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

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Contents

Page

Foreword	iv
Introduction	v
1 Scope	1
2 Normative references	1
3 Terms and definitions	1
4 Principle	2
5 Sampling	2
5.1 Method and equipment.....	2
5.2 Sub-sampling.....	3
5.3 Sample preservation.....	3
5.3.1 Preservation method before analysis.....	3
5.3.2 Preservation method after analysis.....	3
5.4 Recording sample information.....	3
6 Procedures for imaging flow cytometry (morphological analysis)	3
6.1 Pre-processing of samples.....	3
6.2 Observation using imaging flow cytometer.....	3
6.3 Image processing.....	3
7 Procedures for metagenomic analysis (molecular analysis)	4
7.1 General.....	4
7.2 Nucleic acids (DNA/RNA) extraction.....	4
7.3 PCR primers.....	4
7.4 PCR protocol.....	4
7.4.1 DNA.....	4
7.4.2 RNA (cDNA library production).....	4
7.5 Check and purification of PCR amplicon.....	4
7.6 Index PCR.....	5
7.7 Purification of amplicon by magnetic beads.....	5
7.8 Concentration measurement.....	5
7.9 Quality check for amplicon by an automated electrophoresis system using microfluidic chip.....	5
7.10 Sequencing by a next generation sequencer.....	5
7.11 Data processing.....	5
8 Data and sample management	5
9 Combination of morphological analysis and molecular analysis (integrated analysis)	5
Annex A (informative) Example procedure for the analysis of the meiofaunal community by imaging flow cytometry	7
Annex B (informative) Example procedure for analysis of meiofaunal community by NGS	10
Annex C (informative) Example procedure for quantitative analysis of meiofaunal community by NGS	13
Annex D (informative) Useful information for users	16
Bibliography	17

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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; <https://standards.iteh.ai/catalog/standards/sist/4246a3fe-58c6-4cdb-8867-bf95af865ce0/iso-23732-2021>
- 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).

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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.

ISA¹⁾ 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> <https://standards.iteh.ai/catalog/standards/sist/4246a3fe-58c6-4cdb-8867-bf95af865ce0/iso-23732-2021>

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.

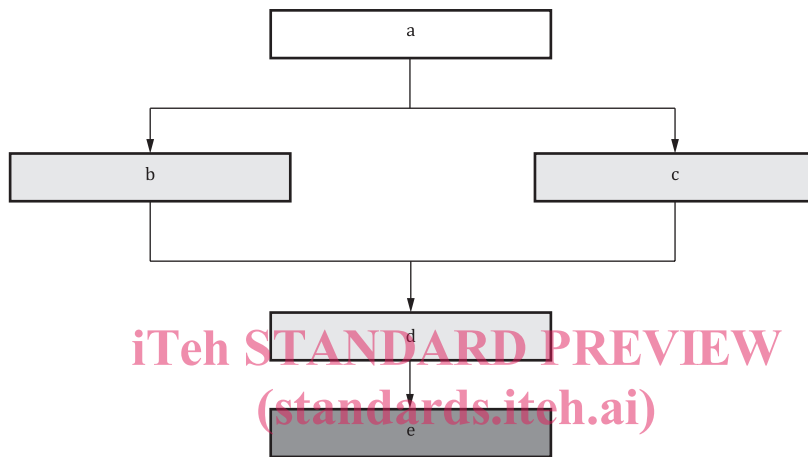
3.3
NGS

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.



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Key

- a Sediment samples. <https://standards.iteh.ai/catalog/standards/sist/4246a3fe-58c6-4cdb-8867-bf95af865ce0/iso-23732-2021>
- 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 ($-80\text{ }^{\circ}\text{C}$), 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 ($-80\text{ }^{\circ}\text{C}$).

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.

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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

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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.

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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