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Marine environment impact assessment (MEIA) — Specification for marine sediments in seabed areas — Survey of interstitial biota

Évaluation de l'impact environnemental marin — Spécifications relatives aux sédiments marins dans les zones de fonds marins — Étude du biote interstitiel

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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 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

Interstitial biota in marine sediments refers to the benthic life forms inhabited or deposited in the interstitial spaces between sediment particles, including marine microorganisms, benthic virus, microbenthos and meiobenthos. They cover the six “kingdoms” of life in the three-domain taxonomic system: Archaea, Bacteria, Fungi, Protista, Plantae and Animalia. Interstitial biota in marine sediments are so small that cannot be obtained and analysed by conventional methods for marine biological survey; they are numerous and complex; they have diverse functions, remarkable ecological significances and rich gene resources; they are ubiquitous and make up the basic components of the life system in marine sediments. Sediment interstitial biotas are the most abundant and complex life groups in the estuaries, intertidal zones, shelf shallow seas and deep sea. They play key roles in the regulation of material and energy flows in benthic ecosystems.

In seabed areas, a number of large international research programs have been carried out, such as the ocean drilling program (ODP) and the international ocean discovery program (IODP). Interstitial biota in marine sediments surveys have been key to solve scientific problems in relevant fields, such as marine biodiversity, oil and gas resource exploration, marine carbon cycle, global change, monsoon rainfall, ice melting, ocean acidification and deep-sea biological resources. But so far the lack of an International Standard leads different countries to use different regulations and technologies on the investigations, resulting in barriers to comparing research results in international cooperation.

This document provides relevant technical approaches for the investigation of sediment interstitial biota in seabed areas. Its purpose is to reflect the recent developments of modern marine science and technology to facilitate international cooperation. It is applicable to investigations and evaluations of marine sediment biodiversity in seabed areas, favouring the development and utilization of marine biological resources, the comprehensive environmental exploration, ecological environment assessment, protection and management, etc. The specifications in this document incorporate technical advances and technological key points reflecting current state-of-the-art and international practice.

Marine environment impact assessment (MEIA) — Specification for marine sediments in seabed areas — Survey of interstitial biota

1 Scope

This document provides requirements and recommendations for conducting marine surveys of interstitial biota in marine sediments. It includes the specification of technical methods for the investigation of marine sediments, foraminifera, ostracoda, radiolaria, diatoms, coccoliths, sedimentary sporopollen, benthic viruses, benthic microbes (including bacteria, archaea and fungi), benthic microalgae, benthic protozoa and metazoan meiobenthos.

This document is applicable to marine surveys in diverse benthic habitats at any seabed, such as benthic sediments of coastal zones, shallow seas, or deep-sea waters.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology 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
marine sediment
substances under the action of crustal surface geology, where the original products such as weathered rocks, metamorphic rocks and pre-existing *sedimentary rocks* (3.2) of the parent rocks (i.e. magmatic rocks, metamorphic rocks and sedimentary rocks) are transported, settled or precipitated by biogenic, volcanic and cosmic phenomena as loose unconsolidated deposits on the sea floor

3.2
sedimentary rock
one of the three major types of rocks that make up the lithosphere (the other two are magmatic rocks and metamorphic rocks), which are formed from the weathering products of a parent rock (or any pre-formed rock), biogenic materials, volcanic material, cosmic material and other original material, and sedimentation after the formation of rock diagenesis

3.3
interstitial biota
benthic life forms that inhabit or are deposited in the interstices between sediment particles

Note 1 to entry: It includes *marine microorganisms* (3.6), benthic viruses, *microbenthos* (3.4), and meiobenthic organisms. In terms of individual sizes, interstitial biota in *marine sediments* (3.1) cover femto-level with a size of less than 0,2 µm, pico-level (0,2 µm to 2 µm), nano-level (2 µm to 20 µm) and micro- and meio-level benthic organisms of more than 20 µm.

3.4
microbenthos
unicellular prokaryotic and eukaryotic microbes living on the surface, and within the interstices, of sediments, which can be trapped by 0,2 µm membrane filtration

Note 1 to entry: Mainly benthic bacteria, benthic microalgae and *benthic protozoa* (3.5). See Figure A.1 for examples of major groups. In terms of sizes of individuals, the microbenthos covers the pico-level of less than 2 µm, the nano-level (2 µm to 20 µm) and the micro-level of more than 20 µm.

3.5
benthic protozoa
unicellular eukaryotes whose life history is entirely or mostly associated with sedimentary environments

Note 1 to entry: It includes heterotrophic flagellates, ciliates, amoebae, etc.

3.6
marine microorganism
microeukaryotes and metazoans included in sedimentary investigations and marine geological surveys, including extant and fossil species of various groups

Note 1 to entry: It includes foraminifera, ostracoda, radiolaria, diatoms, calcareous fossils, sporopollen, pteropoda, ichthyoliths, etc.

3.7
benthic microbe
unicellular and small acellular organism with simple structure and a variety of physiological types that inhabits sedimentary environments

Note 1 to entry: It includes bacteria, archaea and fungi.

3.8
metazoan meiobenthos
metazoan meiofauna
small metazoa and larvae of large metazoans living in sedimentary environments that can pass through a 500 µm aperture mesh but are retained on a 42 µm to 31 µm aperture mesh

Note 1 to entry: The main groups include nematodes, copepods, tardigrades, ostracods, gastrotrichs, priapulid worms, bivalves, arthropods, acarina, polychaetes, kinorhyncha, rotifers, etc. Several major groups are shown in Figure A.2.

4 General

4.1 Technical design

Surveys of interstitial biota in sediments should be designed in terms of survey-related items, including survey section, station, object, detail, method, date, frequency, device, personnel quality, ship, equipment, expected results and survey plan. The establishment of the investigation plan shall refer to the requirements of the related survey plan.

4.2 Basic recommendations for the surveys

4.2.1 Survey object

The survey object can include marine sediments, foraminifera, ostracoda, radiolaria, sedimentary diatoms, coccoliths, sporopollen, benthic viruses, benthic microbes, benthic microalgae, benthic protozoa, and metazoan meiobenthos. Specific objects may be adjusted or designed according to the survey plan.

4.2.2 Auxiliary parameters

Auxiliary observation objects can be added, if necessary, to the survey. These can be designed on the basis of the survey plan.

4.2.3 Recommendations for the sampling equipment

The sampling equipment should follow the recommendations of related clauses in this document.

The common equipment for sediment sampling includes box-corers, multicorers, grab samplers, gravity samplers or drills. Deep-sea sampling equipment can also include remotely-operated vehicles (ROV) and manned submersibles. The main disadvantage of grab sampling is that it is not possible to preserve the seafloor surface sediments, and it rarely preserves depositional sequences.

4.2.4 Auxiliary equipment on board ship

The auxiliary equipment on board ship includes the following:

a) winch systems: a winch system is required for the operation of general multicorers or box-corers; a winch system for armoured cable is required for the operation of television (TV) multicorers and TV box-corers;

b) large A frames: the lifting height should be 4,5 m or more.

4.2.5 Sampling method and scope of application

4.2.5.1 Sediment sampling

Sediment sampling can be used to survey marine sediments for foraminifera, ostracoda, radiolaria, sedimentary diatoms, coccoliths, sporopollen, benthic viruses, benthic microbes, benthic microalgae, benthic protozoa, and metazoan meiobenthos. Sampling should follow the recommendations of the related clauses in this document.

4.2.5.2 Trawl sampling

Trawling can be used for auxiliary sampling of planktonic foraminifera, ostracoda, radiolaria, protozoa, etc.

4.2.5.3 Water sampling

Water sampling can be used to survey planktonic foraminifera, living radiolaria, etc. Sampling shall satisfy the requirements of related clauses in this document.

4.3 Sampling

4.3.1 Sediment sampling

Sediments should be collected by the specified sampler. Staff should strictly abide by the operating procedures and pay attention to the condition of the sampler. Surface samples or core samples shall be collected, treated and stratified according to the requirements of the survey. Sediment sampling procedures in offshore and coastal areas should follow the recommendations in ISO 5667-19:2004, 5.1. Stratigraphic equipment for processing surface samples is shown in Figures B.1 and B.2. In abnormal situations, replicate samples should be collected.

4.3.2 Trawl sampling

Sampling nets can be used in trawl sampling. The speed of net deployment and net withdrawal should be strictly controlled, and the time of net arrival at the target should be determined accurately. The

condition of the net should be noted, and effective measures should be adopted if the situation is abnormal. The net should be washed, and the samples collected carefully, especially biological samples that can stick to the net and pipe.

4.3.3 Water sampling

Water can be collected using a water sampler. Before deployment, check that the cover is open and the faucet is closed. Deploy the water sampler to the target area and maintain its position for a precise time. Water sampling and sample treatments shall satisfy the requirements of related clauses in this document.

4.3.4 Records

All survey objects should be recorded according to the related clauses in this document. Examples of sample record tables are shown in Annex C (Tables C.1, C.2, C.3).

Take additional photos and videos in the case of abnormal phenomena or new discoveries.

4.4 Sample analysis

4.4.1 Sample treatment

Treat the samples according to the related clauses in this document.

4.4.2 Sample measurement

Measurement items should be determined according to the related clauses in this document.

4.4.3 Sample treatment and storage

Samples can be stratified, fixed and dyed on-site according to the related clauses in this document.

Preserve samples after analysis, measurement and identification, either complete or partial, according to the application and academic value. Samples storage should comply with the survey plan.

4.4.4 Sample identification and enumeration

Identify the organisms to the species level wherever possible, and enumerate according to the related requirements in this document. Observe and analyse the samples by microscopy, and record in a table similar to Tables D.1, D.2, D.3, D.4.

4.4.5 Sample data analysis

Data to be analysed are mainly related to community structure, abundance, relative abundance, or dominant groups of interstitial biota in marine sediments. Data analysis can refer to the related clauses in this document.

Analyse data with statistical software. Annex E provides methods of calculation of the community parameters. Table E.1 shows biomass conversion factors.

4.5 Basic recommendations of data organization

4.5.1 Organization of data

4.5.1.1 Quantification and statistics

Quantify and analyse the results of identification and enumeration using the formulas and formatting given in this document.

4.5.1.2 Fill in forms

Fill in the forms according to related provisions of this document.

4.5.1.3 Draw charts

Draw charts to show data according to the requirements of this document.

4.5.1.4 Voyage report

A voyage report, including related requests according to the survey plan, should be completed by the scientist responsible to the object after the survey.

4.5.2 Data archiving, acceptance and achievements appraisal

Data archiving, acceptance and appraisal of achievements should be carried out with related requests in this document.

4.6 Survey results

4.6.1 Original records

Original records made during the survey, including those of sediment, sedimentary rock, organisms, and water samples, field descriptions, location records, etc. shall be retained. This is first-hand information and includes the primary results of the survey.

4.6.2 Maps or drawings

Draw maps or figures on a prescribed scale based on data analysis and calculation.

4.6.3 Investigation report

The investigation report should include the following.

a) Preface, including the survey plan, survey area, content and workload of the objects, work time (indoor and outdoor).

b) Survey and data consolidation, including working methods, station design, quality of original data, methods of data consolidation, accuracy of results.

c) Results, including sediment types, suspension concentration, structure, abundance and distribution of main biota.

d) Data analysis, including method and its basis, distribution characteristics and comprehensive analysis of every element.

e) Conclusions, including suggestions for further work.

4.7 Data archiving

The following data should be archived:

a) survey contract or survey plan;

b) reports, technical design, program report and statements of approval;

c) executable plan and sampling stations;

d) original record of the survey, experiments undertaken and analysis;

e) report and explanation of the results;

f) tables, figures (including base map), photographs with explanatory legends;

g) voyage report and objects summary report;

h) investigation report and acceptance of the results;

i) tables of objects members and reconciliation of budget.

Related requests concerning data archiving, file quality and acceptance of the results can refer to the related clauses in this document.

4.8 Program and quality control

The institution executing the objects can provide the quality prospectus, including quotations of specifications and articles, summary of survey plan, quality target, organization and responsibilities of the execution institution and assurance measures of quality prospectus. Quality control can refer to the related clauses in this document.

Measures for ensuring quality control include the following.

a) Establish a quality control system: In addition to accepting supervision from administrations and technical supervision agency, a process of self-checking or quality control can be adopted. Formulate the quality control systems. Define the duty of quality control and programs of quality supervision and examination. Execute provisions of quality control strictly.

b) Execute quality control: There shall be clear quality requirements in the survey plan. Analyse specific quality of articles and data. Instruments, equipment, tools and materials can conform to the quality standards. Take specific field records for samples and data obtained at sea. Check original samples and data after the survey. Analysis and identification of samples, data consolidation and counts can be based on facts. Archiving of documents, data and results shall fulfil these requirements.

c) Full participation in quality control: Staff participating in the survey can have relevant professional skills. It is the duty of all staff to maintain quality control requirements.

5 Survey of the sediment

5.1 Principle

Analyses of sediment characteristics, including sediment classification, physicochemical characterization and granulometry, to obtain information on the substrate environment for surveying the interstitial biota.

5.2 General provisions

The general provisions include the following:

a) qualitative and/or quantitative analyses should be selected based on the survey plan;

b) the samples shall not be mixed or contaminated;

c) the characteristics surveyed should primarily be the grain size, the type of sediment and its biological components.

5.3 Collection and preservation of the samples

Seafloor surface sediments, columnar cores, or undisturbed drill cores can be collected. A site description of the samples should be made, and certain physicochemical factors of the samples should be measured on site. The samples can be then sealed for refrigeration- or cryo-preservation, depending on the survey plan.

When measuring the physicochemical characteristics and grain sizes, surface sediments and columnar or drill core samples can be collected from various strata at different depths. Each collected sample can be sealed in a polyethylene bag and related information including sampling site, sampling time and location coordinates should be marked on the bag. Parameters including pH, Eh, temperature and Fe^{3+}-to-Fe^{2+} ratios should be measured at the sampling site. For the measurement of other physicochemical characteristics, the samples should be stored in a refrigerator at a temperature of -20 °C and transported to the laboratory for analysis.

5.4 Measurement of environmental factors

The environmental factors measured include suspended particulate matters, moisture content (%), total organic carbon content (%), chlorophyll-a (Chl a) and pheophytin-a (Ph a) content, pH, Eh, temperature and the ratio of Fe^{3+} to Fe^{2+}. The items for measurement can increase or decrease according to the survey plan.

5.5 Measurement of the age of the sediments

As per the requirements of the survey, the sediments of columnar or drilling cores shall be dated by the methods appropriate to each sample.

For sediments from columnar cores and the top of drilling cores, ^{210}Pb or ^{137}Cs dating shall be conducted in accordance with the requirements of the survey plan; for drilling cores, ^{14}C dating, optically stimulated luminescence (OSL) dating, and palaeomagnetic chronology can be selectively conducted depending on the survey plan.

5.6 Measurement of the contents of heavy metals, organic pollutants and oils in the sediments

The survey for heavy metals (Hg, Cu, Pb, Cd, Cr, Zn and As), organic pollutants (arsenite, cyanide, organic-chlorine pesticide and volatile phenol) and oils shall be carried out with related provisions in this document. The specific analyses conducted for each sample can be selected on the basis of the specific conditions of the sea bed.

5.7 Measurement of the grain size of the sediments

The grain size of the sediments should be measured.

5.8 Analysis of the mineral compositions in the sediments

In accordance with the recommendations of the survey, the contents of detrital and clay minerals in the sediments can be selectively measured.

5.9 Classification of the substrate type in offshore sediments

According to the requirements of the survey, the sediments should be classified and named using the ternary diagram classification methods proposed by References [15], [22] and [52].

a) On the basis of the Shepard's ternary diagram, the sediments are classified into nine types: clay, sandy clay, silty clay, clay sand, clay silt, sand, silty sand, sandy silt, and silt. The classification is based on the analysis of the grain size of sediments.

b) Using Folk's detrital-sediment classification method, the sediments are classified into gravel-bearing and gravel-free types. Specifically, through a ternary diagram classification, the gravel-bearing sediments are divided into 14 types: gravel, sandy gravel, argillaceous sandy gravel, argillaceous gravel, gravelly sand, gravelly argillaceous sand, gravelly silty mud, gravel-bearing sand, gravel-bearing argillaceous sand, gravel-bearing mud, sand, argillaceous sand, sandy mud, and mud.

c) Using the ternary diagram method, gravel-free sediments are divided into the following ten types: sand, silty sand, argillaceous sand, clay sand, sandy silt, sandy mud, sandy clay, silt, mud, and clay.

d) In addition to the above classifications, the percentages of gravel, sand, silt and clay components in the sediments are determined based on the results of grain-size analysis.

5.10 Classification of the deep-sea sediments

Deep-sea sediments should be classified and named using the ternary diagram classification method for deep-sea sediments (see Reference [15]).

5.11 Survey of the biological components in the sediments

5.11.1 Summary of the methods

This study can include the identification and analysis of foraminifera, ostracoda, radiolaria, sporopollen, and calcareous nanofossils (such as coccoliths). The identification of these fossils can be carried out regarding the identification of palaeontology in sediments.

The preparation, identification and analysis of the other sedimentary biodetritus, such as ichthyoliths, fish otoliths, stoneworts, corals, bryozoans and pteropods, can be performed regarding the identification of sedimentary palaeontology.

5.11.2 Technical recommendations

The technical recommendations include the following:

a) qualitative and/or quantitative analyses should be selected based on the survey plan;

b) the samples shall not be mixed or contaminated;

c) the abundance of a single species should be denoted as the number per gram of dry sample, or the number per 50 g of dry sample or the number per 10 cm^2;

d) the primary factors to be surveyed are the compositions, abundances and dominant species of major biological components.

5.11.3 Collection and preservation of the samples

According to the requirements for the different objects, seafloor sediments, columnar or undisturbed drilling cores can be selectively collected, sealed on site, and refrigerated or frozen for preservation.

5.12 Organization of data

The organization of data from the surveyed sediments should follow the specific recommendations of this document.

6 Survey of foraminifera

6.1 Principle

Based on the collection, preparation, preservation, identification and analysis of living specimens and tests of the foraminifera, the distribution and preservation of foraminifera in the sediments are investigated to reflect the hydrological and environmental changes in the area.

6.2 General provisions

The general provisions include the following.

a) Design the sampling method and the sampling process according to the survey plan. For sea floor surface sediments, collect from the top 0 cm to 2 cm layer; for deeper sediments, collect at 2 cm intervals from core samples, or at different intervals according to the survey plan.

b) Determine the sampling volume according to the survey plan. Generally, use 20 g to 50 g samples for continental shelf and shallow water depths, and 2 g to 10 g samples for the slope and deeper water depths, because the abundances of foraminifera differ according to depth.

c) Ensure that the samples are not mixed or contaminated. Record the station and sample information

d) Observe the foraminifera specimens (>0,150 mm) under a microscope. Generally, identify the planktonic foraminifera and the dominant species of benthic foraminifera to species level, and identify the others can be to genus level or as ecological categories. Record the abrasion, breakage and dissolution of foraminifera specimens.

e) Count all the specimens if the number of specimens is less than 300. Divide the samples by the riffle or diagonal sample method if the sample volume is too great and identify at least 300 foraminifera for each subsample. About 300 planktonic foraminifera specimens and 150 benthic foraminifera specimens are recommended for microscopic examination.

f) Record the relative abundance of each species, showing its percentage (%), or record its absolute abundance as individuals in per gram dried sample (individuals/g), or record its abundance as individuals in per square centimetre dried sample (individuals/cm^2) for surface sediment sample.

6.3 Collection and preservation of the samples

6.3.1 Sampling and sample processing

Based on different marine sediment types, divide the sampling and processing methods into two types, as follows.

a) Offshore and shoal water type: these sediments are composed mainly of terrigenous clast that have fast deposition rates and low foraminifera content. For obtaining foraminifera specimens, the sediment is placed in a beaker and soaked in clean water. Sodium hexametaphosphate is added for dispersing the sediment particles. After heating and disaggregation, the organic matter is dissolved by adding a moderate amount of hydrogen peroxide. The sample is then washed using

a 0,063 mm sieve. Clean foraminifera specimens are separated from coarse particles by flotation in tetrachloromethane. Foraminifera tests are suspended by stirring and collected by passing through a 0,063 mm filter paper.

b) Slope and deeper sea type: these sediments have less terrigenous clast, obvious fine sediment particles, relative slow deposition rates, and a higher foraminifera content. The sediment is placed in a beaker and soaked in distilled water or tap water for dispersing the sediment particles. After this process, the remnant is washed through a 0,063 mm sieve and oven-dried for specimen preparation.

6.3.2 Collection and preservation of the sedimentary tests

Collect the foraminifera tests using a sediment sampler (box-corer, multicorer, gravity sampler, etc.) on the survey ship. The samples can be preserved by cold storage or treated in the field (dyed with Rose Bengal to distinguish living specimens from empty tests) then seal and preserve at 4 °C or according to the survey plan.

6.3.3 Collection and preservation of living foraminifera

For surveying living foraminifera, collect the surface sediment sample (usually 0 cm to 2 cm of sediment from seafloor, but can be 0 cm to 15 cm according to the survey plan) and treat with ethanol in the field. For distinguishing living foraminifera from empty tests, stain the sample with Rose Bengal solution within 48 h then process and analyse.

For molecular identification of living foraminifera, the samples shall be refrigerated or cryopreserved and stored for further processing and analysis in the laboratory.

6.4 Tools and reagents

The tools and reagents include the following:

a) major equipment: quantitative layering sampler, thermostatic drier box, stereomicroscope, reefer, refrigerator;

b) other tools: mesh sieve (0,063 mm, 0,150 mm), crystallizing dish (50 ml, 100 ml), straw, funnel, glass rod, filter paper, beaker, bottle, brush, writing brush, riffle sampler, glass plate, blade, sampling needle, specimen slice, specimen box;

c) reagents: sodium hexametaphosphate, ethanol, hydrogen peroxide, tetrachloromethane, Rose Bengal, sodium salt ($C_{20}H_2Cl_4I_4Na_2O_5$), distilled water.

6.5 Processing and analysis of the samples

6.5.1 Numbering and weighing the crystallizing dish or beaker

The procedure for numbering and weighing the crystallizing dish includes the following steps:

a) clean the crystallizing dish;

b) dry the crystallizing dish at 60 °C in an oven;

c) number and weigh the crystallizing dish (accurate to 0,01 g);

d) record the mass of the crystallizing dish and the corresponding sample number on the record sheet (Table F.1).

6.5.2 Drying and weighing the sediment sample

The procedure for drying and weighing the sediment sample includes:

a) put the original sediment sample into the pre-weighed crystallizing dish and number it;

b) record the wet mass of the sample;

c) dry the sample at 60 °C in an oven, then weigh and determine the dry mass.

6.5.3 Sample soaking

The procedure for sample soaking includes:

a) add distilled water or tap water to the beaker containing the sediment sample and soak for 1 or 2 days in order to disperse the sediment particles and foraminifera tests;

b) for sediment with high organic content, soak the sample with 3 % hydrogen peroxide for 2 h then wash with clean water (for offshore sediments, add a small amount of sodium hexametaphosphate to make the sample more loose. This step is not suitable for deep-sea sediments or for living foraminifera).

6.5.4 Washing and drying

The procedure for washing and drying includes:

a) transfer the sample into a 0,063 mm sieve and wash repeatedly with running water;

b) transfer the washed sample to a numbered crystallizing dish;

c) dry the crystallizing dish in an oven at 60 °C;

d) record the mass of the coarse fraction of the dried sample.

6.5.5 Suspension and concentration

The procedure for suspension and concentration includes:

a) number the beaker according to the sample number;

b) suspend with tetrachloromethane;

c) pour the sediment residue into a crystallizing dish dry and number the dish;

d) bag and label the sediment residue after microscopic examination.

6.5.6 Bottling and sealing

The procedure for bottling and sealing includes:

a) sieve the weighed and dried samples through 0,150 mm and 0,063 mm meshes respectively;

b) bottle and label the two samples and mark the sample layer and size fraction.

6.5.7 Specimen preparation and analysis

Apply the following procedure:

a) examine the specimens (>0,150 mm) by light microscopy;

b) select 150 to 300 tests at random for specimen preparation;

c) carry out the classification and biocoenosis statistics (species number, abundance, diversity and group ratio, etc.) according to the survey plan;

d) for offshore sediment samples, carry out the classification and statistics of 0,063 mm specimens selectively according to the survey plan;

e) fill in the tables for the identification and statistics of foraminifera (tables are designed according to the survey plan).

7 Survey of ostracoda

7.1 Principle

Based on the collection, preparation, preservation, identification and analysis of living specimens and tests, the distribution and preservation of ostracods in sediments are investigated to reflect the hydrological and environmental changes in the area.

7.2 General provisions

The general provisions include the following.

a) Design the sampling method and the sampling process according to the survey plan. For sea floor surface sediments, collect from the top 0 cm to 2 cm layer; for deeper sediments, collect at 2 cm intervals from core samples, or at different intervals according to the survey plan.

b) Determine the sampling volume according to the survey plan. Generally, use 20 g to 50 g samples for continental shelf and shallow water depths, and 2 g to 10 g samples for the slope and deeper water depths, because the abundances of ostracods differ according to depth.

c) Ensure that the samples are not mixed or contaminated. Record the station and sample information.

d) Identify ostracods with a stereomicroscope, where possible to species level but otherwise to genus level. Record any abrasion, breakage or dissolution of ostracod specimens.

e) For statistical analyses of biocoenosis, identify all the specimens if the number present is less than 100. If the volume is large, divide the samples by the riffle or diagonal sample method and identify at least 100 specimens for each subsample.

f) Record the relative abundance of each species, showing its percentage (%), or record its absolute abundance as individuals per gram dried sample (individuals/g), or record its abundance as individuals per square centimetre dried sample (individuals/cm^2) for surface sediment sample.

7.3 Collection and preservation of the samples

7.3.1 Sampling and sample processing

Based on different marine sediment types, divide the sampling and processing methods into two types, as follows.

a) Offshore and shoal water type: these sediments are composed mainly of terrigenous clast that have fast deposition rates and low ostracod content. For obtaining ostracod specimens, the sediment is placed in a beaker and soaked in clean water. Sodium hexametaphosphate is added for dispersing the ostracods and the sediment particles. After heating and disaggregation, the organic matter is dissolved by adding a moderate amount of hydrogen peroxide. The sample is then washed using a 0,063 mm sieve. Clean ostracod specimens are separated from coarse particles by flotation in tetrachloromethane. Ostracods are suspended by stirring and collected by passing through a 0,063 mm filter paper.

b) Slope and deeper sea type: these sediments have less terrigenous clast, obvious fine sediment particles, relative slow deposition rates, and a higher ostracod content. The sediment is placed in a beaker and soaked in distilled water or tap water for dispersing the ostracods and sediment particles. After this process, the remnant is washed through a 0,063 mm sieve and oven-dried for specimen preparation.

7.3.2 Collection and preservation of dead ostracod tests

Collect dead ostracod tests by sediment sampler (box-corer, multicorer, gravity sampler, etc.) on the survey ship.

The samples can be preserved by cold storage or treated in the field (dyed with Rose Bengal to distinguish living specimens from empty tests) then sealed and preserved (4 °C) according to the survey plan.

7.3.3 Collection and preservation of living ostracods

For surveying living ostracods, collect the surface sediment samples (usually 0 cm to 2 cm of sediment from seafloor, but can be 0 cm to 10 cm according to the survey plan) and treat with ethanol in the field. For distinguishing living ostracods from empty tests, stain the samples with Rose Bengal solution within 48 h then process and analyse.

7.4 Tools and reagents

The tools and reagents include the following:

a) major equipment: quantitative layering sampler, thermostatic drier box, stereomicroscope, reefer, refrigerator;

b) other tools: mesh sieve (0,063 mm, 0,150 mm), crystallizing dish (50 ml, 100 ml), straw, funnel, glass rod, filter paper, beaker, bottle, brush, writing brush, riffle sampler, glass plate, blade, sampling needle, specimen slice, specimen box;

c) reagents: sodium hexametaphosphate, ethanol, hydrogen peroxide, tetrachloromethane, Rose Bengal sodium salt ($C_{20}H_2Cl_4I_4Na_2O_5$), distilled water.

7.5 Processing and analysis of the samples

7.5.1 Numbering and weighing the crystallizing dish or beaker

Apply the following procedure:

a) clean the crystallizing dish;

b) dry the crystallizing dish in an oven at 60 °C;

c) number and weigh the crystallizing dish (accurate to 0,01 g);

d) record the mass of the crystallizing dish and the corresponding sample number on the record sheet (Table F.1).

7.5.2 Drying and weighing

Apply the following procedure:

a) place the original sediment sample into the weighed crystallizing dish and number it;

b) record the wet mass of the sample;

c) dry the sample at 60 °C in an oven, then weigh and calculate the dry mass.

7.5.3 Sample soaking

Apply the following procedure:

a) add distilled water or tap water to the beaker containing the sediment sample and soak for 1 or 2 days in order to disperse the sediment particles and ostracods;

b) soak the sample with 3 % hydrogen peroxide for 2 h then wash with clean water if samples contented high organic matter;

c) for offshore sediments, add a small amount of sodium hexametaphosphate to make the samples more loose. This step is not suitable for deep-sea sediments or for living ostracods.

7.5.4 Washing and drying

Apply the following procedure:

a) transfer the sample into a 0,063 mm sieve and wash repeatedly with running water;

b) transfer the washed sample to a crystallizing dish and number it;

c) dry the crystallizing dishes at 60 °C in an oven;

d) record the mass of the coarse fraction of the dried samples.

7.5.5 Suspension and concentration

Apply the following procedure:

a) number the beaker according to the sample number;

b) suspend with tetrachloromethane;

c) pour the sediment residue into a crystallizing dish and number it;

d) bag the sediment residue and record the sampling information during microscopic examination.

7.5.6 Bottling and sealing

Apply the following procedure:

a) pour the weighed and dried sample through 0,150 mm and 0,063 mm meshes respectively;

b) bottle and label the two subsamples with information of sample layer and size fraction.

7.5.7 Microscopic examination and enumeration

Apply the following procedure:

a) examine the specimens by microscopy (>0,150 mm);

b) select 100 to 200 ostracods for specimen preparation, or select all if there are <100;

c) carry out the identification, classification and biocoenosis statistics (species number, abundance, diversity, etc.) according to the survey plan;

d) for offshore sediments, carry out the identification, classification and statistics of the 0,063 mm fraction selectively according to the survey plan;

e) fill in the tables (designed according to the survey plan) for the identification and statistics of ostracods.

8 Survey of radiolaria

8.1 Principle

Survey of the preservation status, abundance and community structure of planktonic radiolarians deposited in the sediment of related sea area.

8.2 General provisions

The general provisions include the following.

a) Design the sampling method and the sampling process according to the survey plan. For sea floor surface sediments, collect from the top 0 cm to 2 cm layer; for deeper sediments, collect at 2 cm intervals from core samples, or at different intervals according to the survey plan.

b) Determine the sampling volume according to the survey plan. Generally, use 5 g to 10 g samples for continental shelf and shallow water depths, and 1 g to 2 g or 5 g samples for the slope and deeper water depths.

c) Identification of radiolarian specimens is carried out using diascoptic lighting under a biomicroscope. In general, identify to species level for the 60 dominant or common species, other specimens can be enumerated only for statistical analysis. But if the survey plan requires species diversity data, identify all specimens to species level.

d) For biocoenosis statistics, all samples should be counted if the total number of shells is less than 300. If the number of shells is large, sample can be divided by a sampler or a diagonal sampler.

e) Calculate the relative abundance of each species and record its percentage (%) or record the absolute abundance as individuals in per gram dried sample (individuals/g).

8.3 Collection and preservation of the samples

8.3.1 Sample processing

Based on the sediment type, samples should be processed in one of the two following ways.

a) Shelf and shoal water type: these sediments are composed mainly of terrigenous clast and organisms with calcareous shells; they have a fast deposition rate and the radiolarian content is low. For obtaining radiolarian specimens, sediment is soaked in a beaker with clean water. Tetrasodium pyrophosphate is added for dispersing the particles. After heating and disaggregation, organic matter and calcareous fractions are eliminated and dissolved by adding moderate amounts of hydrogen peroxide and hydrochloric acid. The sample is then washed over a 0,063 mm sieve and oven-dried. In order to separate the clean radiolarian specimens from coarse particles, flotation in tetrachloromethane while stirring is carried out. Radiolarian shells in suspension are poured onto a boulting cloth of 0,063 mm mesh or a filter paper.

b) Slope and deeper sea type: these sediments have less terrigenous clast, fine sediment particles, relatively slow deposition rates, and a higher radiolarian content. The sediment is soaked in a beaker with clean water. Tetrasodium pyrophosphate is added for dispersing the particles. Based on the content of calcareous particles, superadd moderate amounts of hydrogen peroxide and hydrochloric acid in order to dissolve organic matter and calcareous fractions. Continue until the reactions stop. The remnant is washed through a 0,063 mm sieve and oven-dried. The dried material may be directly used for making radiolarian specimen slides for species identification and enumeration.

8.3.2 Collection and conservation of deposited empty shells

Empty radiolarian shells deposited on the surface of the sediment are collected from the top 0 cm to 2 cm layer using a box-corer, multicorer or gravity sampler. The sample is stored in a plastic bag, plastic box or glass bottle either at 4 °C or at ambient temperature.

8.4 Tools and reagents

The tools and reagents include the following.

a) Equipment: thermostatic drier box, ultrasonic oscillator, heating plate, beaker, straw, mesh sieve, filter paper, tweezers, glass slide, coverslip and glass rod.

b) Reagents: tetrasodium pyrophosphate, hydrogen peroxide, hydrochloric acid, tetrachloromethane, ethyl alcohol, neutral balsam, xylene (used for dilution or clearing remnant balsam).

8.5 Processing and analysis of the samples

8.5.1 Sample pretreatment

Apply the following procedure.

a) Weigh the dried sample (1 g to 2 g or 5 g), place into a 300 ml beaker, add 200 ml of clean water and 0,5 g to 1 g of tetrasodium pyrophosphate, soak about 10 h.

b) After dispersion of the sediment in water, add a moderate amount of H_2O_2 (a volume fraction of 30 %, analytical pure), place in a water bath at 80 °C, soak for several minutes until the reaction finishes. Monitor and adjust the temperature to avoid loss of radiolarian specimens by foam overflow.

c) Add a moderate amount of analytical pure a volume fraction of 36 % HCl according to the content of calcareous materials and leave for about 10 min or until the reaction stops.

d) Add clean water and allow to stand for about 5 min. Pour off the supernatant. Repeat this process 2 to 3 times in order to eliminate redundant HCl and H_2O_2 and reduce the acidity of the water.

e) Place the beaker in an ultrasonic oscillator for 1 min to 2 min in order to remove clay from the surface of the radiolarian shells.

f) Wash specimens over a 0,063 mm mesh copper sieve. Adjust water flow to avoid loss of specimens by overflowing.

g) Transfer specimens into a small beaker and allow to dry.

8.5.2 Preparation of microscope slide specimens

The preparation of microscope slide specimens includes the following steps.

a) Preparation of dry samples: take all (or a proportion of) specimens of every sample and place in the centre of a glass microscope slide. Add several drops of alcohol to disperse specimens and use a needle to spread the samples evenly over an area similar to that of a cover glass (24 mm × 50 mm). Allow the alcohol to volatilize, or place onto a hot plate to accelerate drying. When completely dry, add 3 to 5 drops of neutral balsam and apply a cover glass with light pressure causing the balsam to spread slowly over the whole area. Number the slide.

b) Preparation of specimens in water: Using a small pipette, mix the sample, place a drop onto a glass microscope slide, and spread evenly over an area similar to that of a cover glass. Place onto a hot plate for drying. Continue as above.

c) The temperature of the hot plate or thermostatic drier box should be maintained at about 60 °C to 80 °C.

d) Allow the slide to dry naturally, or place in a thermostatic drier box (60 °C to 80 °C, being careful to avoid bubbling). Remove surplus balsam from the slide by using xylene after balsam consolidation and hardening.

e) Label the slide.

9 Survey of sedimentary diatom

9.1 Principle

Based on the collection, identification and analysis of community composition of diatom shells in the sediment, the distribution and preservation of diatoms are investigated to reflect the hydrological, climatic and environmental information in the area.

9.2 General provisions

The general provisions include the following.

a) Design the sampling method and the sampling process according to the survey plan. For sea floor surface sediments, collect from the top 0 cm to 2 cm layer; for deeper sediments, collect at 2 cm intervals from core samples, or at different intervals according to the survey plan.

b) For wet samples, take about 5 g to 10 g, for dry samples take about 1 g to 5 g.

c) Diatom identification is carried out using transmitted light microscope. Each sample is observed by random row number. It is suggested to identify the species or variant, otherwise identify to genus level. For incomplete diatom frustules, if more than half of the central diatom is complete, or if the longitudinal furrow side of feathery diatoms is complete, the specimen should be identified. Dominant or common species can be selected for identification with other specimens only being enumerated, however, if the survey plan requires details of species diversity, all species in each sample should be identified.

d) For statistical analyses, at least 300 specimens per gram of dry sample or per square centimetre should be identified for every sample. If there are fewer than 300 diatom specimens in the sample, all should be identified. The relative abundance of each species should be recorded.

9.3 Collection and preservation of the samples

9.3.1 Sampling and treatment

According to differences in diatom content in different types of seabed sediments, sample collection and processing are divided into two types, as follows.

a) Continental shelf and shallow water type: these sediments are composed mainly of terrigenous clast and organisms with calcareous shells; they have a fast deposition rates and the diatom content is relatively low. Dispersion of diatom specimens is by soaking the samples in distilled water. Appropriate amounts of hydrogen peroxide and hydrochloric acid are added in order to remove calcareous particles and organic matter. Allow to stand until the reaction stops. Add zinc bromide or other heavy liquids with a specific gravity of about 2,4 in order to separate the diatoms from other particles by flotation. Stir to keep the diatoms in suspension. These can be used directly for making specimen sheets.

b) Slope and deep-sea type: these sediments have less terrigenous detritus, fine sediment particles, relatively slow deposition rates, and a higher diatom content. Diatoms are dispersed by soaking in distilled water. Based on the calcium content, appropriate amounts of hydrogen peroxide and

hydrochloric acid are added in order to remove organic matter and calcium particles. Allow to stand until the reaction stops. This material can be directly applied to the preparation of specimen slices.

9.3.2 Collection and preservation of sedimentary remains

Surface sediments: scrape sediments from the top 0 cm to 2 cm of newly collected samples using a plastic knife or spoon. Place about 5 g of fresh sediment into a polyethylene bag and seal, label with site numbers and layer positions and store in a sample box in the shade until for further processing.

Core sediments: collect about 5 g fresh sample, or 1 g dry sample, from each layer. Place the sample into a polyethylene bag, seal, label with site number and layer position and store in a sample box in the shade until further processing.

9.4 Tools and reagents

The tools and reagents include the following.

a) Equipment: long-handled spoon, balance, glass beaker, centrifuge, electric heating plate, oven, slides, glass, glass rod, tube, cylinder, tweezers, ventilation cabinet.

b) Reagents: hydrogen peroxide, hydrochloric acid, alcohol, distilled water, neutral gum, sodium pyrophosphate, and zinc bromide.

9.5 Processing and analysis of the samples

9.5.1 Sample pretreatment

9.5.1.1 Centrifuge method

The centrifuge method includes the following steps.

a) Place about 2 g of wet sediment ample into a 5 ml tube with a long-handled spoon. Add about three times the volume of 10 % HCl to that of the sample in order to remove calcium. Allow to stand until the reaction stops, i.e. until no bubbled form. Note that the duration of this reaction depends on the calcium content in the sediment.

b) Centrifuge at 2 500 r/min for 5 min. Remove residual HCl and wash 3 times with distilled water.

c) To remove organic matter, add about 3 ml to 4 ml volume fraction of 30 % H_2O_2, and place in a constant temperature water bath at 60 °C for 1 h to 2 h, i.e. until no bubbles form.

d) Centrifuge at 2 500 r/min for 5 min. Remove residual H_2O_2 and wash 3 times with distilled water.

NOTE If time allows, the sediment can be separated from the supernatant by static precipitation (see 9.5.1.2).

9.5.1.2 Static precipitation method

The static precipitation method includes the following steps.

a) Place about 1 g to 2 g of sample into a beaker. Add a volume fraction of 10 % to 15 % hydrochloric acid. When the reaction stops, distribute the mixture evenly and place for 12 h to 24 h.

b) Remove the residual hydrochloric acid. Add distilled water and place for at least 24 h and pour off the supernatant. Repeat 3 times.

c) Add a volume fraction of 30 % H_2O_2 and place in a constant temperature water bath at 60 °C for 1 h to 2 h.

d) Wash 3 times with distilled water.

If the sample is dry, it is advisable to take about 1 g to 5 g dry sample, put it into 300 ml beaker, add 200 ml of distilled water and 0,5 g to 1 g of sodium pyrophosphate and allow to stand for about 10 h. After the sediment has dispersed, continue as above.

9.5.2 Preparation of microscope slide specimens

The procedure for the preparation of microscope slide specimens includes the following.

a) Immerse coverslips in a volume fraction of 10 % to 20 % HCl solution for at least 24 h, and then immerse in alcohol to remove HCl, before use.

b) Spread diatom suspension evenly on coverslip with a glass rod and allow to dry. Add 1 or 2 drops of neutral resin onto the glass slide. Place the coated coverslip gently onto the resin drop, avoiding air bubbles.

c) Slide can be dried naturally or in an oven (45 °C to 55 °C, temperature should not be too high so as to avoid bubbling) for 48 h.

d) Label the slide and store it in a sample box.

e) Prepare replicate slides for each sample for identification and analysis.

9.6 Organization of data

Identification of diatoms is made by observing under a microscope at 1 000× magnification. Enumeration can be carried out at 400× magnification. At least 300 diatom frustules (not including resting spores and auxospore) per sample should be identified. The relative abundance (%) of each species relative to the total number of diatom frustules (i.e. not including resting spores and auxospore) should be recorded for each sample.

Data processing should meet the relevant provisions of this document. For the calculation of biocoenosis analysis, refer to Annex E.

10 Survey of coccoliths

10.1 Principle

The objectives of investigating coccoliths in sediments are: to meet the need of chronostratigraphy and to date geological age of sediments by analysis and identification of coccoliths; to explore their oceanographical/palaeoceanographical significance through analysis of characteristics of coccolith assemblages; to infer sedimentary environments (including sediment sources) or diagenesis based on analysis and evaluation of coccolith preservation status.

10.2 General provisions

The general provisions include the following.

a) Sampling quality: the sample shall not be contaminated for example by cross-sampling, mixing with another sample, re-using disposable tools for sampling.

b) Sampling depth or depth interval: the topmost 0 cm to 2 cm layer of sediment should be collected for seafloor surface samples; depth intervals between 2 cm to 10 cm, or other intervals in accordance with the requirements of a survey plan can be selectively collected from box-corer, multicorer or gravity sampler, for subsurface sediment samples. For undisturbed sediments and well-preserved sediment sequences, a grab sampler can be used from which vertical tube subcores can be taken. Sampling can then continue in the same way as by multicorer.

c) Sampling amount: take about 5 g to 10 g of wet sediment for sampling shallow water regions of continental shelf; take about 1 g to 2 g of wet sediment for sampling continental slope and deep-water areas. The dry mass of samples should be calculated. It is suggested that the sampling volume be at least 3 cm^3, or the sampling mass more than 5 g, so that the amount of sediment is sufficient to meet the needs of the clean procedure (which includes removing the outer layer of the sample), and the needs for multiple slide-making or scanning electron microscopy.

d) Requirements of identification and classification: identify dominant taxa at species or genus level.

e) Statistical requirements: all coccoliths within 5 fields of view (at a magnification of 1 000×) on a microscope slide shall be enumerated. At least 300 individuals should be identified and enumerated. At least 100 fields of view should be observed if there are fewer than 300 individuals on a slide.

f) Characteristic parameters of fossil community: the parameters include the number of species, dominant and common species, relative abundance and species diversity.

10.3 Collection and preservation of the samples

The common methods of sediment sampling are mainly carried out by box-corer, multicorer, grab samplers, gravity piston corer or drilling. After the sampling, the samples can be stored in sealed plastic bags, plastic boxes, or glass bottle; according to the conditions, the samples can be stored either at 4 °C or in a sample store room at room temperature.

10.4 Tools and reagents

The tools and reagents include the following.

a) Equipment for slide preparation: beaker (commonly 50 ml, 20 ml), wash bottle, electric heating plate, glass microscope slides, cover slips, straws (large and small), glass rods (large and small), ultrasonic cleaner, centrifuge, tweezers, toothpicks, labels, markers, kitchen paper or paper towels, microscope slide box.

b) Reagents and solvents: distilled water, ammonia (preparation of alkaline buffer of pH 9: add a few drops of ammonia into a bottle filled with 1 l distilled water, shake the bottle, test pH with pH test paper), a volume fraction of 10 % hydrochloric acid.

c) Slide mounting medium: the mounting medium should have a refractivity lower than or close to that of calcite, namely between 1,658 to 1,486; neutral balsam (or Canada balsam), or Norland optical adhesive (ultraviolet light is needed to dry it), or abienic balsam.

d) Observation instruments: a polarizing microscope (with magnifications of 200×, 400×, 1 000×). The use of contrast device on the microscope depends on the need. A scanning electron microscope or transmission electron microscope can be used depending on the need.

10.5 Processing and analysis of the samples

10.5.1 Pre-preparation of the equipment and the samples

As coccoliths are very small, the samples are prone to be contaminated. The following precautions should always be taken during sample preparation.

a) Clean the test bed: cover the preparation area of the test bed with disposable paper, replace with new paper after each usage, avoid solution spatters out during preparation.

b) Use fresh samples: scrape off the outer surface of the sample and subsample the interior sediments. Avoid touching the samples directly; wear disposable gloves or use disposable paper while manipulating the samples.

c) Clean the containers and equipment: use disposable tools (such as toothpicks, paper) where possible; glass containers and reusable equipment should be washed after each usage, soaked for 10 min in dilute hydrochloric acid (a volume fraction of 10 %), and rinsed.

10.5.2 Method of preparing simple smear slides

The procedure is as follows.

a) Mark a glass microscope slide.

b) Scrape small fraction of the clean and fresh raw sediment (about the size of a mung bean) and place on a cover slip or glass slide. Add a few drops of distilled water to the sample and use a toothpick to mix into a slurry.

c) Use the toothpick to smear the sediment slurry and form a thin sample layer on the cover slip or glass slide. During the smearing process, large-sized or coarse particles should be moved to the edge of the glass slide and then discard. Put a cover slip or glass slide onto a heating plate (usually set at 70 °C to 90 °C) and allow to dry.

d) Place an appropriate amount of mounting medium onto the cover slip or glass slide. Place the cover slip onto a glass slide, or apply a cover slip onto the glass slide, and place onto the heating plate. Use a glass rod to gently compact the cover slip, squeezing any air bubbles out of the mounting medium. Remove from the heating plate and allow to cool. When Norland optical adhesive is used, place the slide under an ultraviolet light for at least 5 min to allow the adhesive to cure.

The above second step can be substituted as follows.

b) Put the sample into a beaker filled with distilled, buffered water, mix well and allow to soak in order to disperse the coccoliths. Place 1 or 2 drops of the sediment suspension onto a cover slip or glass slide.

10.5.3 Pre-concentration of coccolith samples

For sediments from shallow seas or deep-sea gravity flow environments, sorting and concentration methods are needed. The procedure is as follows.

a) Soak and disaggregate sediment sample: take about 1 g of sample, place it in a beaker, add an appropriate amount of buffer (pH 9), and soak. To disperse particles, either stir, or place the beaker in an ultrasonic bath for 10 s to 15 s.

b) Sort and separate coarse fractions: stir the sample solution, allow the solution to settle for 5 min to 10 min, so that coarse particles (sand and coarse silt) gradually precipitate onto the bottom of the beaker; pour the supernatant into another beaker and discard the settled coarse debris.

c) Sort and separate fine clay fractions: cover the beaker containing the supernatant and allow to settle for 15 h to 20 h. Pour off the supernatant liquid, which contains mainly clay minerals; stir the settled residue in the bottom of the beaker, and take a few drops to make a smear slide. If the sample contains abundant clay fraction, repeat this step 2 to 3 times. Centrifugation can also be used in this step, however, a pre-experiment should be conducted in order to determine suitable speed and time of centrifugation.

10.6 Organization of data

Data analysis and record documentation should meet the relevant provisions of this document. The procedure includes the following.

a) Identification and classification of coccolith species: observe and identify the coccoliths, mainly using polarized light microscopy at a magnification of 1 000×. Generally, coccolith fossils should be identified to species or genus level, for a very small species, identification should be made using a scanning electron microscope at a magnification of at least 10 000×.

b) Evaluation of coccolith preservation status: provide a qualitative and quantitative estimate of the preservation status of coccoliths, e.g. if they have been damaged by chemical etching, physical breakage, or by secondary crystallization/recrystallization (Table G.1). Relative coccolith abundance in sediments is the proportion of coccoliths relative to the sediment clastic components and is expressed as a percentage (Table G.2). It is determined by referring to sediment particle abundance charts[50]. The evaluation should be made based on observations of at least 10 fields of view at a magnification of 1 000×. Observation of at least 100 fields of view is needed if sediments contain very few coccoliths.

c) Enumeration of coccoliths. There are two minimum requirements; at least 300 coccoliths shall be observed and counted for each sample; and at least more than 5 fields of view (at a magnification of 1 000×) randomly located on the slide should be observed. Very commonly, the sediment samples analysed contain abundant coccoliths, and the number of coccoliths within one field of view is more than 100. In this case, a subdivision of one field of view into 4 parts can be made by using the cross-hairs in the eyepiece, and coccoliths within 1/4 field of view can be counted. In this case, at least 5 fields of view shall be observed. If a sediment sample contains very few coccoliths, at least 100 fields of view shall be observed.

d) Analysis of coccolith assemblage abundance: for basic marine geological survey objects, it is recommended to estimate the relative abundance of coccoliths.

e) There are several semi-quantitative estimation methods. For example, the method suggested by Reference [5], it is to obtain the number of coccoliths per area on a sample slide, with the purpose of analysing some selected coccolith species that have significance for ecology or geological age diagnosis. There are also several absolute quantitative methods of analysis to obtain the number of coccoliths per mass in the sample. For example, the random settling method that was suggested by Reference [7] and by Reference [55], the spray method suggested by Reference [8] and the microbeads method suggested by Reference [47]. These methods are commonly applied in palaeoceanographic studies, however, they need more time for sample preparation than simple smear slides. These methods can be applied if necessary in the marine survey objects.

11 Survey of sporopollen

11.1 Principle

The outer coat (exine) of pollen and spore is mainly composed of tough, resistant organic compounds, namely sporopollenin $(C_{10}H_{16}O_3)_x$ and chitin, which protect the sporopollen from desiccation and oxidation. The sporopollen assemblage in sediments reflects the community characteristics of the original vegetation and provides information on the temperature and humidity of terrestrial habitats around sedimentary basins. The methods employed for investigating sporopollen in sediments include sample collection, sample processing by both physical and chemical treatments in order to extract the sporopollen by removing organic materials, carbonate and siliceous minerals, the identification and enumeration of sporopollen and data analysis. Based on the sporopollen data, it is possible to reconstruct past climate change in the terrestrial habitats surrounding sedimentary basins.

11.2 General provisions

The general provisions include the following.

a) All samples should be collected and processed without contamination; only filtered or distilled water can be used; laboratory windows should be kept closed when the pollen filtration system is in operation; there should be no other sporopollen source in the laboratory. Detailed information of the field site and samples shall be recorded.

b) Samples should be collected from the upper 2 cm layer of the sea surface sediments, and at 2 cm intervals for the core sediments, or as required by the investigation program.

c) The investigation should include the identification of the sporopollen components and their quantity, and the total sporopollen concentration.

d) The nomenclature of sporopollen should follow modern classification systems for extant plants. All sporopollen should be identified to genus level (or family level if genus identification is not possible).

e) At least five slides should be observed to enumerate the sporopollen when the sporopollen is low in quantity (less than 100 grains). Otherwise, a minimum of 200 grains (spores not included) should be counted and a total of 100 grains for non-pine pollen is suggested.

11.3 Collection and preservation of the samples

The procedure for collection and preservation of samples include the following steps.

a) Samples of surface or core sediments can be collected, packed and sealed in plastic bags with labels showing the site location, water depth, core number and depth, and sample number. All samples should be kept in a refrigerator or a cool room between 2 °C and 5 °C, or frozen. Samples can be taken in laboratory if the surface sediments and cores are whole packed and stored in refrigerator or frozen.

b) The volume of the sediments for sporopollen investigation is 10 ml or 10 g to 20 g, based on the lithology. A large sample volume is usually needed when the site location is far from the shore or the samples are made up of coarser components.

11.4 Tools and reagents

The tools and reagents include the following.

a) Equipment: balance, polytetrafluoroethylene (PTFE) or polypropylene beaker, glass beaker, funnel, ultrasonicator (40 kHz), standard sieve (7 μm and 180 μm), centrifuge, hot plate, water bath, microscope slides, cover slips (0.150 mm or thinner), watch glasses (Φ=20 cm), pipe, wooden spatulas, stainless steel or polytetrafluoroethylene (PTFE) spatulas and rods, glass rods, stereo microscope.

b) Reagents: *Lycopodium* tablets, glycerine, phenol, gelatine, Canada balsam or nail polish, sodium pyrophosphate ($Na_4P_2O_7$, 5 %), potassium hydroxide or sodium hydroxide (KOH or NaOH, 10 %), hydrochloric acid (HCl, 10 %), hydrofluoric acid (HF, 40 % or conc.), glacial acetic acid (CH_3COOH), mixture of acetolysis anhydride [$(CH_3CO)_2O$] and sulphuric acid (H_2SO_4, conc.) (9: 1 in volume).

11.5 Processing and analysis of the samples

11.5.1 Sample disaggregation

The procedure for sample disaggregation includes the following.

a) Record the site location, water depth, core number and depth, registered the sample number and water depth. Label the beaker.

b) Place the sediment (10 ml, or 10 g dry sample) and one tablet of *Lycopodium* into the beaker. Record the mass or volume of the sample.

c) Add a volume fraction of 5 % sodium pyrophosphate (two times the volume of the sediment sample). Place in a water bath at 100 °C for 30 min, stirring with a rod. The sediment particles are dispersed to maximize the reactive surface area. If the sediments are quite loose, this step can be skipped.

11.5.2 Alkali-soluble digestion

Add a volume fraction of 10 % potassium hydroxide (two times the volume of the sample). Place in a water bath at 100 °C for 10 min, stirring with a rod. This process dissolves humic materials.

11.5.3 Sieving

Add distilled water and suspend the sediments. Wash through a stainless-steel wire sieve (180 µm) into another labelled beaker. The sieving step removes large clasts and organic debris, which makes the reaction in subsequent treatments more efficient when digesting extraneous material. Transfer the suspension by passing through the sieve (180 µm) into a centrifuge tube and centrifuge for 5 min at 3 000 r/min. Decant the supernatant.

11.5.4 Carbonate digestion

Transfer the sediment to a beaker. Add 10 % hydrochloric acid (three times the volume of the sediment sample, or in excess until all obvious reaction ceases). Place at room temperature for 30 min, stirring with a rod. If the carbonate content is high, the acid can be added very slowly and monitored for effervescence to prevent sample loss. Transfer into centrifuge tubes and centrifuge for 5 min at 3 000 r/min. Decant the supernatant. Wash with distilled water and centrifuge for 3 min at 3 000 r/min. Decant the supernatant.

NOTE The addition of hydrochloric acid is necessary to remove carbonate before HF digestion, otherwise the carbonate reacts with HF to form the insoluble precipitate (CaF_2).

11.5.5 Silicate digestion

The procedure for silicate digestion includes the following steps.

a) Transfer the matrix to a polytetrafluoroethylene (PTFE) or polypropylene beaker. Add a volume fraction of 40 % hydrofluoric acid (or up to 70 % conc., two times the volume of the sediment sample). Cover the beaker or seal with a plastic cloth. Place in a fume hood at room temperature for 2 days shaking occasionally. In case there are large amount of silt and sand, HF treatment can be prolonged or repeated until these are completely removed. Digestion can be monitored by rubbing the residue of the reaction against the beaker wall with a spatula or rod. If silts and sands are still present, a distinct gritty texture can be felt.

b) Unseal the beaker and add distilled water. Transfer to a polytetrafluoroethylene (PTFE) or polypropylene centrifuge tube, centrifuge for 5 min at 3 000 r/min. Decant the supernatant. Wash with distilled water and centrifuge for 3 min at 3 000 r/min. Decant the supernatant. Repeat washing procedure.

c) Add a volume fraction of 10 % hydrochloric acid (one or two times the volume of the sediment sample), stirring with a rod. Place at room temperature for 30 min or until the solution turns transparent. This remove any silicofluorides produced during the HF treatment. Centrifuge for 5 min at 3 000 r/min. Decant the supernatant. Wash with the distilled water and centrifuge for 3 min at 3 000 r/min. Decant the supernatant. Wash two or three times or until neutral after the litmus test.

11.5.6 Ultrasonic cleaning and sieving

Clean ultrasonically for 30 s. Wash through a stainless-steel wire sieve (7 µm). Collect and transfer the fraction larger than 7 µm into glass centrifuge tubes. Centrifuge for 5 min at 3 000 r/min. Decant the supernatant.

11.5.7 Acetolysis

This process removes cellulose. The texture of the sporopollen will be clearer under the microscope after the acetolysis. This step is optional. The procedure includes the following.

a) Add a 1/2 tube of glacial acetic acid, stir the mixture, and centrifuge for 5 min at 3 000 r/min. Decant the supernatant. This treatment is to dehydrate the sporopollen sample before acetolysis. Otherwise, the reagents used in acetolysis, i.e. acetolysis anhydride and concentrated sulphuric acid, will react vigorously with the water.

b) Add a 1/2 tube of a solution of acetolysis anhydride and concentrated sulphuric acid (9:1). Stir carefully with a rod. Incubate in a water bath at 100 °C for 3 min to 5 min. Remove and cool the centrifuge tubes. Centrifuge for 5 min at 3 000 r/min with pairing samples or glacial acetic acid. Decant the supernatant. The H_2SO_4 acts as a catalyst for the acetolysis reaction, in which the cellulose is esterified to form cellulose triacetate. This product is soluble in acetic acid (another reaction product in the acetolysis). If the cellulose content in the sample is high, repeat this treatment.

c) Add glacial acetic acid, stir and centrifuge for 5 min at 3 000 r/min. Decant the supernatant. Wash with distilled water. Allow to stand for 5 min before centrifuging for 3 min at 3 000 r/min. Repeat washing procedure.

11.5.8 Storage of processed sample

For temporary storage, add a little distilled water. For indefinite storage, add glycerine (with 1 % to 2 % phenol, two times the volume of the pellet).

NOTE If time permits, the centrifuge stages in the above steps can be substituted by the natural sedimentation in the fume hood for 4 h or longer.

11.5.9 Mounting sporopollen specimens

Three to five monitor or permanent slides shall be mounted based on the requirements of the survey plan. Engrave and label slides and add relevant information to the worksheet.

a) Monitor slide preparation

Dispense a drop of glycerine and water (4: 1 in volume) solution onto the centre of the slide. Add a tip of the prepared sporopollen sample and mix evenly. Apply a cover slip and press gently to give an extremely thin layer of mixture.

b) Permanent slide preparation

Melt a small piece of glycerine jelly and place in the centre of the slide. Add a tip of the prepared sporopollen sample, and mix while heating on a hot plate to degas. Apply a cover slip and press gently to give an extremely thin layer of mixture. Invert the slide so that as much as possible of the sporopollen sediment adheres to the coverslip. Allow to stand at room temperature until the jelly solidifies. Seal the slides with Canada balsam or nail polish. Use as little glycerine jelly as possible in order to minimize amount of sporopollen suspended in the jelly.

11.5.10 Glycerine jelly preparation

Add 7 g of gelatine, 19 g of distilled water, 30 g of glycerine and 1 g of phenol into a beaker. Melt and stir gently. Keep heating until no obvious effervescence. Filter through glass wool in a funnel. The liquid mixture becomes solid glycerine jelly after cooling. Glycerine jelly should be kept in the dark.

11.5.11 Precautions

Be especially careful during the HF treatment. HF is particularly dangerous if inhaled or if it comes into contact with skin or mucus membranes. Only use stainless steel, polytetrafluoroethylene (PTFE)

or polypropylene ware in this step. The acetolysis solution can be explosive when it comes in contact with water. Be familiar with the property of the chemicals and the specification for experimental procedures, such as using protective clothing, gloves and an eye shield. Note the location of the first aid kit, the shower, the eye-wash station and the burn blanket, and know how to use them. All procedures shall be carried out in a fume hood.

11.6 Organization of data

Data of the sporopollen investigation in sediments shall conform to the requirements in this document. The sporopollen concentrations can be calculated using the exotic *Lycopodium* tablet technique and be expressed in numbers of grains per gram dry mass of sediment (N/g), or per millilitre of the sediment (N/ml). The relative abundances of individual sporopollen taxa can be estimated on their group totals (arboreal and herbaceous pollen) and expressed as percentages of the total.

12 Survey of benthic viruses

12.1 Principle

Sediment samples are frozen directly without fixation or fixed with 0,02 µm-filtered seawater containing 2 % formalin or 2 % glutaraldehyde (for fluorescence microscopy) or 0,5 % glutaraldehyde (for flow cytometry). Benthic viruses are enumerated by epifluorescence microscopy (or flow cytometry) after extraction, centrifugation, dilution, filtration, staining and slides preparation (see flow diagrams in Figure H.1).

12.2 General provisions

The general provisions include the following:

a) seawater and MilliQ®[1)] water should be filtered with 0,02 µm membrane after sterilization;

b) reagents and solutions should be filtered with 0,2 µm membrane;

c) individual abundance should be presented as individuals per gram of sediment dry mass;

d) biomass should be presented as micrograms of carbon per 10 square centimetre or microgram of carbon per cubic centimetre;

e) investigation elements should include determining the abundance (or biomass) of viruses.

12.3 Collection and preservation of the samples

12.3.1 Samples for epifluorescence microscopy

Samples for epifluorescence microscopy include the following.

a) Fixed samples: three replicate samples each of 0,5 ml are collected from the top 1 cm layer of undisturbed sediment core and transferred into 5 ml storage tubes. To each replicate, add 3 ml of 2 % formalin (or glutaraldehyde) made using 0,02 µm-filtered seawater. Shake gently and place in the dark at 4 °C for 15 min to 30 min. Samples are then quick-frozen in liquid nitrogen and stored at -80 °C.

b) Non-fixed samples: three replicate samples each of 0,5 ml are collected from the top 0,5 cm or 1 cm layer of undisturbed sediment core and transferred into 5 ml storage tubes. To each replicate, add 3 ml of 0,02 µm-filtered sterile seawater. Samples are then quick-frozen in liquid nitrogen, and then stored at -80 °C.

1) MilliQ® water 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.

c) Samples for stratification analysis: sediment cores are sliced into layers at of 2 cm thickness or according to the experimental design. Each layer of sediment is homogenized and three replicate samples (0,5 ml) of each are collected in triplicate and preserved as described above for fixed and non-fixed samples.

Samples with or without fixation should be analysed immediately after sampling, or prepared as dye-stained slides immediately and stored at -20 °C, or snap frozen in liquid nitrogen and stored at -80 °C, until analysed.

12.3.2 Samples for flow cytometry

Undisturbed sediments of 0,5 ml are collected from the top 1 cm in triplicate and transferred into 5 ml storage tubes. To each replicate, add 3 ml of 0,02 µm-filtered 0,5 % glutaraldehyde (or 1 % paraformaldehyde) made using by 0,02 µm-filtered seawater. Shake well and place in the dark at 4 °C for 15 min to 30 min. Samples are then snap-frozen in liquid nitrogen and stored at -80 °C.

12.3.3 Samples for environmental measurements and molecular diversity of viruses

Sediment samples are collected from the top or sliced layers using 2,9 cm diameter cut-off sterile syringes. Samples are placed into sterile ziplock bags and stored at -80 °C.

12.4 Tools and reagents

12.4.1 Equipment and reagents for epifluorescence microscopy

The equipment and reagents for epifluorescence microscopy include the following:

a) vacuum filtration unit, refrigerator, ultra-low temperature freezer, liquid nitrogen container, centrifuge, ultrasonic processor, vortexers, epifluorescence microscope;

b) 0,02 µm-filtered sterile seawater containing 2 % formalin or 2 % glutaraldehyde ($C_5H_8O_2$);

c) 0,02 µm-filtered paraformaldehyde ($(CH_2O)_n$), the volume fraction of which is 37 % to 40 %, stored at -20 °C;

d) 0,02 µm-filtered sterile MilliQ® water;

e) 0,02 µm-filtered sterile seawater;

f) 55 mmol/l tetrasodium pyrophosphate stock solution made up in 0,02 µm-filtered sterile MilliQ® water, stored in dark at 4 °C;

g) SYBR® Green I[2)] or SYBR® Gold[2)] manufacturer's stock solution (deoxyribonucleic acid specific fluorescent stain), which can be aliquoted in small volumes and stored in dark at -20 °C;

h) antifade solution:

— phosphate buffered saline (PBS) glycerol stock solution:

  prepare a solution of 50 % PBS (0,05 mol/l Na_2HPO_4, 0,85 % NaCl (mass/vol), pH 7,5) and 50 % glycerol, which can be aliquoted in small volumes and stored in the dark at -20 °C;

— 10 % (mass/vol) p-phenylenediamine stock solution, which can be aliquoted in small volumes and stored in the dark at -20 °C;

i) 0,02 µm-filtered ethanol;

2) SYBR® Green I and SYBR Gold® 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.

j) fluorescence free immersion oil.

12.4.2 Equipment and reagents for flow cytometry

The equipment and reagents for flow cytometry include the following:

a) sheath fluid: 0,02 µm-filtered sterile in situ seawater or MilliQ® water;

b) internal standard: 1 002 µm yellow-green fluorescent beads (Polysciences Ref 71825), density $10^8/cm^3$ in 0,02 µm-filtered sterile seawater.

12.4.3 Equipment and tools for molecular diversity of viruses

The equipment and tools for molecular diversity of viruses include the following:

a) equipment: polymerase chain reaction system, water-bath heater, ultracentrifuge, gel electrophoresis imaging system, benchtop, horizontal rotators, constant temperature incubator, vortex mixer, ultrasonic oscillometer;

b) tools and disposables: micropipettor (ranges 0,2 µl to 2 µl, 2 µl to 20 µl, 20 µl to 200 µl, 200 µl to 1 000 µl), 1,5 ml centrifuge tubes, polymerase chain reaction (PCR) tubes, 1 000 µl pipette tips, 200 µl pipette tips, 10 µl pipette tips, Petri dishes.

12.5 Processing and analysis of samples

12.5.1 Enumeration of viruses by epifluorescence microscopy

The procedure includes the following steps.

a) Thaw the -80 °C stored samples at ambient temperature (25 °C) and place on ice until processed.

b) Surfactant processing: shake the sample and transfer into a 10 ml centrifuge tube. Rinse the storage tube with 1,5 ml of 0,02 µm-filtered sterile MilliQ® water and transfer the liquid into the centrifuge tube. Add 500 µl of tetrasodium pyrophosphate stock solution (5 mmol/l final concentration) into the sediment slurry, shake and incubate in the dark at ambient temperature for 15 min.

c) Sonication processing: shake the samples and sonicate in an ice bath for 3 × 1 min (100 W, 40 kHz). During intervals between sonication, shake manually for 30 s to avoid overheating.

d) Centrifugation: add 0,02 µm-filtered sterile seawater into the centrifuge tube to give a total volume of 10 ml. Shake well and centrifuge at 800 *g* for 1 min.

e) Setup the filtration apparatus: the funnel should be soaked in 10 % HCl overnight before use, rinsed with 0,02 µm-filtered sterile MilliQ® water and autoclaved. Wash the funnel with 0,02 µm-filtered sterile MilliQ® water on the day of the experiment and dry the funnel with lint-free paper (the funnel should be cleaned between samples in the same way; the support membrane does not need to be removed). Place a 0,45 µm or 0,8 µm pore, 25 mm diameter, membrane onto the centre of the filter holder as a support membrane (the membrane can be reused until it is broken). Wet the support membrane with 0,02 µm-filtered sterile MilliQ® water and place a 0,02 µm pore, 25 mm diameter Anodisc filter on top, and assemble the filter tower. No bubbles should be present between the two membranes.

f) Preparation of the stain: take out and dilute the SYBR® Green I (or SYBR® Gold) stock stain solution 20-fold with 0,02 µm-filtered sterile MilliQ® water (for example, add 50 µl of stock solution into 950 µl MilliQ® water). Stains should always be handled in low light and placed on ice before use. The rest of the stock solution should be stored in the dark at -20 °C immediately. Repeatedly thawing and freezing adversely affects the stain, so it is necessary to control the stock volume according to the experimental need.

g) Preparation of the antifade solution (0,1 % p-phenylenediamine solution): thaw the p-phenylenediamine stock solution before use. Add 10 µl into 990 µl glycerol/PBS stock solution and mix adequately. The working solution should be kept in low light and placed on ice. The rest of the stock solution should be frozen again immediately and should be discarded after freezing and thawing three times. If the p-phenylenediamine stock solution or the antifade solution has a brownish tint it should not be used.

h) Add the sample: determine the dilution multiple based on sediment type, and filter the corresponding volume of the 10 ml supernatant containing viral particles. 100 µl can be filtered if the dilution multiple is 100-fold, and 20 µl can be filtrated if the dilution multiple is 500-fold, and so on. Samples of sandy sediment can be diluted for 100 to 500 fold; samples of silty sediment can be diluted for 500 to 1 000 fold. Add 1 ml to 2 ml of 0,02 µm-filtered sterile seawater into the funnel before adding the sample. Gently mix the liquid with the pipette tip to distribute the virus particles evenly on the membrane. The tip should not touch the membrane.

i) Filtration: draw the sample through the filter membrane under the pressure of less than 250 mm Hg VAC. Remove the filter funnel.

j) Drying the filter: gently rub the reverse side (not the sample side) of the filter with lint-free paper. Place the filter membrane on the lint-free paper in the dark to air-dry completely (the filter membrane is dry when no translucent areas remain and there is no liquid on the surface). Be careful not to touch the sample side of the membrane filter.

k) Staining: place a 20 µl droplet of SYBR® Green I working solution onto the Petri dish and avoid the light. Place the filter membrane, reverse side down, onto the droplet and allow to stain for 15 min to 20 min in the dark. Rinse the reverse side of the filter membrane with 500 µl of 0,02 µm-filtered sterile MilliQ® water to remove excess stain. Repeat the filter-drying procedure described in step j).

l) Preparation of the slide: place a 10 µl droplet of antifade working solution onto the middle of a glass microscope slide and lay the filter membrane on top (sample side up). Add 20 µl of antifade onto a coverslip and fully cover the filter membrane. No bubbles should be present on or under the filter membrane. The slide can be stored at -20 °C for weeks or months, although counting immediately is the best option.

m) Enumeration: virus particles are distinctly shaped ‘pinpricks’ and fluoresce bright green under blue excitation using an oil-emersion objective. Randomly select 10 to 50 fields of view and count at least 400 virus particles for each filter.

n) Blank controls should be prepared every time on a different day of the measurement, no more than one virus particle should be present in each field of view.

o) Calculate the virus abundance in the sample using Formula (1):

$$N = \frac{N_a \cdot S \cdot D \cdot F_c}{S_f \cdot M} \quad (1)$$

where

N is the abundance of virus particles in the sample, expressed in individuals per gram of dry mass of sediment;

N_a is the average number of virus particles counted in each field view;

S is the filtration area, expressed in square millimetres (mm^2);

D is the dilution fold of the sample;

F_c is the correction factor (computed by repeating the extraction 3 times and adding up all counts of the slides to give the total virus abundance. The correction factor is the ratio of the total abundance to the count of the first extraction);

S_f is the area of the filed of view, expressed in square millimetres (mm^2);

M is the mass of dried 0,5 ml sediment, expressed in grams.

p) Record the results of the analysis on the lab record (refer to the Table D.1).

q) The virus biomass can be estimated by using the factor of 0,2 fg/particles.

12.5.2 Procedure for flow cytometry

The procedure includes the following steps:

a) Prepare the sheath fluid and turn on the apparatus. The sheath fluid filter inside the apparatus cannot be used if filtered seawater is used as the sheath fluid, otherwise the sheath fluid filter should be changed.

b) Carry out for the self-correction procedure for the apparatus. Check the sensitivity and accuracy of the apparatus according to the method provided by the instrument manufacturer (in general, the CV value should be less than 2,0) and ensure the apparatus is in good condition.

c) Thaw the sample quickly in a 37 °C water bath. Add 10 mm^3 of bead solution and 1 cm^3 of sample into a labelled flow cytometry sample tube and mix well with the vortex mixer.

d) Set the parameters of each fluorescent channel of the apparatus: set the threshold value on the red fluorescent channel to make it suitable for the determination of the virus particles in the collected area, record the parameters and collect the log signal.

e) Select and calibrate the flow rate: set the flow rate in the range 10 cm^3/min to 30 cm^3/min and the collection speed in the range 50 to100 particles per second.

f) Data collection: input the sample and begin collecting data after at least 15 s of stability. Extract the data using list mode. The quantity of virus particles collected should be greater than 10 000. Record the setup parameters of the apparatus during sample analysis.

g) Measure the speed of the sample flow: measure the volume of the sample before and after the sample inputting, record the duration of sample inputting, and calculate the speed of the sample flow.

h) Record the results of the analysis on the lab record (refer to Table D.2).

12.5.3 Estimation of the molecular diversity of viruses

12.5.3.1 Overview of the method

The study on the molecular diversity of viruses in sediments is different from that of cellular organisms, in that the viruses should be isolated and purified before DNA (deoxyribonucleic acid) extraction; as there is no universal marker gene for viruses, the total viral DNA should be fragmented randomly; construct the corresponding library and sequence it. After obtaining the sequence information, it is compared with the existing viral genome to determine the group it belongs to.

12.5.3.2 Preparation for the isolation of viruses

The procedure includes the following steps:

a) the experiment glassware should be soaked in 10 % HCl overnight, fully cleaned with distilled water and subsequently sterilized in an autoclave by high pressure steam (120 °C for 20 min);

b) sterilize the centrifuge tube, pipette tips and plastic material with high pressure steam (120 °C for 20 min);

c) filter the distilled water and seawater through a 0,22 µm pore membrane, and sterilize in an autoclave with high pressure steam (120 °C for 20 min), then filter the sterilized water through a 0,02 µm pore membrane;

d) prepare the reagents using the distilled water treated with above treatment, and filter the reagents through 0,22 µm pore membranes.

12.5.3.3 Isolation of viruses

The procedure for isolation of viruses includes the following steps:

a) thaw the -80 °C preserved sample at room temperature (25 °C) and place a 0,5 ml subsample into a centrifuge tube;

b) centrifuge at 8 000 *g* for 10 min, and collect the supernatant;

c) ultrasonic treatment: add 1 ml of filtered seawater into the remaining sediments shake well and sonicated the mixture in water bath by for 1 min (100 W, 47 kHz);

d) centrifugation: centrifuge at 8 000 *g* for 1 min and collect the supernatant;

e) repeat c) to d) five times;

f) collect all the supernatants and filter through a 100 µm pore membrane to remove the large particles;

g) collect the flow-through and centrifuge at 8 000 *g* for 10 min;

h) collect the supernatant and filter through a 0,22 µm pore membrane to remove bacteria;

i) collect the flow-through, centrifuge using caesium chloride and concentrate to a volume of 1,35 ml to 1,5 ml;

j) the concentrate contains most of the virus particles, which can be used for subsequent DNA extraction.

12.5.3.4 Extraction of viral DNA

The procedure for extraction of viral DNA includes the following steps:

a) place 0,5 ml of virus concentrate particles into a sterile 1,5 ml tube;

b) add 1 ml of DNA extraction buffer and 10 µl of 10 mg/ml proteinase K;

c) incubate at 37 °C in a water bath for 1 h and shake using a vortex mixer every 3 min;

d) add 150 µl of 10 % sodium dodecyl sulphate (SDS);

e) incubate at 65 °C in a water bath for 2 h with gentle reverse blending every 15 min to 20 min;

f) centrifuge at 6 000 *g* for 10 min at room temperature and collect the supernatant;

g) add 450 µl of DNA extraction buffer and 50 µl of 20 % SDS into the residue. Shake 10 s using a vortex mixer and incubate at 65 °C for 10 min in water bath;

h) centrifuge at 6 000 *g* for 10 min at room temperature and collect the supernatant. Divide all the supernatant (containing the supernatant from step f) into two 1,5 ml tubes;

i) add 10 µg/ml of RNase according to the volume of the supernatant (1 ml supernatant add 1 µl of RNase) and incubate at 37 °C in a water bath for at least 30 min;

j) add an equal volume of chloroform-isoamyl alcohol (24:1 volume fraction), mix and centrifuge at 12 000 r/min at 4 °C for 10 min;

k) collect the supernatant and add 0,6 volume of precooled isopropanol and place at room temperature for 1 h;

l) centrifuge at 16 000 *g* at 4 °C for 20 min and discard the supernatant;

m) add 70 % ethanol, mix and centrifuge at 16 000 *g* at 4 °C for 10 min, and then discard the supernatant;

n) repeat step m), and invert the tube on a lint-free paper to remove the ethanol;

o) add 50 µl of sterile deionized water and store at -20 °C for the subsequent analysis.

12.5.3.5 Linker-amplified shotgun library

The procedure includes the following steps:

a) randomly shear the obtained viral DNA;

b) carry out end-repairing of the DNA fragments;

c) ligate the dsDNA linkers;

d) randomly amplify the fragments using high-fidelity DNA polymerase;

e) ligate the resulting fragments into the pSMART vector;

f) electroporate into MC12 cells;

g) cloning and culture;

h) sequence.

12.5.3.6 Data analysis

Data analysis includes the following:

a) remove low-quality sequences;

b) annotate the sequences against the NCBI database;

c) construct phylogenetic trees to reveal taxonomic affiliations.

13 Survey of benthic microbes

13.1 Principle

The survey can include bacteria, archaea and fungi from the sediments. Genomic DNA is extracted directly from oceanic sediments. The benthic microbial diversity and community structure are determined using molecular biological techniques such as polymerase chain reaction (PCR), clone library, and high-throughput sequencing. The abundance of benthic microbes are determined by fluorescence microscopy and real-time PCR.

13.2 General provisions

The general provisions include the following:

a) sediment samples should be collected with minimal disturbance;

b) the assessments include diversity and abundance of benthic microbes;

c) sampling tools shall be sterilized or treated with 70 % ethanol prior to use;

d) Eppendorf tubes, pipette tips and solutions for laboratory analysis shall be sterilized and cooled down before use. Reagents shall be filtered through sterile 0,22 µm pore membranes if they cannot be autoclaved;

e) microbial abundances measured by fluorescence microscopy are expressed in particles/ml or particles/g (dry mass);

f) microbial abundances measured by real time-PCR are expressed in copies/g (wet mass).

The technical requirements shall be designed according to the survey plan. A fluid-volume metric for the sediments can be designed according to the survey plan requirements.

13.3 Collection and preservation of the samples

13.3.1 Sampling stations and sample collection

The sampling stations and sample collection include the following.

a) The sampling stations can be aligned with other studies.

b) Surface sediments across the study area can be collected for profiling of marine benthic microbes. To document vertical distribution patterns, a sediment core can be sectioned at intervals of 2 cm. The depth intervals can be adjusted in order to meet the needs of the survey plan.

13.3.2 On-site sample process

13.3.2.1 On-site processing of sediment samples collected by a box-corer

The procedure includes the following steps:

a) remove any disturbed portions of sediment from the surface sediment samples;

b) insert a sterilized sampling tube no less than 2 cm from the side of the box-corer;

c) push the sample out of the sampling tube and section at intervals according to the needs of the survey plan. Remove 1 cm from the surface and keep the middle portion.

13.3.2.2 On-site process of sediments collected by a multi-corer

The procedure includes the following steps:

a) remove surface water from the sampling tube;

b) push the sample out of the sampling tube and section according to the needs of the survey plan. Remove 1 cm from the surface and keep the middle portion.

13.3.2.3 On-site process of sediments collected by pushcore

Follow the working procedure of 13.3.2.2.

13.3.3 Sample storage

The procedure for sample storage includes the following steps:

a) store the samples at -20 °C or -80 °C for microbial diversity analysis and real-time PCR;

b) For fluorescence microscopy, dilute a 1 ml (or 1 cm^{-3}) aliquot of the sediment sample with 0,22 µm-filtered seawater to give a total volume of 10 ml. Add 0,2 ml of glutaraldehyde to a final concentration of a volume fraction of 2 %. Flash freeze in liquid nitrogen and store at -20 °C or -80 °C. Prepare three replicates for each sample.

13.4 Tools and reagents

13.4.1 Equipment

The equipment includes the following:

a) instruments: water bath, analytical balance, stirring plate, ice maker, laminar flow hood, vortex, ultracentrifuge, bench-top centrifuge, PCR thermo cycler, real-time PCR instrument, gel imaging system, electrophoresis systems, orbital shaker, incubator, ultraviolet (UV) spectrometer, vacuum filtration, sonicator, fluorescence microscope, refrigerator, ultra-low temperature freezer, autoclave, pH meter;

b) laboratory consumables: pipettes (0,2 µl to 2 µl, 1 µl to 10 µl, 2 µl to 20 µl, 10 µl to 100 µl, 20 µl to 200 µl, and 200 µl to 1 000 µl), inoculation loop, culture tubes, Petri dishes, PCR tubes, centrifuge tubes (1,5 ml, 50 ml), forceps, tooth picks, glass plates, coverslips, mortar, anodisc filters (0,22 µm), filters (0,22 µm, 0,45 µm, and 0,8 µm), and pipette tips (10 µl, 200 µl, and 1 000 µl).

13.4.2 Reagents

13.4.2.1 Reagents for molecular diversity and real-time PCR

The reagents for molecular diversity and real-time PCR include the following.

a) DNA extraction reagents: liquid nitrogen, DNA extraction buffer, proteinase K (10 mg/ml), lysozyme (50 mg/ml), 10 % SDS, DNA gel extraction kits, chloroform/isoamyl alcohol (24: 1), isopropanol, ethanol, double-distilled water.

b) PCR reagents: 2× PCR buffer (with DNA polymerase), PCR primers, double-distilled water, agarose, PCR product purification kits, DNA gel extraction kits, ethidium bromide (EB).

c) DNA ligation and transformation reagents: DNA ligase, cloning vectors, ligation buffer, double-distilled water.

d) Clone culture reagents: Luria-Bertani (LB) medium, ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), competent cells.

e) Real-time PCR reagents: 2× SYBR® Green PCR mix (with DNA polymerase), PCR primers, double-distilled water, agarose, plasmid extraction kit, restriction endonuclease.

f) DNA extraction buffer: weigh and dissolve 37,3 g of $Na_2EDTA{\cdot}2H_2O$, 17,8 g of $Na_2HPO_4{\cdot}2H_2O$, 15,6 g of $NaH_2PO_4{\cdot}2H_2O$, 87,8 g of NaCl, and 10 g of hexadecyl trimethyl ammonium bromide (CTAB) in 700 ml water. Add 100 mmol/l of tris-HCl buffer (100 mm, pH 8,0). Adjust pH to 8,0. QS to 1 l with double-distilled water.

NOTE QS, "quantify sufficient", means adding enough solvent to bring to the total volume.

g) LB broth (1 l): dissolve 10 g of NaCl, 10 g of peptone and 5 g of yeast extract in 1 l water. Adjust pH to 7,0. Autoclave at 121 °C for 20 min. For LB agar medium, add 1,5 % agar.

h) 50× tris-acetate-ethylene diamine tetraacetic acid (TAE) buffer stock: add 242 g of tris and 37,2 g $Na_2EDTA{\cdot}2H_2O$ to 800 ml of double-distilled water. Mix well to dissolve. Add 57,1 ml of acetic acid and mix well. QS with double-distilled water to 1 l. Store the stock buffer at room temperature. Dilute to 1× TAE with double-distilled water before use.

i) DNA markers: λ-Hind III marker, D2000 DNA marker, 100 bp DNA marker.

13.4.2.2 Reagents for fluorescence microscopy

The reagents for fluorescence microscopy include the following.

a) Phosphate buffered saline (PBS): 0,27 g KH_2PO_4, 1,42 g Na_2HPO_4, 8 g NaCl and 0,2 g KCl. Add 800 ml of double-distilled water, mix well. Adjust pH to 7,4 with hydrochloric acid. QS to 1 l with double-distilled water.

b) 4',6-diamidino-2-phenylindole (DAPI): prepare a DAPI solution to final concentration 1 µg/ml to 5 µg/ml.

c) SYBR® Green I (DNA specific dye) stain stock; store at -20 °C in dark.

d) Mounting solution: 2,4 g polyvinylalcohol 4-88 is added to 6 g glycerol and vigorously mixed at room temperature for 30 min. Add 6 ml of double-distilled water and stir for 2 h. Add 14 ml of PBS solution and mix for 2 h at 50 °C until completely dissolved and the solution is clear. Filter through a 0,22 µm pore sterilized membrane. Store aliquots of 200 µl to 1 000 µl at -20 °C.

e) Antioxidant: freshly prepare 1 mol/l ascorbic acid solution in PBS.

f) Staining solution: dilute SYRB Green I stock solution with mounting solution (1:100) and add the antioxidant solution to a final concentration of 1 %. Adjust pH to 7,4 at room temperature and store at 4 °C. The staining solution can be kept at 4 °C for several weeks.

g) Other reagents: methanol, glutaraldehyde, fluorescence microscopic immersion oil, nail polish.

13.4.2.3 PCR primers

The PCR primers include the following.

a) PCR primers for bacterial 16S ribosomal RNA (16 rRNA):

27F: 5'-GAGTTTGATCCTGGCTCAG-3'

338F: 5'-ACTCCTACGGGAGGCAGCAG-3'

341F: 5'-CCTACGGGAGGCAGCAG-3'

515F: 5'-GTGCCAGCMGCCGCGG-3'

518 R: 5'-ATTACCGCGGCTGCTGG-3'

806R: 5'-GGACTACYVGGGTATCTAAT-3'

907R: 5'-CCGTCAATTCMTTTRAGTTT-3'

1492R: 5'-GGTTACCTTGTTACGACTT-3'

b) PCR primers for archaeal 16S rRNA:

Arch16F: 5'-CTGGTTGATCCTGCCAG-3'

Arch21F: 5'-TTCCGGTTGATCCYGCCGGA-3'

Arch109F: 5'-ACKGCTCAGTAACACGT-3'

Arch349F: 5'-CCCTACGGGGTGCASCAG-3'

Arch519F: 5'-CAGCCGCCGCGGTAA-3'

Arch344R: 5'-TTCGCGCCTGSTGCRCCCCG-3'

Arch806R: 5'-GGACTACVSGGGTATCTAAT-3'

Arch915R: 5'-GTGCTCCCCCGCCAATTCCT-3'

Arch958R: 5'-YCCGGCGTTGAMTCCAATT-3'

c) PCR primers for fungal internal transcribed spacer (ITS), 18S rRNA and 28S rRNA:

ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'

ITS2: 5'-GCTGCGTTCTTCATCGATGC-3'

ITS3: 5'-GCATCGATGAAGAACGCAGC-3'

ITS4: 5'-CGTTACTRRGGCAATCCCTGTTG-3'

1380F: 5'-GCCTCCCTCGCGCCATCAG-3'

1510R: 5'-GCCTTGCCAGCCCGCTCAGC-3'

NL1F: 5'-ATATCAATAAGCGGAGGAAAAG-3'

LS2R: 5'-ATTCCCAAACAACTCGACTC-3'

d) PCR primers for positive check:

M13F: 5'-TGAAAACGACGGCCAGT-3'

M13R: 5'-CAGGAAACAGCTATGACC-3'

NOTE See 13.5.1.2.1, 13.5.1.4.3 and 13.5.2.2.2 for targeting amplification area from the above PCR primers.

13.5 Processing and analysis of the samples

13.5.1 Assessment of molecular diversities of benthic microbes

13.5.1.1 Extraction of genomic DNA from sediments

The procedure for the extraction of genomic DNA from sediments includes the following.

a) Weigh 5 g (wet mass) sediment sample and grind in liquid nitrogen. Add 13,5 ml of DNA extraction buffer and vortex to mix well. Freeze at -80 °C for 3 h. Mix in 65 °C water bath for 10 min to 15 min. Repeat freeze-and-thaw 3 times.

b) Add 50 µl of proteinase K (10 mg/ml) and 2,5 ml lysozyme (50 mg/ml). Shake at 37 °C and 200 r/min for 30 min.

c) Add 3 ml of 10 % SDS to the mixture and mix well. Warm in 65 °C water bath for 2 h (invert gently every 10 min to 20 min).

d) Centrifuge at 6 000 *g* for 15 min. Transfer the supernatant to a new centrifuge tube.

e) To the pellet, add 4,5 ml of DNA extraction buffer and 1 ml of 10 % SDS. Mix well and place in water bath at 65 °C for 1 h. Mix every 10 min to 20 min. Centrifuge at 6 000 *g* for 15 min and retain the supernatant.

f) Combine the supernatants and add an equal volume of chloroform/isoamyl alcohol. Mix well. Centrifuge at 4 °C, 16 000 *g* for 20 min.

g) Transfer the supernatant to a new centrifuge tube. Repeat step f).

h) Transfer the supernatant to a new centrifuge tube and add 0,6 volume isopropyl alcohol. Mix gently, and precipitate at room temperature for 2 h. Centrifuge at room temperature at 16 000 *g*

for 15 min. Discard the supernatant. Wash the pellet twice with cold 75 % ethanol. Centrifuge at 16 000 *g* for 5 min. Air dry. Add 100 µl of double-distilled water to dissolve the pellet. Store in -20 °C freezer.

i) Run the total DNA from the sediment on 1 % agarose gel electrophoresis with λ-Hind III marker as the standard. Check the EB stained agarose gel, and take an image with gel imaging system. The main DNA band can be cut out from the 1,5 % agarose gel and recovered by the kit.

NOTE Other than this method, sediments DNA can also be extracted using commercial kits.

13.5.1.2 PCR amplification

13.5.1.2.1 PCR primers

The PCR primers are shown in Table 1.

Table 1 — PCR primers for benthic microbes

Targeting organism	Targeting region	PCR primers
Bacteria	16S rRNA	27F/1492R
Archaea	16S rRNA	Arch21F/Arch958R Arch109F/Arch958R
Fungi	ITS	ITS1/ITS4

NOTE PCR primers can be selected depending on the needs of the survey plan.

13.5.1.2.2 PCR reaction system

It includes (total volume 50 µl) the following:

a) 2× PCR reaction buffer (with DNA polymerase), 25 µl;

b) primers 10 µm, 1 µl of each forward and reverse primer;

c) DNA template 2 µl;

d) add double-distilled water to 50 µl.

13.5.1.2.3 PCR reaction cycles

13.5.1.2.3.1 Reaction cycles for bacteria

The procedure includes:

a) pre-denaturation: 95 °C 4 min;

b) denaturation: 95 °C 1 min;

c) annealing: 59 °C 1 min;

d) extension: 72 °C 2 min;

e) repeat step b) to d) for 35 cycles;

f) extension: 72 °C 7 min;

g) end at 4 °C.

13.5.1.2.3.2 Reaction cycles for archaea

The procedure includes:

a) pre-denaturation: 95 °C 4 min;

b) denaturation: 95 °C 1 min;

c) annealing: 60 °C 1 min;

d) extension: 72 °C 1 min;

e) repeat step b) to d) for 35 cycles;

f) extension: 72 °C 7 min;

g) end at 4 °C.

13.5.1.2.3.3 Reaction cycles for fungi

The procedure includes:

a) pre-denaturation: 95 °C 4 min;

b) denaturation: 95 °C 1 min;

c) annealing: 55 °C 1 min;

d) extension: 72 °C 1,5 min;

e) repeat step b) to d) for 35 cycles;

f) extension: 72 °C 7 min;

g) end at 4 °C.

13.5.1.2.4 Detection of PCR products

Apply a negative control using sterilized deionized water for all the PCR reactions. Analyse PCR products with 1 % agarose gel electrophoresis with a D2000 DNA marker or 100 bp DNA marker as the standards. Check for negative/positive and the size of the amplified DNA. Replicate 3 times for each sample. Upon confirmation, mix the PCR products from the 3 replicates for constructing the clone library.

13.5.1.3 Clone library

13.5.1.3.1 Purification of the PCR products

Purify positive PCR products using a purification kit or DNA gel recovery kit.

13.5.1.3.2 Ligation reaction system

The ligation reaction system includes the following:

a) DNA ligase;

b) cloning vector;

c) ligation buffer;

d) purified PCR product;

e) sterilized double-distilled water.

13.5.1.3.3 Ligation

The procedure for ligation includes:

a) place the ligation product at 16 °C overnight;

b) the ligation product can be transformed to competent cells or store at 4 °C.

13.5.1.3.4 Transformation

The procedure for transformation includes:

a) thaw competent cells on ice;

b) add about 3 µl to 4 µl of ligation mixture to competent cells;

c) place the mixture on ice-water for 30 min;

d) heat stock at 42 °C for 90 s;

e) place the mixture on ice for 2 min without mixing;

f) add 700 µl of LB medium;

g) incubate in water bath at 37 °C for 20 min;

h) incubate at 37 °C for 40 min while shaking at 135 r/min;

i) spread 100 µl to 200 µl of cell suspension onto a LB plate containing anti-ampicillin, IPTG and X-Gal;

j) incubate the plate at 37 °C for 12 h to 16 h;

k) place the plate at 4 °C for several hours prior to staining to display the colonies.

13.5.1.3.5 Positive clone detection and sequencing

13.5.1.3.5.1 Positive clone check

The procedure for positive clone check includes:

a) use vector common primers of M13F/M13R;

b) randomly pick white colonies from the plate and check by PCR for positive clones;

13.5.1.3.5.2 Colony PCR reaction mixture

The procedure for colony PCR reaction mixture include (total volume 25 µl):

a) 2× PCR reaction buffer (with DNA polymerase), 12,5 µl;

b) forward and reverse primers (10 µmol/l), 1 µl of each;

c) DNA template: 1 µl;

d) add double-distilled water to 25 µl.

13.5.1.3.5.3 Colony PCR reaction cycle

Apply 13.5.1.2.3.

13.5.1.3.5.4 Detection and sequencing of Colony PCR products

The procedure includes the following steps:

a) check the PCR product on 1 % agarose gel electrophoresis to verify the insert length is correct;

b) inoculate the correct colony to 1 ml of LB medium containing 150 µg/ml ampicillin. Shake at 37 °C overnight;

c) in each library, pick about 50 to 300 positive clones for sequencing, send for sequencing.

13.5.1.3.6 Clone library data analysis

The procedure for clone library data analysis includes:

a) Check sequencing results using BioEdit software. Remove vector portion to obtain the target sequences with DNA star software.

b) Remove chimeras in Check-Chimera utility (Ribosomal Database Project).

c) Classify operational taxonomic units (OTUs) and plot rarefaction curves in Mothur and PHYLIP. Calculate abundance, diversity, distribution and coverage of each clone library.

d) Search the representative OTU sequences in the National Center for Biotechnology Information (NCBI) Blast (www.ncbi.nlm.nih.gov/BLAST) to obtain the closest reference sequences. Determine the microbial species composition and ratios between the species in the sample.

e) Align the sequences in Clustal X. Phylogenetic trees are constructed in software MEGA using the Neighbor-Joining method.

13.5.1.4 High-throughput sequencing

13.5.1.4.1 DNA extraction

Follow 13.5.1.1 to extract the sediment genomic DNA.

13.5.1.4.2 DNA quality check

13.5.1.4.2.1 Requirements of DNA quality

DNA samples should be at least 50 ng/µl and their total amount more than 3 µg. The sample can have an optical density (OD) 260/280 ratio between 1,8 to 2,0 without DNA degradation. The DNA sample can be stored in liquid nitrogen.

13.5.1.4.2.2 Check method

The DNA quality can be determined using the following two approaches:

a) DNA purity and integrity can be analysed by agarose gel electrophoresis;

b) DNA purity (OD260/280) and concentration can be analysed by micro-spectrometry.

13.5.1.4.3 PCR primers

The PCR primers are shown in Table 2.

Table 2 — Primers for high-throughput sequencing

Targeting organisms	Target region	PCR primer
Bacteria	16S rRNA: V4	515F/806R
	16S rRNA: V3	338F/518R
	16S rRNA: V3 and V4	341F/806R
	16S rRNA: V4 and V5	515F/907R
Archaea	16S rRNA: V4	Arch519F/Arch806R
	16S rRNA: V3 and V4	Arch349F/Arch806R
	16S rRNA: V4 and V5	Arch519F/Arch915R
Fungi	ITS: ITS1	ITS1/ITS2
	ITS: ITS2	ITS3/ITS4
	18S rRNA: V4	1380F/1510R

13.5.1.4.4 Data treatment and analysis

The procedure for data treatment and analysis includes:

a) classify OTUs according to sequence similarities;

b) compare to the current database to determine microbial species composition and the ratio between species in the sample;

c) plot rarefaction curves for different samples; calculate the abundance and diversity of benthic microbes in different samples.

13.5.2 Benthic microbial abundance

13.5.2.1 Cell counting by fluorescence microscopy

The procedure includes the following steps.

a) Place the frozen sample (storage at -20 °C / -80 °C) on ice after thawing at room temperature.

b) Vacuum filtration assembly: assemble the filter with the holder. Fill the holder with 0,22 µm of sterilized distilled water and vacuum-filter. Repeat 2 or 3 times. When using on the same day, rinse the filter before each sample without removing the backing filter. Take off the filter holder. Place an Anodisc filter (0,22 µm pore size, 25 mm diameter) on top of a backing filter (0,45 µm or 0,8 µm pore size, 25 mm diameter). The backing filter can be used for multiple samples on the same day. Place the filter holder back.

c) Sample treatment: to 10 ml fixed sediment sample, add 0,22 µm of sterile filtered methanol to give a final concentration of 10 %. Sonicate 15 min in 35 °C water bath.

d) Sample filtration: centrifuge the sonicated samples at 190 *g* for 1 min. Dilute 1 ml of supernatant with PBS to 100 ml. Add 1 ml of diluted supernatant to the filter. Gently filter the samples under negative pressure of 15 kPa to 20 kPa until the membrane is completely dry. Add 1 ml of PBS to wash the membrane and filter. Repeat 3 times.

e) Staining: remove the Anodisc filter with forceps under negative pressure. Mount the filter membrane on a glass slide and apply a coverslip (18 mm × 18 mm) with a 6 µl drop of staining solution. There should be no air bubbles on top or below the filter membrane. Seal the slide with nail polish. Although slides can be kept at -20 °C for 1 month, it is recommended counting immediately.

f) Cell counting: observe the slides under fluorescence microscope using oil immersion. Bacterial cells can show bright green colour using the blue filter. Randomly count the cells from 10 to 20 fields. No less than 200 cells should be counted for each sample.

g) A blank control should be checked at the same time. No more than 1 cell should be observed in each field.

h) Use Formula (2) to calculate the total cell counts in the sample:

$$N_B = \frac{N_a \cdot S \cdot D}{S_f \cdot V} \quad (2)$$

where

N_B is the cell count in the sample (particles/ml);

N_a is the average cell count in the microscopic field (particles);

D is the sample dilution factor;

S is the filter area (mm^2);

S_f is the area of the field of view (mm^2);

V is the filtered sample volume (ml).

13.5.2.2 Real-time PCR method

13.5.2.2.1 Extraction of sediment genomic DNA

Follow 13.5.1.1.

13.5.2.2.2 Real-time PCR primers

The real-time PCR primers are shown in Table 3.

Table 3 — Real-time PCR primers

Targeting organism	Target region	PCR primer
Bacteria	16S rRNA	338F/518R
Archaea	16S rRNA	Arch16F/Arch344R
Fungi	ITS	ITS1/ITS2
	28S rRNA	NL1F/LS2R

13.5.2.2.3 Preparation of standard plasmid for real-time PCR

The procedure includes the following steps.

a) Sediment genomic DNA is amplified using real-time PCR primers to build bacterial and archaea 16S rRNA clone libraries and fungal ITS or 28S rDNA clone libraries. Construct the clone library according to 13.5.1.3.

b) Select and sequence a positive clone from each clone library to determine the base pairs of the target gene. Inoculate this clone into 1 ml of LB liquid medium with 150 µg/ml ampicillin. Shake at 37 °C overnight. Extract the standard plasmid with plasmid extraction kit.

c) Linearize the standard plasmid with a restriction enzyme that cuts outside the intended PCR target.

d) Determine the concentration of the linearized plasmid containing the target gene by spectrophotometry.

e) Determine the copy number per microlitre of the plasmid based on molar concentration using Formula (3):

$$C = 6{,}02\times10^{23}\times\left(C_0\times10^{-9}\ /\ M\right) \quad (3)$$

where

C is the plasmid copy number per microlitre (copies/µl);

C_0 is the plasmid concentration (ng/µl);

M is the formula mass of the standard plasmid containing the target gene (plasmid size in base pairs × 660).

f) Carry out a serial dilution of 10-folds in sterilized double distilled water to prepare standard plasmid from 10^2 to 10^9 copies per microlitre.

13.5.2.2.4 Real-time PCR reaction mixture

The real-time PCR reaction mixture includes the following (total volume 25 µl):

a) 2× SYBR® Green PCR Mix (containing DNA polymerase), 12,5 µl;

b) forward and reverse primers (10 µmol/l), 1 µl of each;

c) DNA template, 1 µl;

d) add double-distilled water to 25 µl.

13.5.2.2.5 Real-time PCR reaction cycle

13.5.2.2.5.1 Real-time PCR reaction cycle for bacteria

The procedure includes the following steps:

a) pre-denaturation: 95 °C for 10 min;

b) denaturation: 95 °C for 20 s;

c) annealing: 53 °C 20 s;

d) extension: 72 °C 20 s;

e) repeat steps b) to d) for 40 cycles;

f) collect the fluorescence signal at 85 °C for 10 min.

13.5.2.2.5.2 Real-time PCR reaction cycle for archaea

The procedure includes the following steps:

a) pre-denaturation: 95 °C 10 min;

b) denaturation: 95 °C 20 s;

c) annealing: 61 °C 20 s;

d) extension: 72 °C 20 s;

e) repeat steps b) to d) for 40 cycles;

f) collect the fluorescence signal at 85 °C for 10 min.

13.5.2.2.5.3 Real-time PCR reaction cycle for fungi

The procedure includes the following steps:

a) pre-denaturation: 95 °C 10 min;

b) denaturation: 95 °C 20 s;

c) annealing: 55 °C 20 s;

d) extension: 72 °C 20 s;

e) repeat steps b) to d) for 40 cycles;

f) collect the fluorescence signal at 85 °C for 10 min.

13.5.2.2.6 Real-time PCR and data analysis

The procedure includes the following steps.

a) Perform a real-time PCR reaction with standard plasmid and examine the sample at the same time.

b) Determine the baseline of the real-time PCR reaction. Usually, this refers to the signal level during the initial cycles of PCR, usually cycles 3 to 15, in which there is little change in the fluorescence signal.

c) Set the threshold at the exponential phase of the real-time PCR reaction. Usually, real-time PCR instrument software automatically sets the threshold at 10 times the standard deviation of the fluorescence value of the baseline.

d) Determine the threshold cycle (C_t) of standard plasmid and the examined samples. C_t is the cycle number at which the fluorescence signal of the reaction crosses the threshold.

e) The log of each known copy number in the dilution series of the standard plasmid (X-axis) is plotted against the C_t value for that concentration (Y-axis) to generate a standard curve.

f) The C_t values of the examined sample are compared to the standard curve to determine their copy number. The abundance of the benthic microbes in the sediments is expressed in copies/g (of wet mass).

14 Survey of benthic microalgae

14.1 Principle

The objects of qualitative investigation are mainly microalgae, and microalgal cysts, in the surface sediment with full body, bright pigments, and detectable pigment content. The objects also can include microalgal cells and cyst that can reproduce normally. The sea areas of the quantitative survey are limited to the shallow waters and areas with a shallow euphotic layer. (See Figures I.1 for experimental facilities. For key technical processes, see Figure J.1.)

14.2 General provisions

The general provisions include the following.

a) Determine the technical requirements for qualitative or quantitative investigations according to the needs of the survey plan.

b) The benthic microalgae investigated can include the main groups of living diatoms and dinoflagellates, coccoliths, and the cysts of dinoflagellates. The parameters measured can include composition of species, habitat density and chlorophyll content and toxins.

c) The habitat density of benthic microalgae is expressed in cell abundance per area or unit mass, i.e. in cells/cm^2 or in cells/g of dry mass (m_d) of sediment, respectively. Data for cysts are expressed in cysts/10 cm^2 or in cysts/g of dry mass of sediment. Chlorophyll a is expressed in µg/dm^2 or in µg/g of dry mass of sediment. Toxins are expressed in ng/g of wet mass sediment.

14.3 Collection and preservation of the samples

14.3.1 Sampling design

The sampling design includes the following.

a) Qualitative sampling: these samples are for determining taxonomic composition, species richness, species diversity, etc. of microalgae in the ocean sediments. There is no need for an accurate measurement of surface area, just part of the surface sediment in the sampler is needed.

b) Quantitative sampling: this should be a sample of undisturbed surface sediment. The undisturbed marker is the surface of the sediment with a certain depth of overlying water. The sampler should be closed tight without spilling.

c) Determining the number of samples: at least two parallel samples should be processed for each determinant.

14.3.2 Sampling methods

The sampling points and sampling tools can be selected according to the needs of the survey plan. The sampling method should follow the recommendations in ISO 5667-12:2017, 4.5. The sampling methods include the following.

a) Intertidal zone sampling is in the selected tidal area. Stations for vertical sampling are selected in order to give a representative cross-section of the intertidal zone (high, middle and low tide). At each station, 2 to 4 core samples are collected using sampling tubes and sampling depth is set according to the needs of the survey plan. For muddy sediments, the sample tube is inserted vertically into the mud and the core sample depth is recommended to be 6 cm to 8 cm, which is divided into 0 cm to 2 cm, 2 cm to 4 cm, and >4 cm sections. For sandy sediments, the recommended core sample depth is 10 cm to 20 cm, which is divided into 0 cm to 4 cm, 4 cm to 8 cm, >8 cm sections. For sandy sediments, the top of the sampling tube needs to be tapped with a rubber hammer to reach the desired depth of sampling. Before the sampling tube is pulled out, the rubber plug can be inserted into the top of the tube. Once the sample tube is removed, the bottom end is sealed, and the rubber plug is removed in order to inspect the surface. If disturbance is found, the sample should be recollected.

b) If sampling with a grab bucket or box-corer, a sample of the core sample (resampling) from the bottom sampler can be obtained by using an organic glass tube. The length of the core sample is 8 cm to 12 cm. The sampling location should be at least 2 cm from the edge of the sampler, and 2 to 4 core samples are randomly collected.

c) Collection of sediment capture samples. A sediment capture device is mainly used for studying dinoflagellates cyst formation and sedimentation. Dormant cysts have specific mandatory dormancy period ranging from a few hours to a few months (mostly two weeks to six months). It is recommended that sediment capture samples be collected every 2 weeks, but the sampling frequency can be increased during red tides in order to accurately reflect the relationship between the growth and decline of red tide organisms and the formation of cysts. Some large sediment capture devices are configured with multiple sample collection bottles. These sampling bottles are arranged in a circle on the bottom of the sampler that can be rotated. The program control interval

(5 days to 1 month) collects the sample sediment into a sampling bottle, and then it is tightly closed and then rotated allowing another sample collection bottle to be deployed.

d) Ensure the sample identification, sample collection site and appearance of the sample (including type, odour, whether there is biological material on the surface, etc) are accurately recorded.

14.3.3 Sample preservation and fixation

The procedure for sample preservation and fixation includes the following.

a) For intertidal zone and offshore surveys, samples can be stored into an insulated box or a refrigerated container on the ship until processed.

b) For investigations of viable microalgae and cysts, samples can be stored at low temperature (4 °C to 8 °C) without light and be processed within one month.

c) Long-term preserved deposits samples are often fixed in neutral formalin (5 % to 10 % final concentration). Formalin is adjusted to final concentration of 2 % to 3 % for sifted samples. Other fixatives can include polyformaldehyde and glutaraldehyde.

14.4 Tools and reagents

The tools and reagents include the following:

a) major equipment: vacuum filter, vortex mixer, ultrasonic processor, fluorescence microscope, fluorescence inverted microscope, fluorometer;

b) other tools: blunt-ended forceps, pipettes, tubes, silicone tube, dissecting needle, tissue culture plates, counting chamber, counter, glass fibber filters (GF/F), 0,22 µm common filters, 0,22 µm black polycarbonate filters;

c) solvents: filtered seawater, sterile seawater, distilled water;

d) fixatives: neutral buffered formalin, paraformaldehyde, glutaraldehyde;

e) stains: DAPI (4', 6-diamidino-2-phenylindole), acridine orange, pyrophosphoric acid-sodium chloride (PPi-NaCl) 0,01 mol/l, fluorescein diacetate-propidium iodide (FDA-PI), trypan blue etc.;

f) other reagents: alcohol, cedar oil, glycerine, Dulbecco's phosphate buffered saline (DPBS).

14.5 Processing and analysis of the samples

14.5.1 Sample processing

14.5.1.1 Interception of the samples

Core sediment samples are divided and processed according to the requirements of the survey plan. Generally, living microalgae and dinoflagellate cysts are distributed on the surface of the sediment. 0 cm to 2 cm subsamples of the sediment cores are used to investigate the formation of the cyst only. For investigation of historical cyst deposition, 2 cm to 5 cm depth subsamples are used.

14.5.1.2 Elutriation and separation of the samples

The procedure for elutriation and separation of the samples include the following.

a) The unfixed qualitative samples shall be diluted with sterile filtered seawater. Large pieces of gravel and debris can be separated from the sediment using a 0,5 mm sieve and discarded. Filtered samples can be separated using pipettes after being mixed evenly and then be used for the analysis and culture.

b) Fixed quantitative samples can be diluted and elutriated with distilled water or sterile filtered seawater. Large pieces of gravel and debris are discarded following sieving (0,5 mm). Then the filtered samples can be separated quantitatively with the pipette after being mixed evenly. The filtered diluted samples are incubated with PPi-NaCl (final concentration 1 mmol/l) for 15 min to 30 min at room temperature in the dark.

c) Dilution and elutriation of samples: distilled water is added to a volume of 10 ml and mixed homogeneously with vortex mixer. A 5 ml sample is transported from the column to another sample bottle; repeat this step eight times, and retain the supernatants. Homogenize the supernatants (total 40 ml). The dilution ratio is calculated by taking 2 ml to 4 ml subsample and staining.

d) The elutriation of cysts differs from that for microalgae. They can be filtered and elutriated using a set of sieves because of their larger size. Samples are divided into two subsamples, one for the determination of the wet mass and moisture content, and one for cyst analysis. The wet mass of the first subsample is determined. The subsample is then dried at 70 °C for at least 24 h until its mass is constant. The moisture content is then calculated. The other subsample is filtered using sieves or processed according to palynology after wet mass determination. The palynology treatment refers to the relevant content of this document.

e) Sediment samples are placed into a breaker with a little filtered seawater and treated in the ultrasound bath for 30 s. The samples are poured into each sieve from the large to small pore size. Samples from each sieve are collected and rinsed. Sieved sediments can be mixed with filtered seawater, then centrifuged at 2 000 r/min for 10 min to separate the cysts from other material. The subsamples are placed into sample bottles, fixed in 2 % to 3 % buffered formalin, and made up to a constant volume using filtered seawater.

14.5.1.3 Ultrasonication

Deep-frozen preserved sediment samples are taken into the centrifuge tube, and then dispersed in the ultrasonic processor. Arenous and silted samples are treated for 180 s with amplitude 109, 50 W and 6 mm miniature transducer, disrupting per 45 s and cooling for 1 min; argillaceous samples are treated for 60 s with amplitude 109, 100 W and 3 mm miniature transducer, disrupting per 30 s and cooling for 1 min.

14.5.1.4 Fluorescence staining

The subsamples are stained for 10 min to 15 min using DAPI (final concentration 1 µg/ml to 5 µg/ml) or acridine (final concentration 10 µg/ml) under low temperature in the dark. Generally, the staining operation is carried out at 4 °C.

The solution of FDA-PI can be prepared in advance as follows. Acetone solution with a concentration of 5 mg/ml fluorescein diacetate (FDA) preserved at 4 °C. The FDA is added to the subsample to a final concentration of 100 µg/ml. A DPBS buffer solution with a concentration of 400 µg/ml is preserved at 4 °C. The final concentration is 60 µg/ml when staining at room temperature without light for 3 min.

14.5.1.5 Filtration and collection of the samples

After mixing thoroughly, the stained sample is filtered through a 0,22 µm black polycarbonate filter membrane. The sample bottles are rinsed several times with PPi-NaCl. The stained material is removed from the solution by filtration.

14.5.1.6 Mounting

The black filter is placed onto a clean glass slide using blunt-ended forceps and observed immediately with a fluorescence microscope. Otherwise, it should be kept by mounting temporarily using the cedar oil or a glycerol:distilled water (1:1) solution. It can be kept temporarily for one month under cryopreservation without light, after which time it can be observed by fluorescence microscopy.

14.5.2 Sample analysis

14.5.2.1 Microscopic observation and analysis

The procedure for microscopic observation and analysis includes the following.

a) Fluorescence microscopy.

   Observation and enumeration are carried out at magnifications of 400× to 1 000×. Heterotrophic and autotrophic micro-algae are counted by UV light and blue light respectively.

b) Different light waves and dyes are used to distinguish between heterotrophic and autotrophic microbenthos. After DAPI staining, heterotrophic microorganisms show a blue colour under UV excitation, whereas autotrophic microorganisms show a red colour when viewed with blue light. Mixotrophic microbes give signals under both wavelengths. Bacteria show red (living) or green (dormant or dead) colour under blue light excitation after acridine orange staining. Living and dead cells can be distinguished with FDA-PI staining. Under blue light (maximum wavelength of 495 nm), living cells are stained bright green by FDA; dead cells are stained red by PI.

c) Optical inverted microscope.

   Cysts can be counted under an optical microscope. Since the cysts are mixed with impurities in the sediment, a dissecting needle can be used to remove the impurities. Cyst analysis is performed under an inverted microscope at a magnification of 400× to 600×.

   The detailed procedure is as follows.

   — Take a certain amount of the treated sediment sample (typically 0,5 ml or 1,0 ml, depending on the sample cleanliness) and place it in a counting chamber.

   — Enumerate the cysts under an inverted microscope. First, find the target cysts at a magnification of 100×, and then magnify to 200× to 600× to observe. Use a dissection needle to remove any impurities from around the target. Observe the cyst shape from multiple sides.

   — The type and amount of cysts are recorded. The living and germinated cysts can be distinguishable, especially when the samples are collected by sediment traps.

14.5.2.2 Enumeration

The procedure for enumeration includes the following steps.

a) Count the microorganisms on the filter by observing 20 to 30 fields randomly, and calculate the average number. If the cell density is low, the entire filter membrane can be observed and all cells counted.

b) Use a 0,5 ml or 1,0 ml counting chamber for enumerating cysts. If the number of cysts observed is less than 100, observations can be repeated until at least 100 cysts are counted per sample.

14.5.3 Abundance and biomass calculation

14.5.3.1 Abundance calculation of benthic microalgae

The abundance of microalgae and cysts in the sediment is calculated according to the number of microalgae in each field of view, the ratio of the area of the field of view to the area of the filter membrane, the dilution factor of the sample, and the initial volume of the sample.

14.5.3.2 Carbon biomass calculation

Calculation is according to the microalgae volume data, with reference to the conversion coefficient of each group (Table E.1).

14.5.3.3 Abundance calculation of cysts

Considering the difference of sedimentation rate and moisture content in different sea areas, the cyst abundance is expressed in cyst density per dry mass of sediment. The cyst density in the sediment is expressed in number of cysts per cubic centimetre or per gram of wet mass of sediment.

Cyst abundance is calculated using Formula (4):

$$C = \frac{N \times V / Q}{W / (1 - R)} \tag{4}$$

where

C is the abundance of cysts (cysts/g of dry mass);

N is the number of cysts observed under microscope;

V is the volume of sediment sample after treatment (ml);

Q is the volume for microscopic counting (ml);

W is the wet mass of sediment used for treatment analysis (g);

R is the moisture content of the sediment.

14.5.3.4 Formation rate of cysts

For the samples collected by the sediment trap, the variety and number of cysts in the sample are obtained by microscopic analysis to calculate the formation rate of cysts. The rate is expressed in number of cysts formed per square centimetre (or square metre) per day.

The rate is calculated using Formula (5):

$$A = \frac{N \times V_1 \times V_3}{V_2 \times Q \times T \times S} \tag{5}$$

where

A is the formation rate of cysts [in cysts/(m^2·d)];

N is the number of cysts observed under microscope;

V_1 is the volume of sample from sediment trap (ml);

V_2 is the volume of sample for analysis (ml);

V_3 is the volume of sample after treatment (ml);

Q is the volume for microscopic counting (ml);

T is time (d);

S is the capture area (m^2).

14.5.4 Cyst culture and identification

14.5.4.1 Isolation of cysts

The procedure for isolation of cysts includes the following.

a) Place one drop of washed sediment onto a glass slide. Find the live cysts (apparent bright of pigment) of the desired species. A small needle can be used to clean the background around the cysts. A capillary is used to collect individual cysts.

b) The proximal end of the capillary is connected to a long silicone tube (40 cm to 60 cm) to control suction. The operator can hold the silicone tube in the mouth and use the capillary to suck up the cyst. The target cyst is immediately transferred to another glass slide with culture medium. Repeat the cyst isolation procedure until only the desired cyst remains on the slide.

c) Transfer the cyst to a multiple well tissue culture plate. Incubate in an illuminated incubator. Observe the germination of the cysts and identify the species of the cysts by vegetative cell identification. The general culture medium is f/2 without silicium or SWII (for culture medium preparation see Tables K.1 and K.2).

14.5.4.2 Cyst germination and identification

Inoculate one cyst into each well of a multi-well plate and seal plate with a cover or film to prevent evaporation. Culture temperature is between 10 °C to 30 °C (depending on species). Generally, incubation conditions are 20 °C with approximately 100 µE illumination and 12 h light/dark cycle. Based on the mandatory resting period, begin to observe cyst germination and the growth of vegetative cells on the second day or second week after inoculation. When cells have proliferated sufficiently, vegetative cells can be cultured in a 12-well culture plate, a small test tube or an erlenmeyer flask. Additionally, it is necessary to observe the morphological feature of the empty cyst and the shape and the location of the archeopyle in order to identify and classify the cyst.

14.5.4.3 Cyst identification

It is difficult to identify dinoflagellate cysts based on cyst morphology. It can thus be necessary to study the lifecycle and ecology of the vegetative cells cultivated following excystment.

14.5.5 Fluorescence determination of demagnesium chlorophyll and chlorophyll a

The procedure includes the following steps.

a) Thaw the frozen sediment at room temperature. Cut the sample into sections at 2 cm intervals or according to the needs of the survey plan. Weigh 2 g of each layer of sample, place in a numbered scintillation vial and record the wet mass of each subsample. If the diameter of the sample vial is small, the chlorophyll-a content of unit area can be used.

b) The dry mass and water content of the sample are calculated after pretreatment at -80 °C for 3 h and freeze-drying for 24 h.

c) Add 10 ml of 90 % acetone mix well and allow to stand for 2 h at 4 °C.

d) Treat in ultrasonic processor (4 °C, 80 Hz) for 30 min and allow to stand for 2 h.

e) Centrifuge at 4 000 r/min, for 20 min.

f) Filter the supernatant through a 0,45 µm pore organic membrane.

g) After the sample reaches room temperature, examine using a fluorimeter.

h) All the processes above should be carried out in the dark.

i) For the calculation of chlorophyll a, refer to EN 16161:2012, Clause 7.

14.5.6 Algal toxins determination

14.5.6.1 Technical considerations

Many benthic dinoflagellates contain toxins, and the content of toxins in the dinoflagellate cysts is higher. Technical considerations include the following:

a) based on the results of the pre-experimental analyses of the species identification and the enumeration of cells, the decision is made whether or not to investigate the algal toxins in the sediment;

b) if the number of toxic algal cell or cysts is above 10^5 cells in sample, the toxins can be investigated.

14.5.6.2 Sample collection

Collect the sediment samples qualitatively or quantitatively according to the needs of the survey plan. Only surface sediments are collected.

14.5.6.3 Sample treatment

The procedure for sample treatment includes the following.

a) Weigh 50 g sediment, add filtered sea water and mix well.

b) Ultrasonic oscillation for 15 min to 30 min.

c) Use a sieve to wash the sample (mesh-sizes of a series of sieves, see 15.3.1). Wash the sediment on the upper sieve carefully with filtered seawater using a wash bottle in order to wash out the adsorbed cysts. Transfer the mixture that passes through a 20 µm sieve into a watch glass.

d) Turn the watch glass to use centrifugal force for separating the cysts and algae cells from sand grains. Transfer the cysts and cells into a centrifuge tube with a pipette. The cysts and algae cells in the tube are the sample for toxin extraction.

14.5.6.4 Determining the type of algal toxin

The benthic toxic dinoflagellates are mainly *Prorocentrum* and *Gambierdiscus*. *Prorocentrum lima* contains diarrhetic shellfish poison (DSP). *Gambierdiscus* spp. contain ciguatera. Planktonic dinoflagellate cysts such as *Alexandrium* spp. contain paralytic shellfish poison (PSP).

15 Survey of benthic protozoa

15.1 Principle

Due to the different densities of organisms and sediment particles, microbenthos (e.g. small-density protozoa) and meiobenthos can be separated from sediment particles by silica sol density centrifugation: micro- and meiobenthos float in the silica sols whereas sediment particles sink to the bottom of the centrifuge tube. The target organisms are harvested and enumerated using either a standard or an inverted light microscope. If possible, the analysis combines the quantitative protargol stain and molecular diversity (Figures J.2, J.3, J.4, J.5).

15.2 General provisions

The general provisions include the following:

a) sampling sediments are undisturbed;

b) the survey contents should include the composition, abundance and dominant species;

c) abundances are expressed in individuals/10 cm^2 or in individuals/cm^3;

d) biomass is expressed in µg C/10 cm^2 or in µg C/cm^3.

15.3 Collection and preservation of the samples

15.3.1 Sampling equipment

Sampling inshore areas can be carried out alongside marine geology and/or marine benthos surveys. Sediment samples are collected in the intertidal area by coring using tubes; in the inshore area by using a box-corer; and in the deep sea by using a 0,25 m^2 large box-corer. If possible, sediment samples can be taken by multicorer.

The inner diameter of sampling tubes can be 2,2 cm, 2,6 cm, 3,4 cm, 3,6 cm or 4 cm. Deep-sea sediment samples are directly taken from the multicore or from a giant box-corer using the sampling tubes. The sampling tubes are as described in the survey of metazoan meiobenthos or designed according to the needs of the survey plan.

15.3.2 Sediment core collection

Intertidal areas: two to four undisturbed core samples are randomly collected at each station in a representative section (high, middle, and low tide zones). If possible, samples can be collected by free-diving in subtidal areas according to survey plan requirements.

Inshore sea areas: two or three sediment cores are randomly taken from undisturbed box core samples or directly from Multicore according to the sediment depth, at a distance of at least 2 cm from the corner edge.

Deep-sea areas: samples are directly taken from the multicore or from the box-corer using sampling tubes.

15.3.3 Sediment layers

The sediment layers are selected according to the needs of the survey plan and experimental requirements.

The sediment cores are sliced into 1 cm to 2 cm layers in the intertidal and inshore area. The deep-sea sediment layers are selected according to experimental requirements. The sediment samples collected by multicore are sliced into different layers by a divider.

The surface layer, and/or other layers with target organisms, are taken for the measurement of environmental factors. Subsamples for the analysis of sediment grain size are immediately stored at 4 °C. Corresponding subsamples for the measurement of sediment water content and the concentration of organic matter, organic nitrogen, chlorophyll a and pheophytin a, are stored at -80 °C or -20 °C.

For the analysis of molecule diversity, the surface 1 cm layer is taken and placed into a sterile plastic bag and stored at ultralow temperature.

15.3.4 Sample fixation and preservation

Subsamples of sediment from the different layers are fixed with an equal volume of 4 % ice-cold glutaraldehyde (a volume fraction of 2 % final concentration), immediately mixed, and stored in the dark at 4 °C.

For the separation and cultivation of living protozoa, the sediment samples are processed on site or taken back to the laboratory.

For the analysis of molecular diversity, the sediment samples are stored at -80 °C before DNA and RNA extraction.

15.3.5 Collection and treatment of living protozoa

15.3.5.1 Separation of living protozoa

The separation of living protozoa includes the following.

a) Cultivation method: cultivate in order to make active protozoa in the sediment reproduce and inactive ones (such as resting cysts) excyst and reproduce.

b) Serial dilution method: separate benthic protozoan by a series of elutriation processes, and collect the supernatant for qualitative and quantitative analysis.

c) Pipetting method: collect target organisms with a suitable micropipette under a dissecting microscope for qualitative analysis.

d) Adhesion onto cover slip: place a cover slip on the top of the sediment, allow protozoa to colonise the cover slip, and collect at different times.

e) Sea-water ice method: place some sea-water ice on the top of a sediment column. Allow the melt-water to gently permeate the sediment by passing through absorbent cotton. Under gravity and the temperature difference, the interstitial fauna migrate actively out of the sediment and aggregate in a culture dish placed under the sediment column.

f) Flushing with $MgCl_2$ solution: collect benthic protozoa by flushing sediments with filtered seawater $MgCl_2$, which narcotises benthic protozoa.

15.3.5.2 Cultivation of fresh samples

Pipette field sample into Petri dish, remove extra sediment particles under dissecting microscope, and add a moderate amount of bait feed (rice or wheat grains, microalgae). Check daily and remove non-target species. It is recommended to establish various cultures under different environmental conditions (e.g. different food, temperature and light intensity, etc.). When the raw culture is established, certain species can be grown in pure culture. Target organism can be adapted to laboratory conditions by gradually increasing the ratio of filtered seawater and sediment extract. Those which are difficult to cultivate can be directly isolated for treatment.

15.3.5.3 Observation and record of fresh samples

For the observation of ciliates and flagellates in vivo, high-resolution differential interference contrast microscopy is recommended in order to observe the arrangement of cilia and flagella and their movement. Scanning electronic microscopy is necessary for very small flagellates for observing important features for identification. For large testate amoebae and foraminiferans, it is recommended to observe their shape and movement under a dissecting microscope and then to use high-resolution light microscopy for detailed structures.

Protocols: pipette organisms onto a glass slide and apply a cover slip supported by vaseline at its four corners to avoid squashing the organisms; observe their shape and movement at low magnifications and then move to high magnifications to observe and record detailed taxonomic features including body colour, size, shape, cell structure (e.g. flagella, cilia, skeleton, extrusomes, holdfast, pigments, food vacuoles, contractile vacuoles, nuclear apparatus).

For those organisms that are difficult to preserve with fixatives (e.g. some flagellates and amoebae), it is recommended to cultivate organisms together with sediment in different kinds of culture media. Organisms can then be observed and enumerated in vivo for qualitative and quantitative investigation.

15.4 Tools and reagents

15.4.1 Equipment and reagents for silica sol centrifugation

The equipment and reagents for silica sol centrifugation include the following:

a) device for separating sediment from organisms (vacuum filtration system, Figure I.1), ultracentrifuge or supercentrifuge, vortex mixer;

b) test tubes (50 ml, 100 ml), graduated cylinder, wash bottle, centrifuge tubes (5 ml, 15 ml, 50 ml), straw;

c) Percoll®[3] silica sols, or Ludox® HS 40[3] silica sols, or Ludox™[3] silica sols, distilled water.

15.4.2 Equipment and reagents of quantitative protargol staining

The equipment and reagents of quantitative protargol staining include the following:

a) equipment: vacuum filtration system, thermostatic heating panel, thermostat, light microscope, electronic balance (sensitive quality: 1 mg);

b) tools and vessels: staining jars or filter holder[53], nitrocellulose membranes (diameter: 25 mm, porosity: 1,2 µm to 3 µm), Eppendof pipette (range: 100 µl to 1 000 µl, 1 000 µl to 5 000 µl), pipette, flat tweezers for filter membrane, single edge blade, pan paper, filter paper (diameter 25 mm), large Petri dishes (diameter 15 cm), 50 ml beakers, glass slides (28 mm × 48 mm), cover slips (24 mm × 24 mm, 24 mm × 32 mm), 2 ml Pasteur pipette;

c) reagents:

— agar embedment: 3 % to 4 % agar, 10 % formaldehyde;

— staining: 0,2 % potassium permanganate, 2,5 % oxalic acid, silver proteinate, sheet copper, tap water, distilled water, developer, 0,5 % gold chloride, 2,5 % sodium thiosulfate;

— developer: 0,5 g sodium sulfite dissolved in 98 ml distilled water plus 0,1 g sodium carbonate and 1 g hydroquinone;

— dehydration and mounting: 30 %, 50 %, 70 %, 100 % isopropanol, isopropanol/dimethylbenzene (1:1), 100 % dimethylbenzene, Canada balsam or neutral balsam.

15.4.3 Equipment and reagents for molecular analysis

15.4.3.1 Equipment

The equipment includes the following:

a) equipment: polymerase chain reaction system, universal mutation detection system, water-bath, ultracentrifuge, superclean bench, gel imaging system, shaking table, incubator, and vortex instrument;

b) disposable consumables: micropipette (range: 0,2 µl to 2 µl, 2 µl to 20 µl, 20 µl to 200 µl, 200 µl to 1 000 µl), 1,5 ml tube, PCR tube, pipette tips (1 000 µl, 200 µl, 10 µl), Petri dish.

3) Percoll®, Ludox® HS 40 and Ludox™ silica sols 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.

15.4.3.2 Reagents

The regents include the following:

a) DNA extraction: DNA extraction buffer, proteinase K (10 μg/ml), RNase (10 μg/ml), chloroform, isoamyl alcohol, isopropanol, 70 % ethanol, double distilled water, 10 % SDS, 20 % SDS;

b) PCR: Polymerase, PCR buffer, deoxyribonucleoside triphosphate (dNTP) mixture, double distilled water, primers, agarose;

c) Denaturing gradient gel electrophoresis (DGGE): acrylamide, bis-acrylamide, urea, deionized-formamide, 10 % ammonium persulfate, N,N,N',N'-Tetramethylethylenediamine (TEMED);

d) DNA ligation: DNA ligase, vector, DNA ligation buffer, double distilled water;

e) Cloning: tryptone, yeast extract, agar, sodium chloride, ampicillin (0,1 mg/ml), X-Gal (5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside), IPTG (isopropyl β-D-1-thiogalactopyranoside);

f) 50× electrophoresis buffer: $Na_2EDTA{\cdot}2H_2O$, tris (2-amino-2-(hydroxymethyl)-1, 3-propanediol), deionized water;

g) DNA stain: dissolve 1,0 g of ethidium bromide in 100 ml deionized water, stir till fully dissolved and transfer to amber glass bottle, store in dark and at room temperature. The final working concentration is 0,5 μg/ml;

h) Primers for PCR: Euk1A: CTGGTTGATCCTGCCAG; Euk516: ACCAGACTTGCCCTCC; Euk528F: GCGGTAATTCCAGCTCCAA; Euk706R: AATCCRAGAATTTCACCTCT; CilF: TGGTAGTGTATTGGACWACCA; CilRI: TCTGATCGTCTTTGATCCCTTA; CilRII: TCTRATCGTCTTTGATCCCCTA; CilRIII; TCTGATTGTCTTTGATCCCCTA; Cer25F: CATATGCTTGTCTCAAAGATTAAGCCA; Cer1256R: GCACCACCACCCAYAGAATCAAGAAAGAWC; For14F: ACGCAMGTGTGAAACTTG; For17R: CGGTCACGTTCGTTGC; GC clamp: CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG.

15.5 Processing and analysis of the samples

15.5.1 Silica sol density centrifugation

15.5.1.1 Salt reduction

To avoid gelling of Ludox®, each of the fixed sediment samples should be elutriated to decrease salinity. The procedure includes the following steps:

a) add sterilized tap water to give a volume of 300 ml and glutaraldehyde with a final concentration 1 %;

b) settle for 24 h to 48 h in a graduated cylinder with sealing film on the top;

c) siphon off the supernatant and leave a final 40 ml sample;

d) the process can be delayed this stage by transferring the final 40 ml sample into a sample bottle and storing at 4 °C in the dark.

15.5.1.2 Add silica sol

Place 8 ml of Ludox® solution into a 15-ml conical centrifuge tube. A 50-ml centrifuge tube can be used for large samples.

15.5.1.3 Inject sediment sample

Fast inject 2 ml sample into the Ludox® solution. This process helps to dilute the sol to an appropriate density gradient and separate organisms from fine sediment particles.

15.5.1.4 Preparation of water film

Carefully add about 2 ml of distilled water on the top of the sample-Ludox® mixture. This is to: (1) prevent gelling of Ludox®; (2) rinse down organisms that adhere to the tube wall; (3) facilitate pipetting of extracted organisms.

15.5.1.5 Centrifugation

After the centrifugation at 4 300 *g* for 15 min, six density layers are roughly recognizable (from top to bottom): the water column, the extracted organisms and light organic matter, the diluted Ludox®, the layer containing heavy organic debris/dead diatoms, the denser Ludox®, and the sediment pellet.

The extracted organisms can be harvested by removing the appropriate layer with a Pasteur pipette.

15.5.1.6 Sample analysis

The extracted organisms contain benthic protozoa such as flagellates, ciliates and foraminiferans and other microbenthos and meiobenthos (e.g. cyanobacteria, diatoms and nematodes). The organisms are identified and enumerated using a light microscope (Table D.3). If possible, a filter membrane can be stained with quantitative protargol stain.

15.5.2 Quantitative protargol stain (QPS)

15.5.2.1 General introduction to the method

The extracted organisms are pipetted and concentrated on cellulose nitrate filter. Specimens on the filters are embedded in agar, stained with protargol and mounted to create a permanent slide for further enumeration and identification. This method can be applied according to the needs of the survey plan (Figure J.3).

15.5.2.2 Procedure

15.5.2.2.1 Concentration of cells on the filter membrane

The concentration of cells on filter membrane includes the following.

a) Place the sample in a millipore filter holder with a smooth fitted glass base for supporting filters and a backing filter for even dispersion.

b) Add distilled water or tap water (aerating for 24 h in the air) to the filter. Filter slowly by applying a suction at <5 mm Hg. Avoid adding the fixed samples directly to the filter.

c) Add the extracted organisms to the filter holder. Before all the liquid is removed, add tap water slowly to the side of the column and apply suction until no fixative or silica sols remain.

d) The organisms can be distributed evenly on the filter before the vacuum filtration system turned off. Suck water from the column until it is empty; otherwise, cells may rupture.

15.5.2.2.2 Agar embedding

The procedure for agar embedding includes the following.

a) Warm the glass slides on a heating plate with the temperature in the range of 50 °C to 60 °C; glass slides should be cleaned and prepared before sample filtering.

b) Remove the filter membrane from the filtering device with forceps and place on a warm glass slide. Keep the filter warm and dry during the agar embedding process in order to avoid air bubbles or cells to become suspended in the agar.

c) Place a large drop of liquid agar onto the middle of a second warm slide.

d) The slide is inverted and carefully placed on the filter-bearing slide, so that the whole filter becomes covered with agar, like a sandwich. The warm agar between the two slides spreads to a thin layer. No further pressure has to be applied.

e) The agar/membrane/glass slide sandwich should be kept warm for about 1 min.

f) Place the sandwich into a dish filled with cold tap water for complete solidification of the agar (15 min).

g) The upper glass slide and excess agar are carefully removed. The embedded filter membrane can be peeled off the lower slide. The edges of the filter should be trimmed to make it fit under a 24 mm × 32 mm coverglass.

15.5.2.2.3 Hardening of agar

The procedure for hardening of agar includes the following.

a) Transfer the filter membrane into 10 % formaldehyde for 10 min to 15 min. This hardens the agar and makes it heat resistant.

b) The process can be delayed at this stage by storing the filter membrane in 30 % isopropyl alcohol for several weeks. Before storage, all formaldehyde shall be removed from the filter membrane by rinsing in tap water three times (3 min to 5 min each time) and once in distilled water.

15.5.2.2.4 Protargol stain

15.5.2.2.4.1 Bleaching

The procedure for bleaching includes the following.

a) Remove the filter membrane from the formaldehyde and rinse in tap water for 10 s.

b) Transfer the filter membrane into a 0,2 % solution of potassium permanganate for 6 min to 7 min. The bleaching time depends on the nature of the sample. When the agar is thicker and high amounts of organic matter are present, the bleaching time is longer.

c) Wash in tap water for 10 s.

d) Place filters in 2,5 % oxalic acid for 5 min to 6 min.

e) Wash with tap water for 10 s (3 to 5 times) and distilled water for 10 s (once).

15.5.2.2.4.2 Protargol stain

The procedure for protargol stain includes the following.

a) prepare a 0,2 % to 0,8 % protargol solution by sprinkling 0,08 g to 0,32 g of silver proteinate powder on the surface of 40 ml distilled water (not deionized water);

b) when the powder has dissolved (without stirring), add approx. 0,5 g of unoxidized copper;

c) leave the filter membrane in protargol for 40 min to 50 min in a 60 °C incubator.

NOTE The concentration of protargol solution ranges from 0,3 % to 0,5 % because of variations in the origin, brand or type protargol. The protargol can also be synthesised and the working concentration adjusted according to different product feature.

15.5.2.2.4.3 Developing

The procedure for developing includes the following:

a) cool the filter membrane to room temperature for 10 min;

b) develop for 10 s to 30 s in a hydroquinone solution (optimum time of development can be tested by observing a filter membrane under the dissection microscope: the recommended time is about 15 s);

c) after the colour of the filter membrane (and cells) has changed to yellow, the development can be stopped by transferring into distilled water;

d) wash the filter with tap water once (10 s).

15.5.2.2.4.4 Increase the stain intensity by gold chloride

The procedure includes the following steps:

a) increase the stain intensity by placing the membrane in 0,5 % gold chloride for 2 s to 3 s;

b) place the filter membrane in a 2,5 % oxalic acid solution for 30 s (optional).

15.5.2.2.4.5 Fixation

The procedure for fixation includes:

a) wash in water for 2 min;

b) place the filter membrane in a 2,5 % sodium thiosulfate solution for 5 min;

c) wash with tap water for 3 min (3 to 5 times).

15.5.2.2.5 Dehydration and mounting

15.5.2.2.5.1 Dehydration in isopropyl alcohol

The procedure for dehydration in isopropyl alcohol include:

a) 30 % isopropyl alcohol (5 min to 10 min);

b) 50 % isopropyl alcohol (5 min to 10 min);

c) 70 % isopropyl alcohol (10 min);

d) 100 % isopropyl alcohol (≥5 min);

e) 100 % isopropyl alcohol (≥5 min);

f) Isopropyl alcohol/xylene (1:1) 5 min;

g) 100 % xylene (≥5 min);

h) 100 % xylene (≥5 min).

15.5.2.2.5.2 Mounting in neutral balsam

The procedure for mounting in neutral balsam includes:

a) place a small drop of neutral balsam onto the middle of a glass slide;

b) place a filter membrane onto the neutral balsam (with organisms on the top); add another drop of neutral balsam or Canada balsam;

c) store in a dry ventilated place for 24 h; dry at 60 °C to 80 °C in an incubator for at least three days;

d) permanent mounting preserves the relative integrity of microbenthic and meiobenthic communities.

15.5.3 Estimation of molecular diversity

15.5.3.1 Summary of the methods

The 18S rRNA gene is amplified by eukaryotic- and group-specific primers. The PCR products are analysed by DGGE, clone library sequencing, and high throughput sequencing to estimate the diversity of protozoa.

NOTE The estimation of protozoan diversity based on the 18S rRNA gene sequencing has been successfully applied to marine environments.

15.5.3.2 DNA extraction

The procedure for DNA extraction includes the following steps:

a) place 1 g of sediment into a 1,5 ml tube;

b) add 1,35 ml of DNA extraction buffer and 10 µl of proteinase K (10 µg/ml);

c) incubate at 37 °C in a water bath for 1 h and shake using a vortex mixer every 3 min;

d) add 150 µl of 10 % SDS;

e) incubate at 65 °C in a water bath for 2 h, with gentle inversion every 15 min to 20 min;

f) centrifuge at 6 000 *g* for 10 min and collect the supernatant;

g) add 450 µl of DNA extraction buffer and 50 µl of 20 % SDS, vortex for 10 s and then incubate at 65 °C in a water bath for 10 min;

h) centrifuge at 6 000 *g* for 10 min, collect the supernatant; divide all the supernatant (containing the supernatant from step f) into two 1,5 ml tubes;

i) add the RNase and incubate at 37 °C in a water bath for at least 30 min;

j) add an equal volume of chloroform-isoamyl alcohol (24:1, volume fraction), mix and centrifuge at 12 000 r/min at 4 °C for 10 min;

k) collect the supernatant and add 0,6 volume of isopropanol at room temperature for 1 h;

l) centrifuge at 16 000 *g* at 4 °C for 20 min;

m) add 70 % alcohol, mix and centrifuge at 16 000 *g* for 10 min, and discard the supernatant;

n) repeat step m);

o) add 50 µl of sterile deionized water and store at -20 °C.

15.5.3.3 PCR

15.5.3.3.1 Primers

The protozoan 18S rRNA gene is amplified by the following primers: Euk1A and Euk516, Euk528F and Euk706R, CilF and CilRI, II, III, Cer25F and Cer1256R, and For14F and For17R. The primers with GC-clamp are used for DGGE analysis.

15.5.3.3.2 PCR mixture

The procedure for PCR mixture includes:

a) 0,3 µmol/l of each primer;

b) 1× PCR buffer;

c) 80 µmol/l dNTP mixture;

d) 3 U DNA polymerase;

e) DNA template;

f) deionized water.

15.5.3.3.3 PCR program for primers Euk1A and Euk516

The procedure includes the following steps:

a) 94 °C for 130 s;

b) 94 °C for 30 s;

c) 56 °C for 45 s;

d) 72 °C for 130 s;

e) repeat b) to d) 34 times;

f) 72 °C for 10 min;

g) 4 °C.

15.5.3.3.4 PCR program for primers Euk528F and Euk706

The procedure includes the following steps:

a) 94 °C for 3 min;

b) 94 °C for 30 s;

c) 60 °C for 30 s;

d) 72 °C for 1 min;

e) repeat b) to d) 30 times;

f) 72 °C for 5 min;

g) 4 °C.

15.5.3.3.5 PCR program for ciliate-specific primers

The procedure includes the following steps:

a) 95 °C for 5 min;

b) 94 °C for 45 s;

c) 58 °C for 1 min;

d) 72 °C for 1 min;

e) repeat b) to d) 35 times;

f) 72 °C for 10 min;

g) 4 °C.

15.5.3.3.6 PCR program for cercozoa-specific primers

The procedure includes the following steps:

a) 95 °C for 5 min;

b) 95 °C for 32 s;

c) 70 °C for 36 s;

d) 72 °C for 3,5 min;

e) repeat b) to d) 30 times;

f) 72 °C for 8 min;

g) 4 °C.

15.5.3.3.7 PCR program for foraminifera-specific primers

The procedure includes the following steps:

a) 94 °C for 1,5 min;

b) 94 °C for 1 min;

c) 50 °C for 1 min;

d) 72 °C for 45 s;

e) repeat b) to d) 25 times;

f) 94 °C for 1 min;

g) 50 °C for 30 s;

h) 72 °C for 2 min;

i) repeat f) to h) 10 times;

j) 72 °C for 5 min;

k) 4 °C.

15.5.3.3.8 Detection of PCR products

PCR products are analysed on a 1 % agarose gel for detection.

15.5.3.4 DGGE

15.5.3.4.1 Casting denaturing gradient gels

The procedure includes the following steps.

a) Place the sandwich assembly in the alignment slot. Tighten the sandwich clamps.

b) Put a syringe on to the cam wheel system and set the volume indicator to 14,5 for 16 cm × 16 cm gels.

c) Prepare the desired amounts of the high- and low-density gel solutions in two disposable test tubes (eukaryotes to 6 % polyacrylamide gels prepared with denaturing gradient ranging from 20 % to 50 %; prokaryotes to 8 % polyacrylamide gels prepared with denaturing gradient ranging from 40 % to 60 %).

d) Add a final concentration of a volume fraction of 0,09 % each of ammonium persulfate and TEMED solutions. Cap and mix by inverting several times.

e) Carefully remove air bubbles from the syringe by turning it upside down (plunger cap towards the bench) and gently tapping. Push the gel solution to the end of the tubing.

f) Place the syringe into the gradient delivery system syringe holder.

g) Rotate the cam wheel slowly and steadily to deliver the gel solution. It is important to cast the gel solution at a steady pace to avoid disturbance between gel solutions within the sandwich.

h) Carefully insert the comb to the desired well depth and straighten. Let the gel polymerize for about 60 min.

15.5.3.4.2 Adding samples

The procedure for adding samples includes:

a) remove the comb and put the gel into the electrophoresis instrument;

b) pre-heat the running buffer;

c) add PCR products.

15.5.3.4.3 Electrophoresis

Electrophoresis is run at 80 V for 16 h at 58 °C.

15.5.3.4.4 Staining

The procedure for staining includes:

a) remove one of plates, and place the gel into deionized water;

b) wash the plate with the gel in the deionized water;

c) remove the deionized water, add 250 ml of fixative (10 % ethanol, 0,5 % acetic acid) and fix for 15 min;

d) remove the fixative and wash in deionized water;

e) add 200 ml of DNA stain and incubate for 30 min;

f) visualize the gel with a visible light illumination.

15.5.3.4.5 Image analysis

Analysis of the DGGE image is performed by the Quantity-One software.

The procedure includes the following steps:

a) image improvement;

b) lane detection;

c) band detection;

d) band and lane matching;

e) concentration analysis;

f) conversion of DGGE profile into a species matrix, enabling the community composition at different stations to be compared.

15.5.3.5 Clone library sequencing

15.5.3.5.1 Ligation mixture

The ligation mixture includes the following:

a) DNA ligase;

b) vector;

c) PCR products;

d) sterilized water.

15.5.3.5.2 Ligation procedure

The ligation procedure includes:

a) place the ligation mixture at 14 °C overnight;

b) centrifuge for 10 s;

c) place in an ice bath for 1 min.

15.5.3.5.3 Transformation procedure

The transformation procedure includes:

a) prepare *Escherichia coli* competent cells;

b) add 1 µl to 2 µl of ligation mixture to the competent cells;

c) mix;

d) incubate in an ice bath for 30 min;

e) incubate in a water bath at 42 °C for 30 s to 60 s;

f) transfer into an ice bath for 2 min;

g) add 250 µl of LB;

h) incubate at 37 °C for 1 h with continuous shaking;

i) add 50 µl to 200 µl of LB to the culture medium;

j) incubate at 37 °C for 16 h.

15.5.3.5.4 Detection of positive clone

The procedure for detection of positive clone includes:

a) collect the white colonies of bacteria for the PCR following the program described in 15.5.3.3;

b) PCR products are analysed on a 1 % agarose gel for detection;

c) collect 100 to 500 colonies for sequencing.

15.5.3.5.5 Data analysis

The procedure for data analysis includes:

a) check for chimeras using the Check-Chimera method;

b) cluster OTUs using the software DOTUR;

c) calculate the alpha diversity index for samples using software DOTUR;

d) construct Neighbor-Joining (NJ) trees to identify taxonomic affiliations.

15.5.3.6 High throughput DNA sequencing

15.5.3.6.1 Preparation for sequencing

The procedure for the preparation for sequencing includes:

a) prepare the ice box;

b) transfer the PCR products into the ice box;

c) send the PCR products to a sequencing company.

15.5.3.6.2 Data analysis

The procedure for data analysis includes:

a) cluster the OTUs according to their sequence similarities;

b) annotate the representative sequences of each OTU and estimate the community composition.

16 Survey of metazoan meiobenthos

16.1 Principle

Sediment samples pretreated on site are centrifuged through silica sol and the metazoan meiobenthos floated on the silica gel column. The biological samples are collected in Petri dishes and placed directly under a stereomicroscope to isolate, identify and enumerate the metazoan individuals. Permanently sealed specimens and quantitative silver staining are used for high-precision qualitative and quantitative analysis of major metazoan groups according to the needs of the survey plan (see Figure A.2). In addition, molecular biology tools are employed to assist in the classification and determining the diversity of the metazoan meiobenthos.

16.2 General provisions

The general provisions include the following.

a) Samples are undisturbed sediment from the corer. The sign for undisturbed sediment is that there is no obvious trace of disturbance on the surface of the sediment. The corer should be tightly closed, there should be no/little leakage of the overlying water, and the sample should feel cold.

b) Three sediment cores are recommended to be sampled at each station. Two of the three cores can be used as replicates for the identification and enumeration of the meiobenthos. The other core can be used for analysing environmental parameters including chlorophyll, total organic carbon and sediment particle size.

c) If the sampler is equipped with imaging equipment, photographs and/or video sequences should be taken before sampling.

d) Abundance is expressed in number of individuals per 10 cm^2 (ind/10 cm^2).

e) Biomass is expressed in units per gram of dry mass or per 10 cm^2.

f) The survey should include analyses of the community composition, abundance and biomass of the main taxa and dominant species.

16.3 Collection and preservation of the samples

16.3.1 Sampling in intertidal zones and shallow waters

This part of the work can be carried out simultaneously with the survey of benthic protozoa.

16.3.2 Deep-sea sampling

16.3.2.1 Sampling preparation

The sampling preparation includes the following:

a) multicorer: it is recommended to carry more than 8 sampling cores (inner diameter ≥9,5 cm and length ≥60 cm) and to use a TV multicorer, if possible;

b) box-corer: area of sediment collected is 500 mm × 500 mm (0,25 m^2); it is recommended to use TV box-corer if possible;

c) remotely-operated vehicles (ROV) and manned submersible: there can be more than 3 pushcore sampling cores (inner diameter ≥6,5 cm and length ≥40 cm).

16.3.2.2 Sampling at sea

16.3.2.2.1 Sampling by using a (TV) multicorer

Take the undisturbed sediment from (TV) multi-corer which can be approximately 15 cm to 45 cm in length. Three sediment cores can be collected at each station. Two of the three cores can be used as replicates for analysis of meiobenthos; the other core can be used for analysing environmental parameters.

16.3.2.2.2 Sampling by using a TV box-corer

The procedure includes the following steps.

a) Overlying water in box-corer can be removed by siphoning onto a sieve with aperture of 0,25 mm in order to filter out the benthos in suspension.

b) Photographic images of the surface of samples, with a scale (ruler) and station information plate, can be taken from front and side view. Features of the surface sediment should be recorded.

c) When subsampling sediment from box-corer, it is recommended that the cores be at least 5 cm away from the inside edge of the case. Three sediment cores can be collected at each station. Two of the three cores can be used as replicates for analysis of meiobenthos; the other core can be used for analysis of environmental parameters.

16.3.2.2.3 Sampling by using a pushcore

Take the sediment from the pushcore. It is recommended that core samples be approximately 15 cm to 30 cm in length. Two sediment cores can be sampled at each station. One can be used for analysis of meiobenthos and the other one can be used for analyses of environmental parameters. If there is insufficient sample material, take one sediment core for the analysis of meiobenthos.

16.3.3 Sample slicing

16.3.3.1 Equipment

The equipment includes the following:

a) filtration device and 20 µm membrane filters;

b) sieve: the sieve mesh-size for on-site filtration of overlying water is 30 µm to 32 µm;

c) devices for sediment subsampling: sediment sample extruders, slicing blade, washing bottle, etc.

16.3.3.2 Slicing of sediment cores for meiobenthos analysis

The procedure includes the following steps:

a) Before slicing the cores, depth (height) of the overlying water and sediment in the cores is measured and recorded (see Table C.2).

b) The overlying water should be removed by siphoning onto a sieve of mesh-size 32 µm to filter out the meiobenthos;

c) Place the bottom of the core into the sediment extruder. Put a casing of the same inner diameter on top of the core. Adjust accurately the lower screw disc of the extruder into position. Push the core down to the lower screw disc and extrude the sediment to the required length. Insert slicing blades between the top of the core and pipe and slice off the sediment sample.

d) The two cores of sediment samples used for the analysis of meiobenthos are sliced into four layers, i.e. 0 cm to 1 cm, 1 cm to 2 cm, 2 cm to 4 cm, 4 cm to 6 cm (additional layers can be added if necessary). Pack together the overlying water and sediment of layer 0 cm to 1 cm from every core. The remaining layers of sediment can be bottled individually.

16.3.3.3 Slicing of subsamples for analyses of environmental parameters

Use the surface sediment for the analysis of environmental parameters. Alternatively, the sediment layer 1 cm to 2 cm may also be used according to the demands of the survey plan.

The measured parameters can include sediment particle size, total organic carbon (%), chlorophyll a (Chl a, µg/g) and pheophytin a (Ph a, µg/g).

16.3.4 Sample fixation and preservation

16.3.4.1 Quantification and morphological identification of meiobenthos samples

Fix one of the replicates using an equal volume of 10 % formalin prepared with seawater, and fix the other in 80 % ethanol. Shake immediately and place in the shade.

16.3.4.2 Subsample for molecular diversity analysis of meiobenthos

Fix in Dess-Martin periodinane solution (see Annex K) for the transport, preparation of permanent slides, and DNA sequencing.

16.3.4.3 Subsamples for analyses of environmental parameters

Sediment subsamples for particle size analyses can be refrigerated at 4 °C. Subsamples for analysis of organic carbon, chlorophyll a and pheophytin a should be kept in plastic bags and placed in a -20 °C freezer.

16.3.5 Labelling

A detailed label should be put onto each bottled or bagged samples, respectively (see Table C.1).

16.3.6 Record

After slicing of the samples from each station, fill in a sample record sheet (see Table C.3). Carefully check the record sheets and the labels on the sample bottles and bags.

16.4 Tools and reagents

The tools and reagents include the following:

a) sieves: 4 nested sieves, with mesh size of 500 µm (offshore samples) or 250 µm (deep-sea samples) on the top, followed by 125 µm, 64 µm and 30 µm to 32 µm;

b) tabletop centrifuge: maximum speed 6 000 r/min;

c) stereomicroscope: magnification should be greater than 90×;

d) reagents: ethanol, Rose Bengal solution, Ludox® HS 40 (or Ludox™), distilled water, etc.

16.5 Processing and analysis of the samples

16.5.1 Quantification and taxa analysis

16.5.1.1 Sample pretreatment

The procedure for the sample pretreatment includes the following steps.

a) Pour the sediment sample onto the top sieve of the 4-layer sieve stack (sieves from top to bottom in the order 250 µm, 125 µm, 64 µm and 30 µm to 32 µm). Rinse gently with filtered tap water to remove formaldehyde, seawater and most of the sediment.

b) Transfer the samples remaining on each sieve to dying vats containing 0,1 % Rose Bengal solution and stain for 1 h.

c) Rinse the stained specimens with water until the colour of the rinse water fades.

16.5.1.2 Silica sol centrifugation technique

The procedure includes the following steps.

a) Prepare a solution of Ludox®: dilute Ludox® HS 40 (or Ludox™) with distilled water until the density reaches 1,19 by hydrometer.

b) Transfer the sediment left on each sieve to a 100 ml centrifuge tube. If the sample volume exceeds 15 ml, it can be divided into two separate centrifuge tubes. Add the Ludox® solution prepared above until the total volume reaches 60 ml, cap tightly and mix thoroughly by shaking.

c) Stand the centrifuge tube for 5 min to allow the sedimentation of the denser particles.

d) Balance the tubes and centrifuge at 1 800 r/min for 5 min. The centrifugation speed can be reached following a gradual increase over 3 min.

e) Pour the supernatant onto a 32 µm sieve (the Ludox® reagent in the tube can be drained).

f) Add same amount of Ludox® solution into the centrifuge tubes. Repeat the above steps 3 times. Rinse the residues from the wall and cap of the centrifuge tube and sieve the rinse water.

g) Rinse the sieve thoroughly with filtered tap water to remove the Ludox® solution. Place the sieve into a dying vat with 1 % Rose Bengal solution and stain for 1 h. After staining, rinse the sieve with distilled water until the colour of the rinse water disappears. Count the meiobenthos under a stereomicroscope.

16.5.1.3 Subsampling

The procedure for subsampling includes the following.

a) If there are a large number of meiobenthos (over 500 individuals) in the sediment, subsamples can be randomly taken for identification and counting.

b) Transfer the sample into the sample splitter and add distilled water to a total volume of 2 dm^3. Close the splitter cap and mix. Allow the solution stand for 1 h. Take 2 or 3 subsamples.

16.5.1.4 Counting

Observe, identify and count the meiobenthos under a stereomicroscope (magnification ≥40×). Record the number of individuals of different taxa in Table D.4.

16.5.2 Molecular analysis of biodiversity

16.5.2.1 DNA extraction

The templates for PCR amplification of the gene of interest from nematodes can be obtained in a variety of methods. The method chosen can provide sufficient DNA as a template for PCR reactions of multiple genotypes, and should be less prone to cross contamination. The following method is recommended, in addition to which the commercial kits for DNA extraction can also be used.

An example of lysis buffer is given in K.13.2. In practice, specialized components can be empirically added to increase the efficiency of lysis. All samples should be processed in a DNA-free environment using a dedicated area that is UV-sterilizable. In addition, all solutions can be treated in the same way and be dispensed in small tubes, which can be discarded after use. The working procedures are as follows.

a) Put the lysis buffer (see Table K.4 for formulation) 20 µl (for individual nematodes) or 50 µl to 100 µl (for 10 to 100 nematodes) into 0,5 ml PCR cores containing the tissue samples.

b) Store at -70 °C for 15 min or more. The lysate can be stored at -70 °C for several days. Repeated cycles of freezing and thawing can increase the efficiency of lysis.

c) Thaw the sample at room temperature and add a drop of mineral oil.

d) Digest at 60 °C for more than 1 h to remove protein and membrane material.

e) Inactivate the protease and nuclease by heating to 95 °C for 15 min.

f) Cool to 4 °C.

g) Vortex gently for 2 s to 3 s or pipette samples repeatedly, and then centrifuge at 6 000 r/min.

h) Take 2 µl of the supernatant for PCR amplification. Set up a negative control.

16.5.2.2 PCR amplification

There is no universal primer applicable to all nematodes. Therefore, it is recommended to modify the primers for specific target sites. There are many taxa of nematodes, and primers can be designed according to the preliminary morphological classification results. Three different sites are recommended for genetic analysis of nematodes: small subunit ribosomal DNA (SSU rDNA), large subunit ribosomal DNA (LSU rDNA), and mitochondrial DNA (e.g. MtCO1). SSU rDNA sequence data can be used for species-level identification of certain nematodes. LSU (D2-D3) can also differentiate nematodes at the level of species. Mitochondrial DNA can be used for intraspecific differentiation of nematodes.

In experiments, a negative control should be set up. All reagents can be dispensed in small volume. PCR and PCR product processing can be performed in the assigned area in order to prevent contamination. Pipette tips, reagents, and experimental areas should not be contaminated with DNA.

16.5.2.3 Sequencing

Sequencing can be performed using standard methods. Routine DNA sequencing can be performed by a professional sequencing company. The sequencing results can include files of standard data, such as sequencing chromatograms.

16.5.2.4 Preservation of DNA material

Samples of extracted DNA can be stored for subsequent research and optimization of quality control/experimental methodology. Lysates of individual nematodes can be stored in a cryogenic refrigerator. FTA paper can also be used for long-term preservation of nucleic acid at room temperature.

16.6 Organization of data

16.6.1 Precision

The precision provisions are as follows:

a) the precision of meiobenthos enumeration is expressed as standard error or confidence limits (95 % C.L.);

b) statistical test of differences in community structure can be carried out.

16.6.2 Abundance

16.6.2.1 Abundance calculation

Use Formula (6) for the abundance calculation:

$$D = \frac{T}{\pi r^2} \tag{6}$$

where

D is the abundance of individuals, expressed in number per 10 cm^2 (ind/10 cm^2);

T is the average number of individuals among replicates of core samples (ind);

r is the radius of the sampling core (cm).

16.6.2.2 Spatial distribution of abundance

Calculate the total abundance of main biological taxa at each station and the average abundance of the surveyed areas. Complete Table D.4.

16.6.2.3 Vertical distribution of abundance

Calculate the relative abundance (%) of organisms, and the composition of main taxa, in each sediment layer. Complete Table D.4.

16.6.3 Biomass

16.6.3.1 Biomass of individual meiobenthos

The biomass can be calculated based on the conversion of volume, which is applicable to main taxa of meiobenthos.

a) Based on the measurements of organisms using camera lucida microscopy, the volume of any individual can be calculated according to Formula (7):

$$V = L \cdot W^2 \cdot C \tag{7}$$

where

V is the volume of one individual (mm^3);

L is the body length of individual (for long-tailed species posterior to conical part; for filiform-tailed species, posterior to anus), expressed in millimetres (mm);

W is the maximum body width (mm);

C is a conversion factor.

b) Calculate the average volume of individuals according to Formula (8):

$$\bar{V} = \sum_{i-1}^{N} V_i / N \tag{8}$$

where

$\bar{V}$ is the average volume of individuals (mm^3/ind);

V_i is the volume of the *i*th individual (mm^3);

N is the number of individuals (ind).

c) Calculate the individual dry mass by conversion, according to Formula (9):

$$D_w = \bar{V} \cdot K \cdot D \tag{9}$$

where

D_w is the average dry biomass of individuals (10^3 μg/ind);

$\bar{V}$ is the average volume of individuals (mm^3/ind);

K is the average gravity, assumed to be 1,13;

D is the dry-wet ratio, assumed to be 0,25.

16.6.3.2 Calculation of the meiobenthos biomass

Calculate the biomass of meiobenthos according to Formula (10):

$$B = \sum_{i-1}^{N} D_w \cdot \bar{D}_i \tag{10}$$

where

B is the total biomass of meiobenthos (10^3 μg/10 cm^2);

D_w is the average individual body mass of the *i*th population (10^3 μg/ind);

$\bar{D}_i$ is the average individual abundance of the *i*th taxon (ind/10 cm^2);

N is the number of taxa.

Calculate the total biomass at each station based on the above Formula (10).

16.6.4 Complete the report

Complete the report according to the relevant provisions in this document.

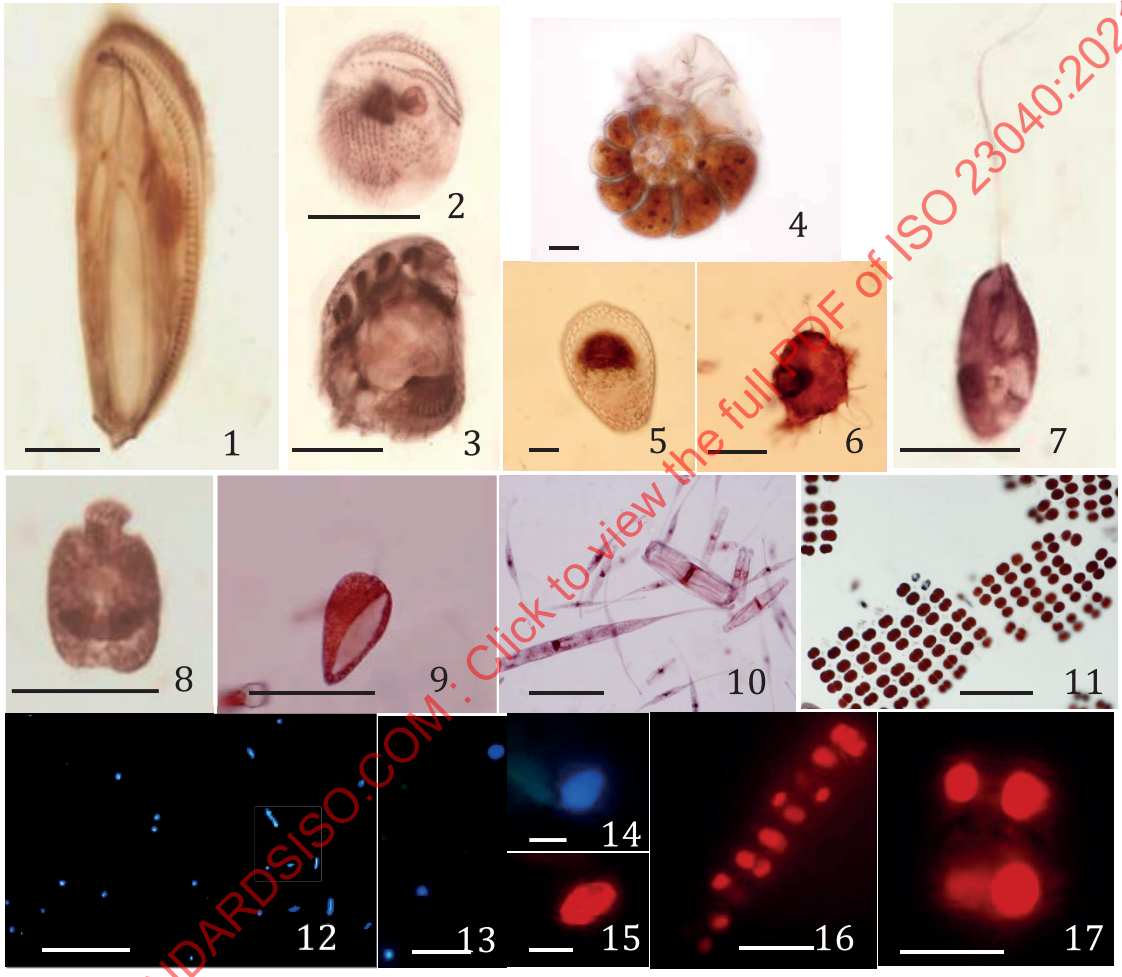
16.6.5 Plotting data

Plot data according to the requirements of the survey plan, such as the percentage composition, spatial distribution and vertical distribution of abundance and biomass of main taxa.

Annex A
(informative)

Photographs of several groups of communities of interstitial biota

Figure A.1 shows pictures of several representative microbenthos. These are only examples of photographs.



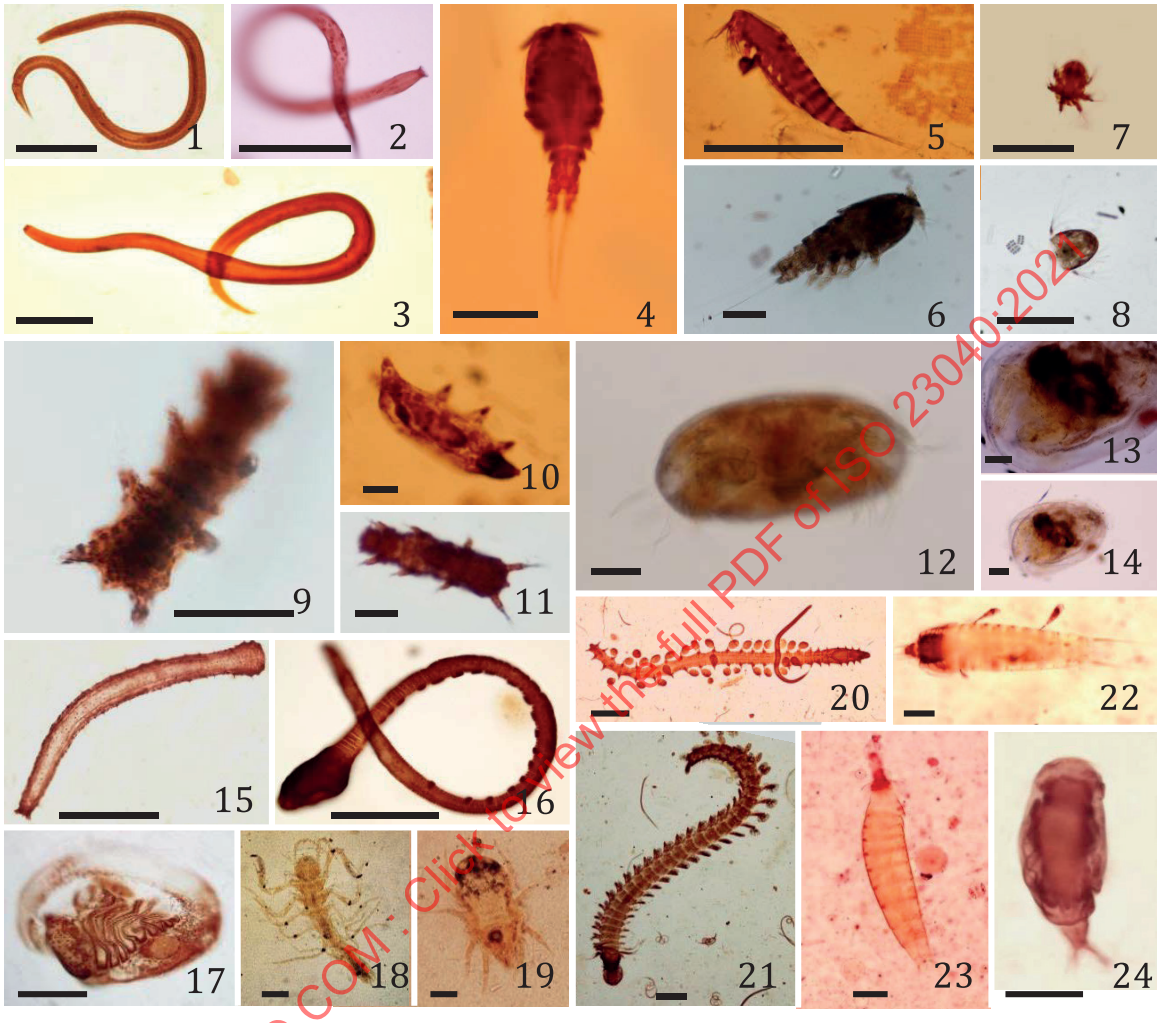
Key

1, 2, 3	ciliates	11	cyanobacteria
4	foraminifera	12	bacteria
5, 6	testate amoeba and gymnamoebida	13, 14	heterotrophic flagellates
7, 8, 9	flagellates	15	autotrophic flagellate
10	diatoms	16, 17	diatoms

NOTE 1 to 11 are after quantitative protargol staining; 12 to 17 are after DAPI staining; bar = 20 µm.

Figure A.1 — Example of representatives of major groups of microbenthos

Figure A.2 shows pictures of several representative metazoan meiobenthos. These are only examples of photographs.



Key

1, 2, 3	nematodes	17	bivalve
4, 5, 6, 7, 8	copepods and copepod nauplius	18	arthropod
9, 10, 11	tardigrades	19	mite
12, 13, 14	ostracoda	20, 21	polychaetes
15	gastrotricha	22, 23	kinorhyncha
16	priapulid worm	24	rotifera

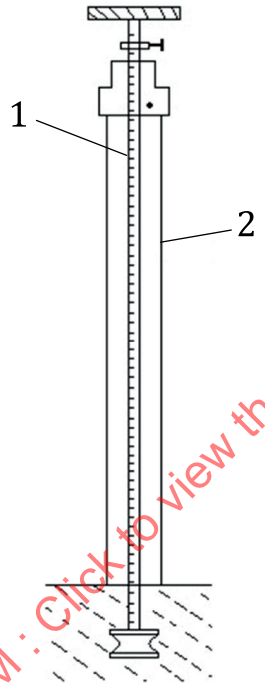
NOTE All pictures are after quantitative protargol staining.

Figure A.2 — Example of representatives of major groups of meiobenthos

Annex B
(informative)

Several stratified sampling devices for the survey of interstitial biota

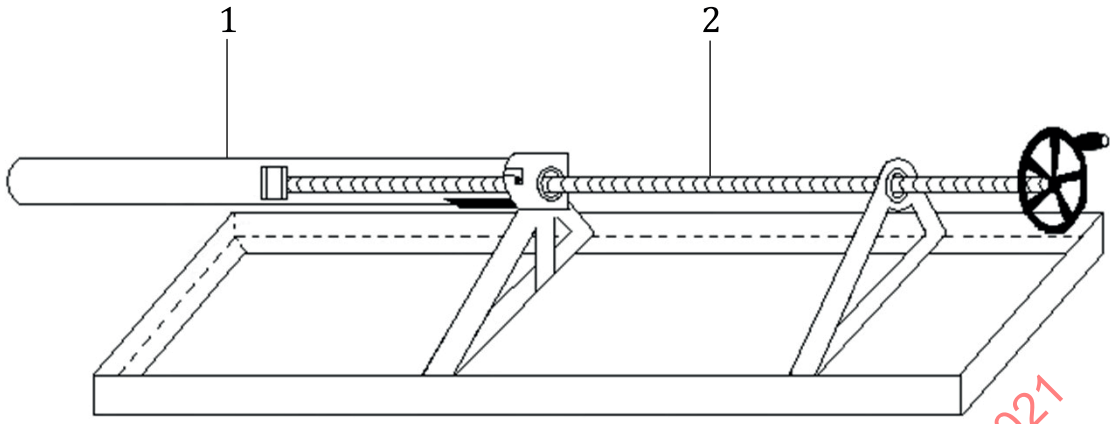
Figure B.1 and Figure B.2 show example of quantitative stratified sampling devices for interstitial biota survey.



Key

1 stratified sampling device
2 cylindrical sampling tube

Figure B.1 — Push-type stratified sampling device



Key

1 cylindrical sampling tube

2 rocker-type stratified sampling device

Figure B.2 — Rocker-type quantitative stratified sampling device

Annex C
(informative)

Tables for sample labelling and sampling record — Examples

See Tables C.1, C.2, C.3 for examples of sample labels and sampling records for interstitial biota.

Table C.1 — Sample label

Station:_____ Date:_____ Time:_____ Weather:_____
Water depth:____m Longitude:_____ Latitude:_____ Layer:_____
Sample condition:
Water temperature: Air temperature:
Sediment texture: Sediment colour: Sediment smell:
Column sediment core: Surface layer sediment:
Note:

Table C.2 — Sampling record table

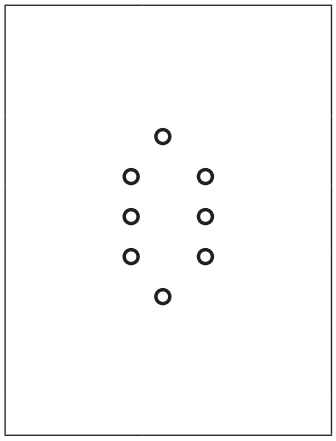
Page_____Total pages_____

Sea area_____**Vessel**_____**Cruise**_____**Station**_____

Longitude of the measured station (E/W)_°_'_'' Latitude (N/S)_°_'_'' Water depth__m Sampling number__

Inner diameter of sampling tube__cm Sampling date (UTC)__Year__Month__Day to__Year__Month__Day

	Deploying into water	Touching the bottom	Retrieving onto deck
Local time			
Latitude (N/S)	° ' "	° ' "	° ' "
Longitude (E/W)	° ' "	° ' "	° ' "
Water depth, m			
Cable length, m	——		——



Core number	Height of overlying water (cm)	Length of sediment (cm)	Length of sampling tube (cm)	Note
1				
2				
3				
4				
5				
6				
7				
8				

Table C.2 *(continued)*

9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
Note:				
Sampling_____Recording_____Proofreading_____				

Table C.3 — Sampling records of meiobenthos

Page_____Total_____Pages

Sea area_____Vessel_____Cruise_____Station_____

Longitude of touching seabed (E/W)___°___'___'' Latitude (N/S)___°___'___'' Water depth____m

Type of sampler_____ Inner diameter of sampling tube_____cm Type of sediment_____

Sampling time (UTC)____Year____Month____Day____Hour____Minute to____Year____Month____Day____Hour____Minute

Touching bottom (UTC)____Year____Month____Day____Hour____Minute to____Year____Month____Day____Hour____Minute

Metazoan meiobenthos				**Chloroplast and organic carbon**	
Layer	Core number:	Core number:	Core number:	Layer	Core number:
				0 cm~1 cm	
0 cm~1 cm				1 cm~2 cm	
1 cm~2 cm				2 cm~3 cm	
2 cm~4 cm				3 cm~4 cm	
4 cm~6 cm				4 cm~5 cm	
6 cm~8 cm				5 cm~6 cm	
8 cm~10 cm				6 cm~7 cm	
10 cm~12 cm				7 cm~8 cm	
12 cm~14 cm				8 cm~9 cm	
14 cm~16 cm				9 cm~10 cm	
16 cm~18 cm				10 cm~11 cm	
18 cm~20 cm				11 cm~12 cm	
Note:					

Sampling_____Recording_____Proofreading

Annex D
(informative)

Tables of microscopy records — Examples

Tables D.1 and D.2 show examples of tables of benthic viruses and bacteria, for counting and testing records.

Microscopy tables of microbenthos and meiobenthos are shown in Tables D.3 and D.4.

Table D.1 — Table of benthic viruses and bacteria: counting by fluorescence microscopy

Total pages_____**Page**_____

Area_____**Name of ship**_____**Voyage**_____**Sampler**_____**Counting method**_____

Position number	**Sample number**	**Water depth** m	**Sampling time**	**Counting time**	**Dilution multiple**	**Particles of field** particles	**Average particles of field** particles/field	**Total particles** particles/g	**Remark**
Event:									

Analyst_____**Recorder**_____**Proofreader**_____

Table D.2 — Table of benthic viruses and bacteria: counting by flow cytometer

Total page_____Page_____

Sea Area_____Name of ship_____Voyage_____Sampler_____Counting method_____

Position number	Sample number	Water depth m	Sample layering	Sampling time	Counting time	Injection volume cm^3	Voltage V					Laser power mW	Flow velocity mm^3/min	Injection time min	The number of cells		Sample volume mm^3		Population density 10^3 cells/cm^3	
							FS	SS	FL1	FL2	FL3/ Threshold value				virus	bacteria	No additives	Have additives	virus	bacteria
Event:																				

Analyst_____Recorder_____Proofreader_____

Table D.3 — Microscopy table of microbenthos

Surveyor: _____Total pages_____Page

Sample position and time (R1):	**Sample position and time (R2):**	**Sample position and time (R3):**
Sample volume (R1):	**Sample volume (R2):**	**Sample volume (R3):**
Remark:	**Remark:**	**Remark:**

Specific name and characteristic (sketch)		**Length×width** μm	**Individual number**	**Remark**
sp. 1			R1:	
			R2:	
			R3:	
sp. 2			R1:	
			R2:	
			R3:	
sp. 3			R1:	
			R2:	
			R3:	
sp. 4			R1:	
			R2:	
			R3:	
sp. 5			R1:	
			R2:	
			R3:	
sp. 6			R1:	
			R2:	
			R3:	
sp. 7			R1:	
			R2:	
			R3:	
sp. 8			R1:	
			R2:	
			R3:	
sp. 9			R1:	
			R2:	
			R3:	
sp. 10			R1:	
			R2:	
			R3:	