit or sequencer.

NOTE 2 Sequence of 'nnnnnnnn' (N8) is random barcode for counting.

C.4.2 SPE reaction

A random sequence (N8) containing a primer (first primer) is used to bind the counting tag sequence of the target gene sequence. As the purpose is to label the quantification sequence, no PCR amplification is performed. Therefore, the primer used in this reaction is the first primer only.

An example of SPE reaction mixture is as follows: total volume 20 µl/PCR tube: 2 µl of 10 × buffer, 1,6 µl of dNTP, 0,4 µ of 10 µM first primer, 0,1 µl of ExTaq (Takara), 1 µl of template DNA (extracted DNA) (0,5 to 1 ng/µl), 14,5 µl of DNase/RNase free distilled water.

NOTE The concentration of extracted DNA is measured in advance, and the concentration of each sample is equalized.

An example thermal conditions for SPE reaction is as follows: 1 min of Taq DNA polymerase activation at 96 °C; next, denaturation at 96 °C for 25 s; annealing at 55 °C for 45 s; extension at 72 °C for 1 min; then final extension at 72 °C for 7 min: by using thermal cycler.

C.4.3 Digestion of first primer

Enzymatic digestion of excess SPE primers (first primer) is performed to avoid incorporation of N8-random sequence during subsequent PCR cycles.

For example, after the SPE reaction, 1 µl of exonuclease I (10 to 20 units) is added to make the total volume of 21 µl, and the excess primer not used for the reaction is digested by incubating at 37 °C for 120 min. The exonuclease is then inactivated by incubation at 80 °C for 30 min.

C.4.4 Check of first primer digestion

After the SPE reaction, it is verified that the exonuclease treatment is properly performed, and whether or not the first primer is completely digested and subjected to PCR amplification by adding only the second primer. If the amplicon is obtained in this PCR, the first primer is still remained in the SPE reacted mixture. In such case, digestion should be performed once again.

An example of PCR reaction mixture is as follows: total volume 20 µl: 2 µl of 10 × buffer, 1,6 µl of dNTP, 0,4 µl of 10 µM second primer, 0,1 µl of ExTaq (Takara), 1 µl of SPE product, 14,5 µl of DNase/RNase free distilled water.

An example of PCR cycle condition is as follows: 1 min of Taq DNA polymerase activation at 96 °C; next, 40-45 cycles, denaturation at 96 °C for 25 s; annealing at 55 °C for 45 s; extension at 72 °C for 1 min; the final extension at 72 °C for 7 min: by using thermal cycler. Increase the number of thermal cycles to avoid missing slight containing of first primer in the SPE product.

See C.4.7 for confirmation of PCR amplification by-products.

PCR amplification by second and third primers is performed using as a template SPE product confirmed to have no undigested first primer (amplification product preparation).

C.4.5 PCR enzyme and component of reaction mixture

An example of PCR reaction mixture is as follows: total volume 20 µl: 2 µl of 10 × buffer, 1,6 µl of dNTP, 0,4 µl of 10 µM third primer, 0,4 µl of 10 µM second primer, 0,1 µl of ExTaq (Takara), 1 µl of template DNA (extracted DNA) (0,5 to 1 ng/µl), 14,5 µl of DNase/RNase free distilled water.

C.4.6 PCR cycle condition

An example of PCR cycle condition is as follows: 1 min of Taq DNA polymerase activation at 96 °C; next, 20–30 cycles, denaturation at 96 °C for 25 s; annealing at 55 °C for 45 s; extension at 72 °C for 1 min; the final extension at 72 °C for 7 min: by using thermal cycler.