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Marine technology — Marine environment impact assessment (MEIA) — On-board bioassay to monitor seawater quality using delayed fluorescence of microalga

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Foreword

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

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

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

Introduction

Mining of offshore mineral resources has attracted much interest. These resources can be utilized as potential mineral resources. However, deep-sea mining of the seafloor can pose potential hazards to deep-sea environments and ecosystems (see ISO 10253 and References [3], [19], [21]). One concern is the toxicity of heavy metals released from excavated minerals. Such heavy metals can be released into the seawater of the deep marine ecosystem[7],[18]. Further, there is a risk of unexpected leakage of the recovered minerals and mining wastewater from the mining plant, which can result in heavy metal contamination of the surface seawater[8].

Considering the above, an appropriate scheme for the monitoring and evaluation of the quality of deep and surface seawater can ideally be introduced at each deep-sea mining site. The International Seabed Authority (ISA) states that environmental impact assessments should address not only areas directly affected by mining, but also the wider region impacted by discharged plume and materials released during mineral transport to the surface[8].

An on-board or onsite method for heavy metal evaluation is essential, as it would allow prompt action in case of an unexpected pollution incident. Rapid evaluation of the nature and extent of pollution provides an opportunity to prevent wider spread of the toxic contaminants and, consequently, minimize idle periods of a mining plant.

Although many chemical analytical methods are available at land-based laboratories, few methods have been developed for on-board application. Deep-sea mineral deposits are inhomogeneous and can be the source of release of various types of metal elements. Therefore, evaluation of mining contaminants requires simultaneous analysis of multiple elements. Special instruments are needed to perform such analyses, such as inductively coupled plasma mass spectrometry. Further, these instruments have to be operated by expert staff, require considerable laboratory space and are expensive to install. Such instrument types are difficult to install at every mining site as standard equipment for environmental monitoring.

Bioassays constitute an alternative approach to specialist equipment, and are commonly used to assess ecological risks of chemical contaminations (see ISO 10253). Bioassays do not provide quantitative information about the contaminating substances, but can be used to detect a wide spectrum of toxicants, including unknown toxicants. This feature is advantageous for the monitoring of water quality during deep-sea mining activities.

General bioassay test protocols that use a variety of aquatic organisms have been published by organisations, such as ISO (see ISO 10253), the Organization for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency (US-EPA). These authorized protocols are accepted in various water quality management fields. However, similarly to chemical analyses, they require a considerable amount of time and space, and are thus not suitable for on-board monitoring. It should also be noted that most protocols have been developed for inland freshwater quality assessments.

This document was developed to address the shortcomings of the currently available bioassays for monitoring seawater quality on-board. It describes a bioassay specifically for on-board determinations.

Marine technology — Marine environment impact assessment (MEIA) — On-board bioassay to monitor seawater quality using delayed fluorescence of microalga

1 Scope

This document specifies a bioassay for the determination of the presence of unknown toxic contaminants in test seawater (see Figure A.1). It is based on the inhibition of photosynthetic activity of the marine cyanobacterium *Cyanobium* sp. (NIES-981) by such toxic contaminants. The inhibition is determined based on delayed fluorescence (DF) intensity.

The method is rapid and requires less laboratory space than standard bioassays. Hence, it can be used on-board to generate basic data for seawater quality management at deep-sea mining sites where time and space are extremely limited.

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 terminological databases for use in standardization at the following addresses:

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

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

3.1
delayed fluorescence
DF
delayed light emission
weak fluorescence signal from photosynthetically active cells that originates upon repopulation of the excited energy states of chlorophyll by stored energy after charge separation

3.2
DF decay curve
time-course change of the *DF* (3.1) intensity of *test algae* (3.7) that had been left in darkness after exposure to light

Note 1 to entry: See Annex E, Table E.1 and Figure E.1.

3.3
effective concentration
ECx
concentration of test substance that results in an x % reduction in specific growth rate relative to the controls

3.4
no observed effect concentration
NOEC
tested concentration below the *LOEC* (3.5) that has no statistically significant effect

3.5
lowest observed effect concentration
LOEC
lowest tested concentration that is significantly different from control

3.6
test seawater
seawater that is tested

3.7
test algae
alga (*Cyanobium* sp., NIES-981) that is used for bioassay

3.8
growth medium
artificial seawater (ASW-SN) containing nutrients and trace metals, commonly used for the culture of *test algae* (3.4)

3.9
test medium
mixture of the *growth medium* (3.8) and *test seawater* (3.6)

3.10
algal stock culture
living or cryopreserved culture of test algae (*Cyanobium* sp., NIES-981) that has been prepared at a land-based laboratory and is carried on board

3.11
algal inoculum culture
culture used in the bioassay, prepared from the *algal stock culture* (3.10) immediately before testing

3.12
cryopreservation
preservation of cells, tissues or organs at a very low temperature for future use

Note 1 to entry: See Annex D.

4 Principle

The described on-board bioassay provides basic data for seawater quality management at deep-sea mining sites using DF (3.1) of the marine cyanobacterium *Cyanobium* sp. (NIES-981). The method is quicker, and requires less laboratory space and equipment, than a standard growth inhibition assay using other algae[15]. First, the test seawater is collected at the target site, e.g. the surface seawater in the vicinity of the mining plant or mining wastewater generated by the mining plant. Then, duplicate cyanobacterium cultures in triplicate are set up in control tubes (growth medium with no test seawater) and tubes containing diluted test seawater [test seawater mixed with growth medium at a volume fraction of 80:20] (see Annex A and B, Figure A.1, A.2 and B.1). After incubation of 24 h, DF is determined using an appropriate detector system for luminescence (see 6.2). Finally, total DF intensities of the test seawater are compared with those of the control (growth medium with no test seawater) using an appropriate statistical test. Significant differences between the test seawater and control indicate that the collected test seawater has been polluted by mining or other activities. The results of the on-board bioassay would support the appropriate environmental safety actions. As an option, effective concentration (ECx), no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values may be determined by assaying additional dilutions of the test seawater in a geometric series (see ISO 10253).

DF is measured as a delay (ms to min) after the cells are transferred from light to darkness[4],[20]. The delay in emission is associated with the repopulation of excited states of chlorophyll by stored energy after charge separation. More specifically, it is the back-reaction of accumulated charges across the thylakoid membrane in the electron transport chain[11]. Because DF is an indicator of the electron

transfer state within the photosynthetic apparatus, it can be used as a sensitive intrinsic index of photosynthetic activity [12],[16],[17].

Bioassays that rely on alga and plant DF are comparable with conventional growth inhibition tests[5],[6],[9],[13],[14],[22].The method described in this document is a new DF-based bioassay system developed specifically for water quality monitoring in offshore environments. It relies on a marine autotrophic cyanobacterium and a modified method[22]. The DF-based bioassay is rapid and requires less extensive sample handling than the standard growth inhibition test. Consequently, it can be used on-board, where time and space are substantially limited.

5 Materials

5.1 Test alga

Axenic culture of the marine cyanobacterium *Cyanobium* sp. (NIES-981). Strain NIES-981 is closely related to the genus *Synechococcus* that is one of the major primary producers in the marine environment. It exhibits stable and high growth under the appropriate conditions. The complete genome of strain NIES-981 has been sequenced. It encodes 3 268 proteins, and harbours 46 tRNA genes and three sets of rRNA genes[23]. These genetic features provide a basis for the development of the ecotoxicological bioassay. Strain NIES-981 can be obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES) (MCC-NIES, https://mcc.nies.go.j).

5.2 Reagents

5.2.1 Water

Deionised, for the preparation of the medium and stocks (nutrient, metal and tris) for the medium.

5.2.2 Growth medium

ASW-SN, optimized to allow sufficient growth of *Cyanobium* (NIES-981) to meet the test quality (Table 1), are used for pre-culture and testing (see 7.1).

5.2.3 Nutrient, metal and tris stock solutions

Stock solutions of nutrients, metals and tris for ASW-SN (see Table 1), are prepared at a land-based laboratory. The stock solutions are also added to the test seawater so that their concentration is the same as in ASW-SN (see Annex B).

Table 1 — Reagents for ASW-SN (left) and stock solutions

ASW-SN	g	a) Stock solution of nutrients	g	b) Stock solution of trace metals	mg	c) Stock solution of tris	g
NaCl	25,0	$NaNO_3$	75	$Na_2EDTA{\cdot}2(H_2O)$	580	Tris	100
$MgCl_2{\cdot}6(H_2O)$	2,0	$K_2HPO_4{\cdot}3H_2O$	3,0	$FeCl_3{\cdot}6(H_2O)$	422	Deionised water	1 000 ml
KCl	0,5	Deionised water	1 000 ml	$ZnSO_4{\cdot}7(H_2O)$	2,93		
$CaCl_2{\cdot}2(H_2O)$	0,5			$CoCl_2{\cdot}6(H_2O)$	1,33		
$MgSO_4{\cdot}7(H_2O)$	3,5			$MnCl_2{\cdot}4(H_2O)$	24,0		
Nutrients a)	10 ml			Na_2SeO_3	2,30		
Trace metal b)	100 µl			$Na_2MoO_4{\cdot}2(H_2O)$	0,839		
Tris c)	10 ml			$NiCl_2{\cdot}6(H_2O)$	0,37		
Deionised water	1 000 ml			Deionised water	100 ml		
pH	8,2					pH	8,2

6 Apparatus

6.1 General

All equipment that comes into contact with the test medium and all solutions used for its preparation are made of glass or a chemically inert material. Glassware that is free of chemical contaminants and sterile is used for culturing and testing. Use general laboratory apparatuses and the following (see 6.2 to 6.6).

6.2 High sensitivity luminometer

Sufficiently sensitive to detect DF of at least 10^6 NIES-981 cells per millilitre, able to count photons at 0,1-s intervals for at least 60 s, and equipped with a red light source (50 $\mu E \cdot m^{-2} \cdot s^{-1}$) for excitation. In addition, the shutter controls the excitation time accurately (see Annex C and Figure C.1).

6.3 Incubator and tube shaker

An incubator that can maintain 23 °C ± 2 °C is recommended, although the assay may also be performed using light equipment in a room controlled at 23 °C ± 2 °C. For culturing and testing, white fluorescent light (60 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 80 $\mu E \cdot m^{-2} \cdot s^{-1}$) is used. Although a white LED lamp can also be used instead of the white fluorescent light, check that it contains two wavelength ranges, red (with a peak between 660 nm and 670 nm) and blue light (with a peak between 450 nm and 460 nm). These are the appropriate wavelengths for algal photosynthesis. An orbital and wheel shaker with a speed controller is recommended for the pre-culturing and testing.

6.4 Apparatus for determining algal cell density

The same device as that used for DF measurements (6.2) can be used for cell density determinations. Algal cell density determination is needed for preparing a living stock culture at a land-based laboratory and for determining the initial cell density at the beginning of testing on-board.

6.5 Culture tubes

Autoclaved or disposable sterile tubes with a capacity between 5 ml and 10 ml (glass tubes are recommended) with air-permeable stoppers are used for pre-culture and testing.

6.6 Clean bench

Sub-culturing of the living stock cultures is ideally performed on a clean bench, but a portable bench with apron covers and a fan unit is sufficient.

7 Preparations at a land-based laboratory

7.1 Preparation of the growth medium

ASW-SN is used for testing and pre-culture of strain NIES-981. ASW-SN is also used for the preparation of the test seawater for the incubation of test algae. The growth medium is ideally prepared in advance at a land-based laboratory, and sent to the offshore site in sterile containers.

— Dissolve ASW-SN reagents and stock solutions in 900 ml of deionised water, as indicated in the left column of Table 1. Prepare the stock solutions of nutrients, metals and tris in advance, according to Table 1.

— Adjust pH to 8,2 using 1,0 N HCl (this requires the addition of approximately 5,5 ml of 1,0 N HCl).

— Add deionised water until the volume equals 1 000 ml.

— Filter the medium through a membrane filter with a pore size of less than 0,22 µm into a sterile container. Alternatively, the medium may be autoclaved (121 °C for 20 min).

7.2 Preparation of the algal stock culture

Either a living stock culture or a cryopreserved stock culture of strain NIES-981 shall be prepared at a land-based laboratory. The stock shall then be used to prepare an inoculum culture on-board before testing. As soon as possible after the living stock culture arrives on-board, it should be placed under culture condition. This step is important, to shorten the preparation time of the alga inoculum on-board.

The density of the living stock culture should be 10^8 algal cells per millilitre in the exponential growth phase. This corresponds to between 1 000 counts and 1 500 counts of photons at 0,1 s after the start of the measurements. The living stock culture should be pre-cultured to obtain the inoculum culture for the bioassay.

First, determine the cell density of original stock culture using a luminometer (the same device as that for DF measurements, see 6.2). Add a sufficient amount of cells from the algal stock culture to 100 ml of the growth medium (7.1) for the cell density to be 2×10^6 algal cells per millilitre, and pre-culture for 3 days. After pre-culturing, the cell density in 10 ml of the living stock culture in the exponential growth phase shall be determined and adjusted to 10^8 algal cells per millilitre. If cell density at that stage is much lower than 10^8 algal cells per millilitre, the culture is not at the exponential growth phase. In that case, repeat the 3-day pre-culturing step until cell density exceeds 10^8 cells per millilitre. The living stock culture shall be incubated under the same conditions as those for the test (8.4). Determine the cell density of the living stock culture immediately before use, to calculate the required culture volume.

If the stock culture is in the stationary phase, at least two or three pre-culturing rounds are necessary to obtain culture in the exponential phase of growth. When older stock cultures are used (more than 3 weeks old), more time may be required for recovery.

For the preparation of the cryopreserved stock culture, see Annex D.

All steps should be performed on a clean bench (6.6).

8 Test procedure on-board

8.1 Preparation of the algal inoculum culture

For reproducible results, the algal inoculum culture shall be in the exponential growth phase, with the biomass increasing ca. 16-fold over 72 h. First, immediately before use, determine cell density of the living stock culture using a luminometer, and add a sufficient amount of cells from the living stock culture to the growth medium (7.1) for a cell density of 10^6 algal cells per millilitre. A three-day pre-culture is necessary to obtain an algal inoculum culture with a cell density of over 10^8 algal cells per millilitre (i.e. sufficient density for starting the test) in the exponential phase of growth. If cell density after pre-culturing is much lower than 10^8 algal cells per millilitre, the cells are not in the exponential growth phase. In that case, repeat the 3-day pre-culturing step until cell concentration exceeds 10^8 cells per millilitre.

8.2 Choice of the test concentrations

Two cyanobacterium cultures (*Cyanobium* sp. NIES-981) in triplicate shall be established in the control tubes (growth medium with no test seawater) and tubes containing diluted test seawater [test seawater mixed with the growth medium at a volume fraction of 80:20]. As an option, additional dilutions of the test seawater in a geometric series may be tested to determine ECx based on the regression analysis.

8.3 Preparation of the test medium

First, test seawater shall be collected at an appropriate site (e.g. surface water, mining wastewater, etc.). A filtrate shall be obtained using an appropriate filtering device and a filter with a pore size of < 0,22 µm. The stock solutions of nutrients, metals and tris shall be added to the filtrate so that their concentration is the same as that in ASW-SN. The mixture shall then be diluted to 80 % volume fraction with the growth medium. As the test medium, aliquots of the diluted filtrate shall be dispensed into three test tubes of appropriate dimensions and optical properties for DF measurements (see Annex B).

Additional aliquots of the growth medium shall be dispensed into three test tubes used as the control.

The aliquot volume to be dispensed shall be the same in all test tubes. Adequate headspace shall remain in all test tubes for continuous algal suspension using a shaker.

8.4 Inoculation and incubation

Reproductive results can be obtained by precisely determining the initial algal cell density. First, determine the cell density of the algal inoculum culture using a luminometer. Add a sufficient amount of cells from the algal inoculum culture to the test medium and the control for a cell density of 10^6 algal cells per millilitre. Working on a clean bench is not indispensable, but the assay should be performed under conditions that are as clean as possible. Caution shall be taken to avoid contamination of the test seawater by marine bacteria and algae, or during handling.

Algal suspensions should be continuously shaken on an orbital and wheel shaker at 23 °C ± 2 °C under continuous white fluorescent light (60 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 80 $\mu E \cdot m^{-2} \cdot s^{-1}$) for 24 h. Although a cabinet or chamber is recommended, it is also possible to perform this using lighting equipment in a room controlled at 23 °C ± 2 °C without a cabinet.

9 DF measurement

The DF of the inoculated samples shall be measured 24 h after the exposure. For the DF measurement, the samples shall be placed in the dark for 60 s and then illuminated for 1 s with red light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After the excitation light is turned off, DF shall be detected using a high sensitivity luminometer at 0,1-s intervals for 1 min.

10 Interpretation of data

10.1 Plotting the DF decay curve

I_{DF}[14], which is the integrated value of DF intensities from 2,0 s to 40 s, is used to estimate the toxicity of the test seawater (see the equation below). In some cases, a crossover point can be apparent in the DF decay curves, ca. for the first 2,0 s (I_{DF} should be higher than that in the control before the crossover point). Considering the crossover point, the DF decay curve from 2,0 s on shall be used for the quantification, as follows:

$$I_{DF} = \sum_{i=2s}^{40s} C_i$$

where

I_{DF} is the integrated value of DF intensities from 2,0 s to 40 s;

i is the measured time point;

C_i is DF intensity (counts of photon) at the measured time point.

Statistical analyses (e.g. Student's *t*-test) may be used to compare the means of the response variables of the control and test sample. If the variances of the two groups are not equal, a *t*-test adjusted for unequal variances should be performed.

10.2 Calculation of per cent inhibition

Calculate the per cent inhibition of I_{DF} using the equation below:

$$I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

where

I_r is the per cent inhibition of I_{DF}

μ_C is the average I_{DF} in the control group

μ_T is the average I_{DF} in the test sample

11 Interpretation of the results

If the I_{DF} of the test seawater is significantly lower ($p < 0{,}01$) than that of the control, the test seawater likely contains substances that inhibit the photosynthetic activity of algae. Inhibition of less than 5 % can be disregarded. The per cent inhibition gives an indication of potential hazards but cannot be used directly to predict the inhibitory effects of growth on algae in the natural environment.

12 Test report

The test report shall contain at least the following information:

a) the test method used, together with a reference to this document, i.e. ISO 23734;

b) all data required for the identification of the test sample, e.g. chemical identification data for the test substance;

c) test organism: species, origin, strain number, method of cultivation;

d) test details:

- start date and duration;
- method of preparation;
- composition of the medium;
- source and salinity of the seawater;
- culturing apparatus and incubation procedure;
- light intensity and quality;
- temperature;
- pH of the test solutions at the start and end of the test;
- DF measurement parameters (light source used for excitation, measured DF interval, etc.);

e) results:

- DF intensity in each test vessel at each measuring point;

- mean I_{DF} for each tested concentration (and control) 24 h after the exposure;
- relationship between the concentration and the effect (percentage inhibition values vs. concentration) in a table or graphical representation, e.g. percentage inhibition vs. concentration, on a logarithmic-scaled abscissa;
- EC_{10} value and method of determination;
- EC_{50} value and method of determination;
- other observed effects;
- if appropriate, results of positive controls, control chart.

Annex A
(informative)

Schematic overview and procedures of the on-board bioassay

A.1 Flow-chart of the procedure

The procedure of the on-board bioassay is conducted as indicated in the flow chart in Figure A.1.

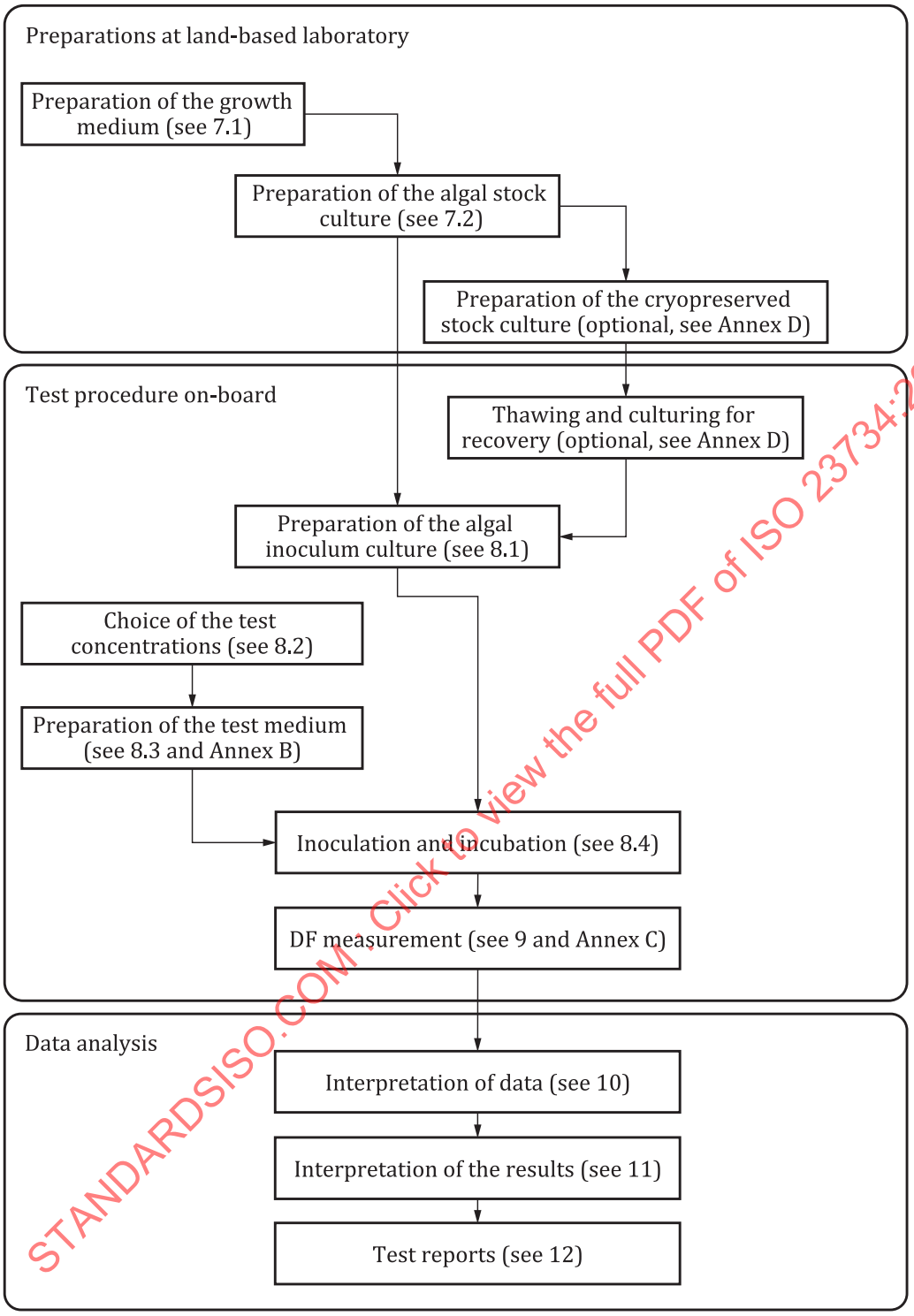
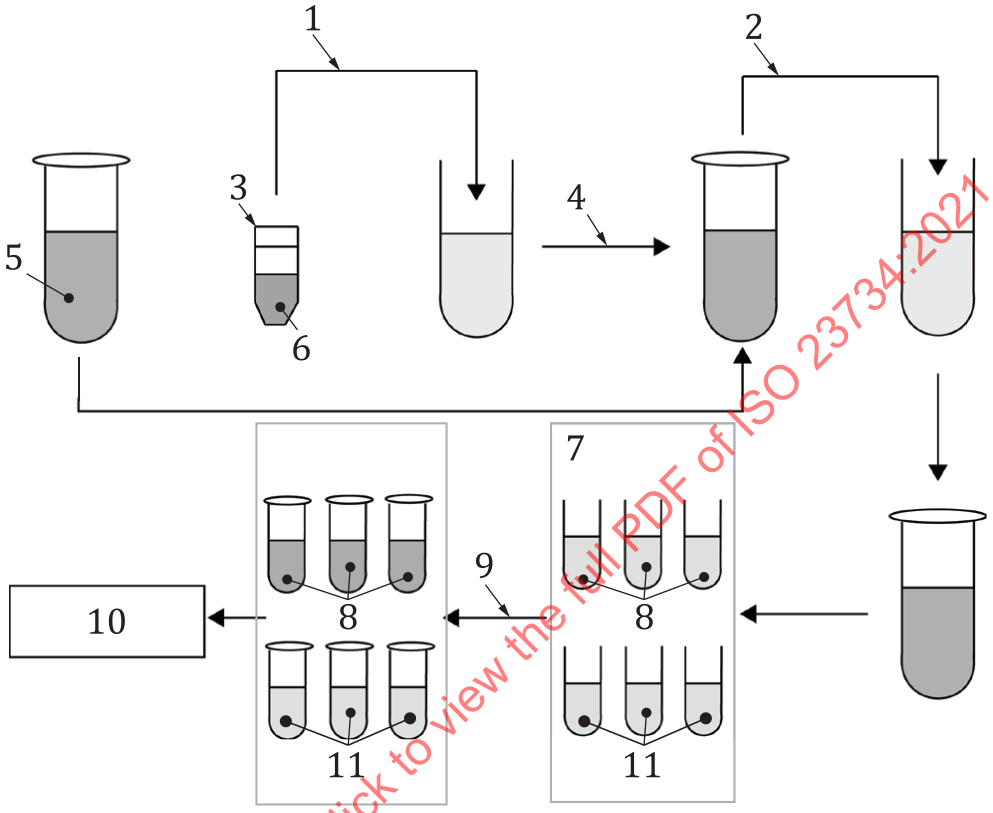


Figure A.1 — Schematic overview of the on-board bioassay to monitor seawater quality using delayed fluorescence of microalgae

A.2 Sample preparation to DF measurements

The procedures for sample preparation to DF measurements is shown in Figure A.2.



Key

1 transfer 1 ml of thawed sample to cuvette containing 9 ml of medium
2 pre-culture: transfer 1 ml of culture to new cuvette
3 cyro-tube
4 culturing for recovery
5 living stock culture
6 thaw cryopreserved cyanobacteria at 40 °C
7 inoculum: transfer 1 ml of pre-culture to cuvette
8 controls
9 culturing
10 DF measurement
11 80 % test medium

Figure A.2 — Schematic procedures of the on-board bioassay, from sample preparation to DF measurements

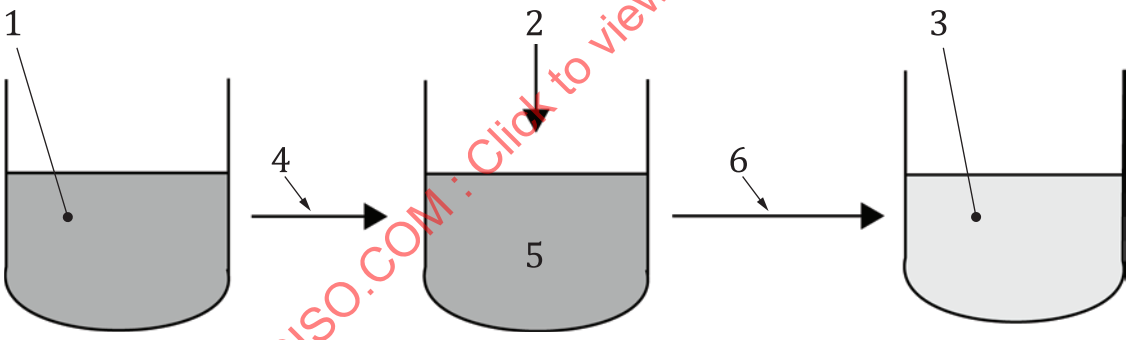
Annex B
(informative)

Preparation of the test medium in seawater

B.1 Preparation of the test medium

When using a seawater collected at an appropriate site (e.g. surface water, mining wastewater, etc.) as a test seawater, a test medium series is prepared by diluting the collected sample with the growth medium (artificial seawater, ASW-SN). In the dilution series, the concentrations of nutrients ($NaNO_3$ and $K_2HPO_4 \cdot 3H_2O$), metals and tris are the same as in the growth medium. If the salinity is appreciably different from that of the growth medium, an appropriate amount of sodium chloride (in the case of low salinity) or deionised water (in the case of high salinity) is added.

1. Filter 1,0 l of the collected sample through a membrane filter with a pore size of < 0,22 µm.
2. Add the same volume of stock solutions of nutrients, metal mix and tris as those used for the growth medium.
3. Adjust the pH to 8,2 by adding 1 N HCl or 1 N NaOH.
4. Dilute the adjusted sample with ASW-SN to the desirable dilution [e.g. mix the test seawater and the growth medium at a volume fraction of 80:20].



Key

1	test sea water	4	filtration
2	add stock solutions of nutrients metals and tris	5	adjust pH to 8,2
3	test medium	6	dilute adjusted sample with ASW-SN

Figure B.1 — Schematic of a procedure for test sample dilution series

Annex C
(informative)

Practical procedures for the on-board bioassay and schematic diagram of the high sensitivity luminometer

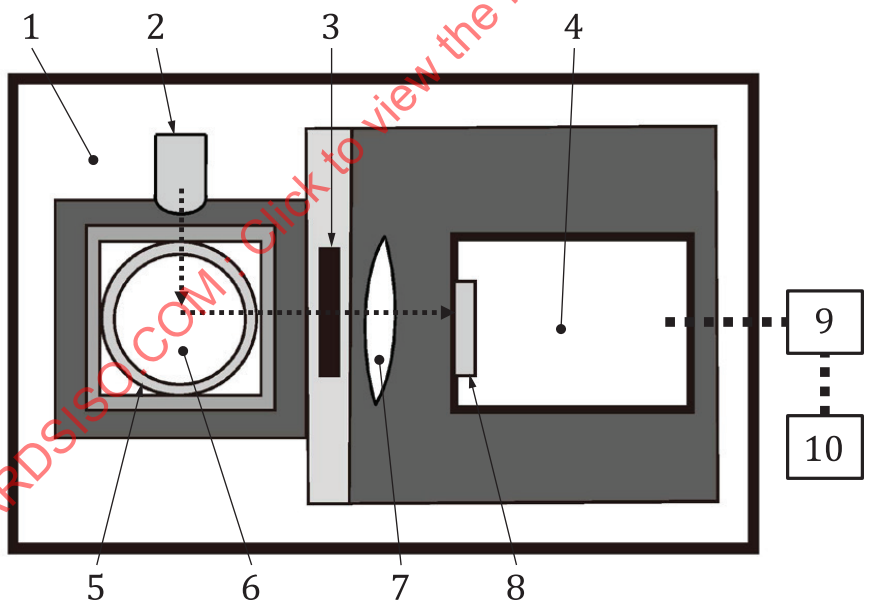
Figure C.1 shows a schematic view and practical flow chart for the on-board bioassay. The bioassay system consists of a high sensitivity luminometer for DF measurements and other required devices. As an example, a tube shaker for algal incubation is commercially available and works well with the on-board bioassay.

Practical steps for the on-board bioassay using a high sensitivity luminometer are as follows.

— Transfer the algal inoculum culture to cuvettes (25 mm in diameter and 85 mm in height) containing fresh test medium and control tubes for a cell density of 10^6 algal cells per millilitre (in a total volume of 10 ml).

— Shake continuously on an orbital and wheel shaker for 24 h at 23 °C ± 2 °C, under white fluorescent light (60 $\mu E \cdot m^{-2} \cdot s^{-1}$ to 80 $\mu E \cdot m^{-2} \cdot s^{-1}$).

— Measure DF using the high sensitivity luminometer.



Key

1	dark box	6	cuvette
2	LED(excitation light)	7	lens
3	shutter	8	photocathode
4	photomultiplier	9	signal counting circuit
5	cuvette holder	10	personal computer

Figure C.1 — Schematic diagram of the DF detector system