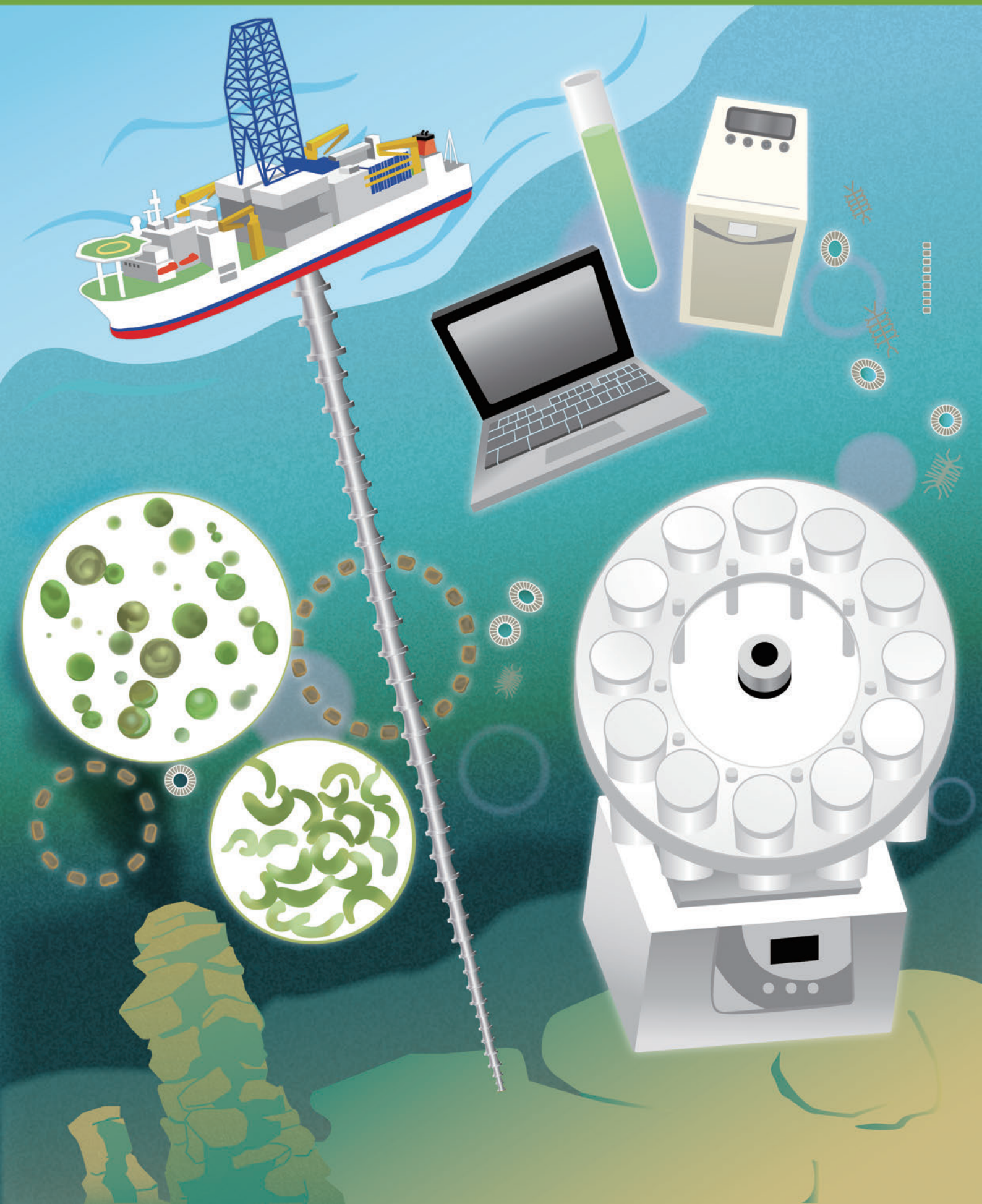
SIP Protocol Series No.6

Onboard bioassay for seawater quality monitoring using delayed fluorescence of microalgae



Cross-ministerial Strategic Innovation Promotion Program

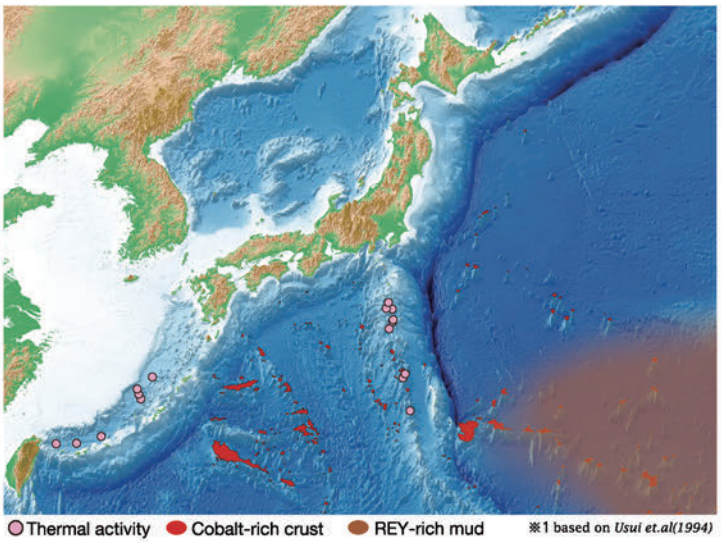
The Strategic Innovation Promotion Program (SIP) was launched by the Council for Science, Technology, and Innovation (CSTI), which oversees projects that target scientific and technological innovation in line with Japanese government directions as stated in the Comprehensive Strategy on Science Technology and Innovation and the Japan Revitalization Strategy. This interdisciplinary program among government agencies, academic institutes and private sectors addresses eleven issues. One of these issues is Next-Generation Technology for Ocean Resources Exploration.

Zipangu in the Ocean Program and Protocols for Environmental Survey Technologies

Zipangu in the Ocean Program is a technical study of the development of submarine mineral deposits that takes into consideration the wise use of these resources.

One research area is the ecological survey of organisms and their long-term monitoring. However, an ecosystem consists of various interrelated factors; thus, in addition to a comprehensive understanding of the system, observation and analysis of each component to its most elemental level are unavoidable. Recently, increased environmental awareness and the necessity of forming a consensus have become key issues in conducting development activities. Growing concern for the environment by the public and the diversification of the use of maritime areas have complicated the interests of stakeholders. To facilitate the formation of a consensus under these conditions, it is important for standardized methods to be implemented. This will ensure that research processes are transparent and that the collection of survey data is objective.

This protocol series aims to introduce more accurate, user-friendly, objective and effective underlying technologies required to understand the environmental impact of submarine mineral resource development. We believe that creating such a technology tool-kit will allow us to develop these resources in a sustainable manner.



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Introduction

1.1. Potential heavy metal pollution by deep-sea mining activity

Mining offshore mineral resources is a major source of potential hazards for deep-sea environments and ecosystems (Ahnert and Borowski, 2000; Thiel, 2003; Smith et al., 2008). One concern is the toxic effect of heavy metals from excavated minerals that are released into seawater in the deep marine ecosystem (Simpson and Spadaro, 2016; Fallon et al., 2017). Additionally, there is a risk of unexpected leakage of recovered minerals and mining wastewater from the mining plant, which may result in heavy metal contamination in surface seawater (Fuchida et al., 2017).

An appropriate monitoring scheme and evaluation method for deep and surface seawater quality should 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 the wider region impacted by discharged plume and materials released when transporting minerals to the surface (ISA/LTC, 2013).

1.2. Requirements for seawater monitoring at mining sites

An onboard or onsite evaluation method for heavy metals is essential for prompt action when there is an unexpected pollution incident. Rapid evaluation would provide an opportunity to prevent wider spread of toxic contaminants, and consequently minimize mining plant idle periods.

1.3. Existing evaluation methods

Although many chemical analytical methods are available at land-based laboratories, there are few methods with onboard application. Deep-sea mineral deposits are inhomogeneous and can release various types of metal elements; therefore, simultaneous analysis of multiple elements is required for evaluation of mining contaminants. To our knowledge, these analyses can only be performed by special instruments, such as inductively coupled plasma mass spectrometry (ICP-MS), which needs an expert to operate, a large space, and is expensive to install. This type of instrument would be difficult to install at each mining site as standard equipment for environmental monitoring.

Bioassays are an alternative approach, and are commonly used to assess and evaluate ecological risks of chemical contaminations. Bioassays do not provide quantitative information of contaminated substances, but can detect a wide spectrum of toxicants, including those that are unknown. This feature is advantageous for safe monitoring of water quality during deep-sea mining activities.

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

1.4. Onboard bioassay method using delayed fluorescence of microalgae

To address obstacles of existing bioassays, we investigated the practicality of a rapid bioassay protocol using delayed fluorescence (DF) of marine phytoplankton (Yamagishi et al., 2016). To do this, we developed a new test organism, the marine cyanobacterium *Cyanobium* sp. NIES-981. *Cyanobium* sp. NIES-981 is closely related to *Synechococcus* and *Prochlorococcus* species, which are major primary producers in the offshore environment.

We demonstrated that EC_{50} values obtained with the DF-based bioassay method corresponded to results using a standard growth inhibition assay, indicating that our DF-based method would be a practical alternative to the standard growth inhibition test.

This method is suitable for onboard use because it requires a smaller amount of sample water and is quicker than the standard bioassay method.

The following document describes an "Onboard Bioassay" method for evaluation of seawater quality at a mining site using DF quantification of the marine cyanobacterium *Cyanobium* sp. NIES-981.

What is delayed fluorescence?

Delayed fluorescence (DF), also known as delayed luminescence or delayed light emission, is very weak fluorescence that is emitted by photosynthetically active cells, and measured as a delay of milliseconds to minutes after cells are transferred from light to dark conditions (Strehler and Arnold, 1951; Arnold and Davidson, 1954). Delay in emission is the result of repopulation of excited states of chlorophyll from stored energy after charge separation; more specifically, it is the back-reaction of accumulated charges across the thylakoid membrane in the electron transport chain (Joliot et al., 1971). Because DF is an indicator of electron transfer state within the photosynthetic apparatus, it can be used as a sensitive intrinsic index of photosynthetic activity (Jursinic, 1986; Schmidt and Senger, 1987a, b).

Delayed fluorescence: A next-generation rapid algal bioassay method?

Multiple studies show that bioassays using DF of algae and plants are comparable to conventional inhibition tests (Gerhardt and Kretsch, 1989; Katsumata et al., 2006; Berden-Zrimec et al., 2010; Breuer et al., 2016). Therefore, bioassays using DF are expected to become one of the next-generation standard methods. Over the last decade, the National Institute for Environmental Studies and Hamamatsu Photonics K.K. jointly developed a rapid DF bioassay system using freshwater algae (Katsumata et al., 2009, 2017). We have now developed a new DF bioassay system specifically for water quality monitoring of offshore environments, using a marine autotrophic cyanobacterium.

General protocol for a rapid onboard bioassay using delayed fluorescence of the marine cyanobacterium *Cyanobium* sp. (NIES-981)

2.1. Scope and outline of method

This onboard bioassay method aims to provide basic data for seawater quality management at deep-sea mining sites.

First, a seawater sample is collected at a target site, such as surface seawater around the mining plant or mining wastewater generated by the mining plant. Then, a pair of triplicated marine cyanobacterium cultures (*Cyanobium* sp. (NIES-981)) are established in a control sample (artificial seawater) and diluted test seawater tubes (test seawater: artificial seawater = 80: 20 (v/v)). After incubation, test samples are subjected to DF measurement with an appropriate detector system for luminescence. Finally, DF quantities or total intensities of seawater samples are compared with control samples using an appropriate statistical test. Significant differences between the seawater sample and control may indicate that the seawater has been affected by mining activities. Results of the onboard bioassay would support appropriate environmental safety actions.

2.2. Technical background

Compared with general ecotoxicological assays, this bioassay method has been simplified to improve practicality for onboard use at mining sites. However, this method has demonstrated similar results to the standard growth inhibition test (OECD TG201); EC_{50} values obtained corresponded to those of a standard growth inhibition assay, indicating that our DF-based method is a viable alternative to the standard growth inhibition test (Fig. 1) (Yamagishi et al., 2016).

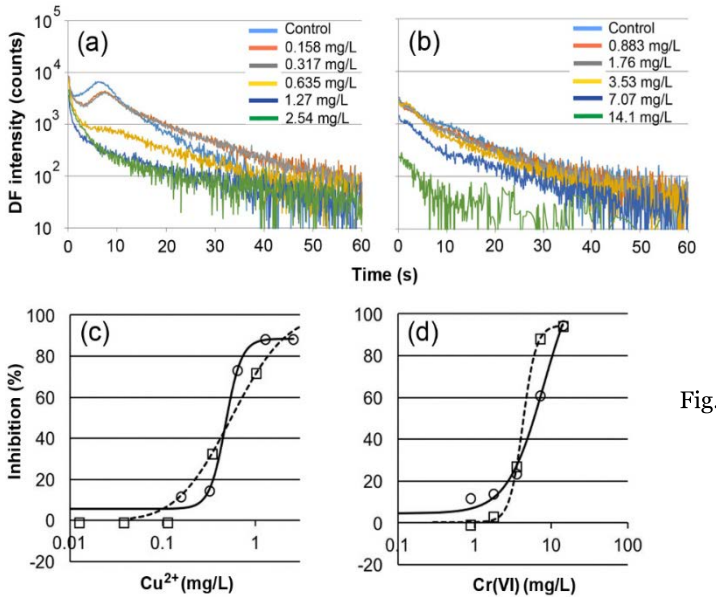

Fig. 1. DF intensity of *Cyanobium* sp. (a,b) and dose-response curves (c,d) after 24 h exposure to Cu^{2+} (a, c) and Cr(VI) (b, d). Dotted line = standard growth inhibition test. Solid line = delayed fluorescence.

2.3. Materials

2.3.1. Test organism

The onboard bioassay uses an axenic culture of marine cyanobacterium, *Cyanobium* sp. (NIES-981). NIES-981 can be obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (NIES) (http://mcc.nies.go.jp/index.html).

The complete genome of NIES-981 has been sequenced, revealing that the genome codes 3,268 proteins with 46 tRNA genes and three sets of rRNA genes (Yamaguchi et al., 2016), providing a basis for developing an ecotoxicological bioassay.

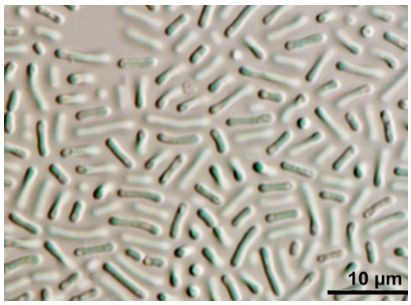

Fig. 2. Light microscopic image of *Cyanobium* sp. (NIES-981).

2.3.2. Cryopreservation of test organism

Cryopreservation can be applied for easy and timely onboard use of NIES-981. Cryopreservation provides access to test organism cultures with the same initial algal concentration, on an as needed basis for multiple tests, and minimizes the need to install equipment for transferring or pre-culturing. Cryopreserved test organisms are prepared in advance at a land-based laboratory, and sent to the offshore site via refrigerated transport.

Cryopreservation Procedure for NIES-981

1. To make a cryoprotectant solution, add 2 mL filter-sterilized dimethyl sulfoxide to 98 mL of sterile artificial seawater medium (final concentration of cryoprotectant agents, 2% v/v dimethyl sulfoxide).
2. Dispense 500-µL aliquots cryoprotectant solution into sterile cryogenic vials (Fig. 3A, Greiner Bio-One GmbH, Frickenhausen, Germany).
3. At a clean bench, add 500 µL of *Cyanobium* sp. NIES-981 cell suspension (concentration, ca. 2×10^8 cells mL^{-1}) to each cryogenic vial containing the cryoprotectant solution (Fig. 3A, final concentration, ca. 10^8 cells mL^{-1}). Incubate the vials for 15 to 30 min at room temperature.
4. Cool the vials to −40 °C at a rate of -1 °C min using a programmable freezer (Fig. 3B, Kryo 320-1.7: Planer PLC, Sunbury-On-Thames, Middlesex, UK).
5. Once the target temperature has been reached, remove the vials from the cooler and immediately submerge in a Dewar flask containing liquid nitrogen for 1 h. Store the vials in the vapor phase of a liquid nitrogen tank until use (Fig. 3C).
6. Before starting the delayed fluorescence-based bioassay, thaw the cryopreserved vials containing cell suspension with a thawing device (e.g. water bath (Fig. 3D) or Allutox Shaker: Hamamatsu Photonics) at 40 °C until the frozen cell suspension is completely thawed. Immediately after thawing, transfer the cell suspension into new medium for pre-culturing.

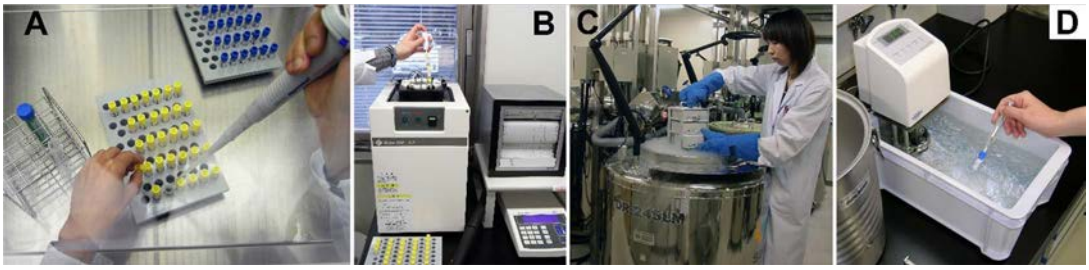

Fig. 3. Equipment and materials for cryopreservation. A: vials and a clean bench, B: programmable freezer, C: liquid nitrogen tank, D: water bath.

2.3.3. Medium

An exclusive artificial seawater medium, ASW-SN, is used for incubating the control and pre-culture of N-981. Macronutrient-enriched ASW-SN is used as the diluent for incubating the test sample. These culture media are prepared in advance at a land-based laboratory, and sent to the offshore site using sterile containers.

Preparation of ASW-SN medium

1. Dissolve ASW-SN reagents in 900 mL deionized or distilled water, as shown in the left column of Table 1. Prepare the metal mix solution in advance, according to the right column of Table 1.
2. Adjust pH to 8.2 using 1.0 N HCl.
3. Add deionized or distilled water until the medium equals 1000 mL.
4. Filter the medium through a membrane filter with pore size of 0.22 μm into a sterile container. Autoclave at 120°C for 15 min.

Table 1. Reagents for ASW-SN (left) and Metal mix solution (right).

ASW-SN		Metal mix	
NaCl	25 g/L	Na_2EDTA	0.58 g
$MgCl_2 \cdot 6H_2O$	2.0 g/L	$FeCl_3 \cdot 6H_2O$	0.422 g
KCl	0.5 g/L	$ZnSO_4 \cdot 7H_2O$	2.933 mg
$NaNO_3$	0.75 g/L	$CoCl_2 \cdot 6H_2O$	1.332 mg
$K_2HPO_4 \cdot 3H_2O$	0.03 g/L	$MnCl_2 \cdot 4H_2O$	24 mg
$MgSO_4 \cdot 7H_2O$	3.5 g/L	$Na_2MoO_4 \cdot 2H_2O$	0.839 g
$CaCl_2 \cdot 2H_2O$	0.5 g/L	Na_2SeO_3	2.306 mg
Tris	1.0 g/L	$NiCl_2 \cdot 6H_2O$	0.37 mg
Metal mix	100 μL	D.W.	100 mL
D.W.	1000 mL		

Preparation of macro nutrients-enriched ASW-SN medium

The preparation method and reagents for the macronutrient-enriched ASW-SN medium are the same as the ASW-SN medium (Table 1), except it is prepared using 5-fold amounts of $NaNO_3$,·K_2HPO_4·$3H_2O$, and Tris, compared with the normal ASW-SN medium.

2.3.4. Preparation of inoculum culture

To obtain reproducible results, test algae must be in the exponential growth stage, with 16-fold growth ability for at least 72 h. When using a cryopreserved organism, which may be partially damaged during preservation processes, preparing a pre-culture 3–4 days after thawing is recommended to ensure exponential growth stage.

2.4. Incubation test and procedure

2.4.1. Preparation of test and control samples

First, a water sample is collected from an appropriate site (e.g. surface water, mining wastewater). The filtrate is obtained using an appropriate filtering device and filter with a pore size of < 0.22 µm. The filtrate is diluted to 80% with macronutrient-enriched ASW-SN media. Aliquots of the diluted filtrate are dispensed into three test tubes with appropriate dimensions and optical properties for DF measurement, and are referred to as "test samples."

Additional aliquots of the ASW-SN medium are dispensed into three test tubes as "control samples."

Aliquot volume should be the same in all test tubes and leaving adequate headspace for continuous algal suspension when in the shaker.

2.4.2. Inoculation and incubation

The same amount of inoculum culture of N-981 is added to test and control sample tubes, which will be the initial algal concentration (around 10^7 cells ml^{-1}). Cell density of the pre-culture (3-4 days after thawing) may be equivalent to 10^8 to 10^9 cells ml^{-1}.

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

2.4.3. Measurement of delayed fluorescence

After 24 h light exposure, the inoculated samples are placed in the dark for 60 s, and then illuminated for 1 s with red and infrared light (50 µmol $m^{-2}s^{-1}$). After the excitation light is turned off, delayed fluorescence is detected with highly-sensitive luminometers at 0.1-s intervals for 1 minute.

2.4.4. Analytical determination and evaluation

Changes in the shape of the DF decay curve, from 2.0-40 s (ΔDF), were quantified using the equation below (Leunert et al., 2013; Yamagishi et al., 2016).

$$\Delta DF = \sum_{i=2s}^{40s} \left| \frac{t_i}{c_2} - \frac{c_i}{c_2} \right|$$

t_i = test samples at the measured time point
c_i = control samples at the measured time point
c_2 = start value of the control

Delayed fluorescence intensity (DFI) (Katsumata et al., 2006), which is the integrated value of DF values from 2.0-40 s, can also be used (see equation below).

$$DFI = \sum_{i=2s}^{40s} t_i$$

To compare means of the control and test sample response variables, statistical analyses can be used (e.g. Student's t-test). If variances of the two groups are unequal, a t-test adjusted for unequal variances should be performed.

A practical example using a commercially-available delayed fluorescence system, adopted by NIES

Below are a schematic view and practical flow chart for our onboard bioassay procedure (Fig. 4). We acquired a rapid algal bioassay system using delayed fluorescence, supplied by Hamamatsu Photonics, K.K., Japan. The bioassay system is a commercially-available instrument that works well with our onboard bioassay procedure. Hamamatsu Photonics, K.K. also supplies other devices necessary for this bioassay, such as a thawing device for cryopreserved algae and a tube shaker for algal incubation.

Practical steps for the onboard bioassay using a Type 7100DF detector system

1. Thaw vials of cryopreserved cyanobacteria at 37 °C until the cell suspension is completely thawed, using the thawing device (Warming block: Hamamatsu Photonics, K.K., Shizuoka, Japan).
2. Transfer thawed sample to cuvettes (φ 25 × 85 mm) (Hamamatsu Photonics) containing 9.0 mL of fresh culture medium.
3. Shake continuously with the tube shaker (Hamamatsu Photonics) for 72 h at 23 ± 1 °C, under white fluorescent light (10 to 20 $\mu E \cdot m^{-2} s^{-1}$), to promote recovery from freezing damage.
4. Transfer recovered algal sample (1 mL) to cuvettes (φ 25 × 85 mm) (Hamamatsu Photonics) containing 9.0 mL of control and test medium.
5. Shake continuously with the tube shaker (Hamamatsu Photonics) for 24 h at 23 ± 2 °C, under white fluorescent light (60 to 80 $\mu E \cdot m^{-2} s^{-1}$).
6. Measure delayed fluorescence of test samples with high sensitivity luminometer (Type 7100, Hamamatsu Photonics).
7. Calculate ΔDF or DFI values to quantify changes in shape of the DF decay curve and compared with the control.

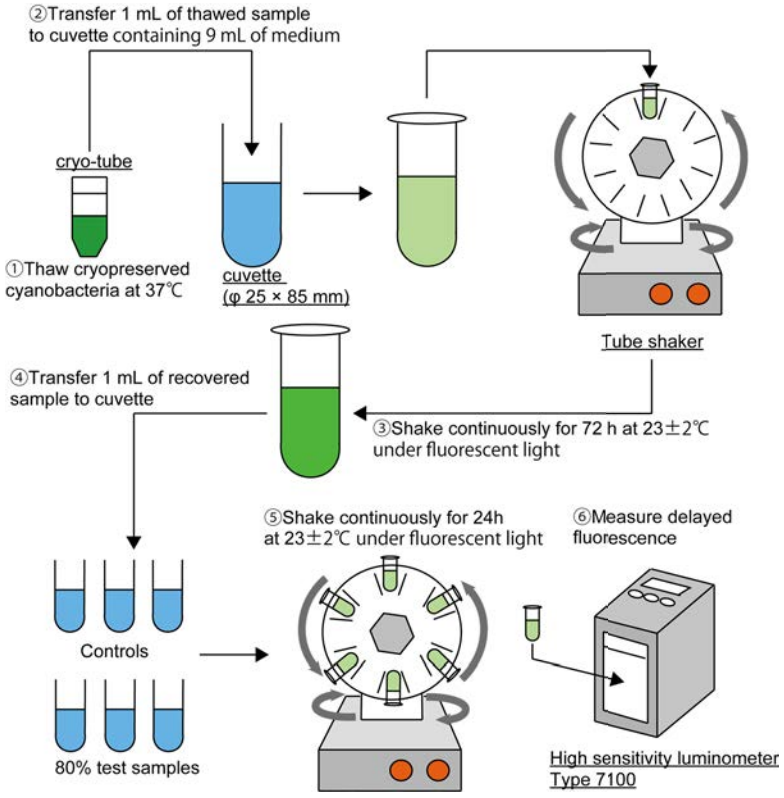

Fig. 4. Practical steps for the onboard bioassay using delayed fluorescence.

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Onboard bioassay for seawater quality monitoring using delayed fluorescence of microalgae

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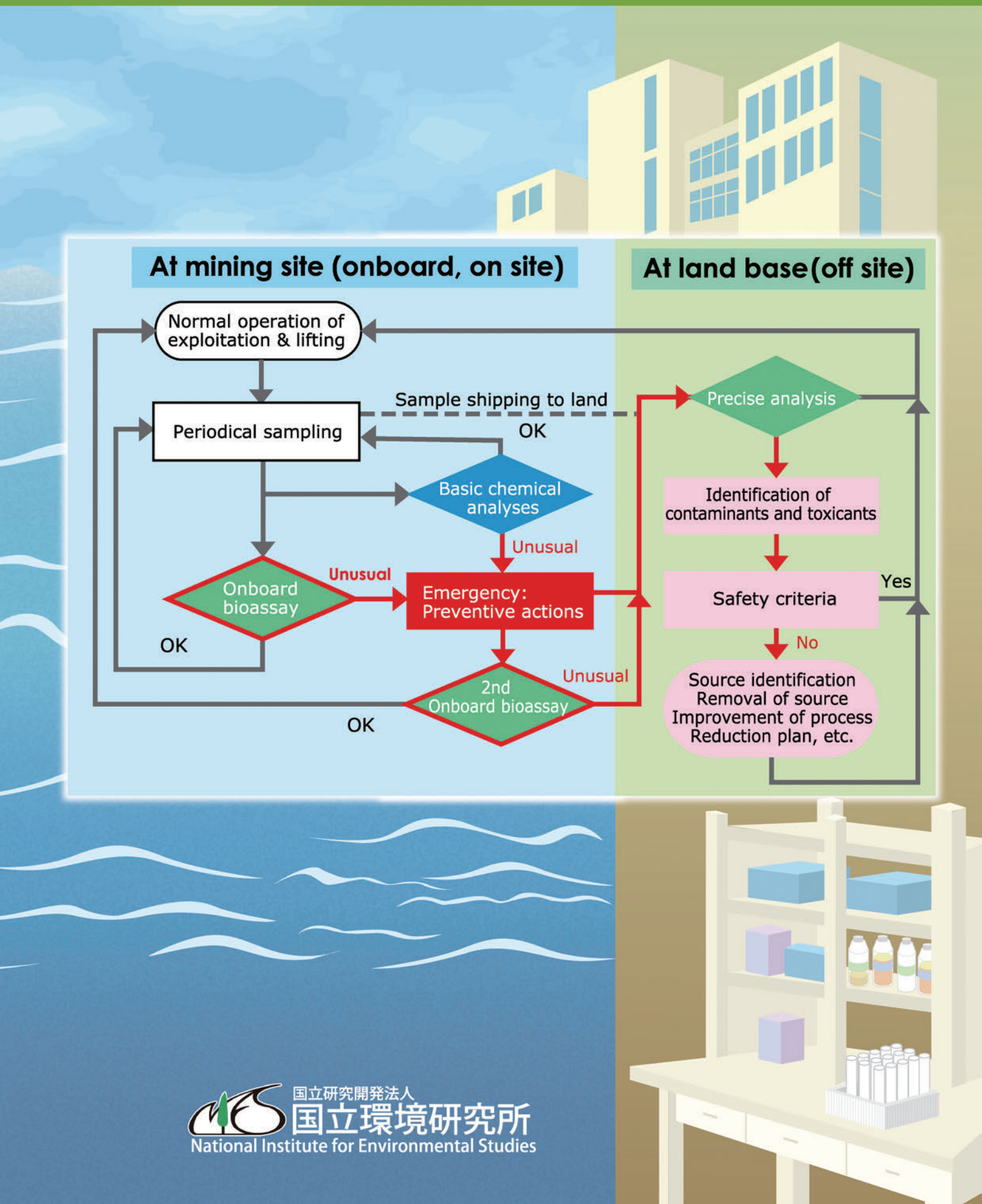

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