Bull. Fac. Fish. Hokkaido Univ.
51(3), 153-157, 2000.

An Evaluation of Viable Staining Dyes Suitable for Marine Phytoplankton

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Abstract

To evaluate a rapid and simple technique for determining viability of marine phytoplanktons, three staining dyes (neutral red (NR), Evans blue (EB), and fluorescein diacetate (FDA)) were applied to the live intact cells, and formalin killed cells. FDA was the best dye for determining live cells of all phytoplankton species tested in this study. NR- and EB-staining were also available, but their applications were limited in live cells of *Tetraselmis* sp., *Skeletonema costatum*, *Alexandrium catenella* and *Prorocentrum minimum* for NR, and in dead cells of *S. costatum*, *A. catenella*, *Gymnodinium mikimotoi*, and *Heterosigma akashiwo* for EB.

Key words: Marine phytoplankton, Viable staining, Neutral red, Evans blue, Fluorescein diacetate

Introduction

Since the field of marine biotechnology, tissue culture and genetic engineering, of macroalgae has been developed (Saga et al., 1991), an accurate evaluation method for determining cell viability was needed. At least six dyes were applied to differentiate live and dead cells of green (*Enteromorpha intestinalis*), red (*Porphyra ezoensis*), and brown (*Macrocystis pyrifera*) macroalgae. It was reported that suitable dye for determining live cells was neutral red or fluoresceine diacetate (FDA), that for dead cells was Evans blue (Saga et al., 1987; Saga, 1989; Saga et al., 1989).

On the other hand, there was little information about viable staining techniques for marine phytoplankton, although ecological impact of the phytoplankton in ocean has been focused. Availability of viable staining methods for some marine phytoplanktons has been investigated on some phytoplanktons due to an ecological aspect (Dorsey et al., 1989), or environmental pollution studies (Bentley-Mowat, 1982). In the cases, FDA was successful dye for determining viability of some Bacillariophycea, Chlorophyceae, Prasinophyceae, Dinophyceae and so on.

We recently found that virus-like growth suppression agents of dinoflagellates, *A. catenella* and *G. mikimotoi*, existed in coastal seawater collected on Usujiri located at the Mouth of Funka Bay, Japan (Onji et al. 1999; Onji et al. 2000). The agent suppressing the growth of *A. catenella* was observed to be a virus-like particle

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with 70 nm hexagonal shape (unpublished data). However, as the infection mechanism is still unclear, methods for the assessment of cell viability of the virus-like agent infected *A. catenella* cells are needed. Applicability of the viable staining dyes was never reported in red tide microalgae species, especially *A. catenella* and *G. mikimotoi*. In this paper, we evaluate their applicability of three dyes, neutral red, Evans blue and FDA for determining cell viability of ten species of marine phytoplanktons including *A. catenella* and *G. mikimotoi*.

Materials and Methods

Culturing marine phytoplanktons

Culture of ten marine phytoplanktons, *Tetraselmis* sp. FK1, *Phaeodactylum tricornutum* strain PD1, *Chaetoceros sociale* strain NIES377, *Skeletonema costatum* strain NIES323, *Alexandrium catenella* strain TN-7, *Gymnodinium mikimotoi* strain 34, *Heterosigma akashiwo* strain NIES4, *Prorocentrum minimum* strain 14, *Prorocentrum micans* strain 13, and *Chattonella marina* strain NIES3 were used in this study. *A. catenella* strain TN-7 were kindly provided from Dr. Sako, Kyoto University. All strains were cultured in f/2 medium (Watanabe and Nozaki, 1994) under cool white light irradiation at about 45.6 mmol photon m^{-2} s^{-1} with a 14 : 10 LD cycle at 15°C for *Tetraselmis* sp. FK1, *P. tricornutum* strain PD1, *C. sociale* strain NIES377, *S. costatum* strain NIES323, and at 20°C for others. The dead cells were prepared by the addition of one-tenth volume of 10% formalin.

Viable staining

FDA (ICN Pharmaceuticals, Inc.) was dissolved in dimethyl sulfoxide (DMSO) as 5 mg/ml, and stored at 4°C before use (Dorsey et al., 1989). FDA solution was added to each phytoplankton culture as 1 μl/ml and incubated for 20 min at a suitable culture temperature for each phytoplankton with light irradiation. Neutral red (NR; Kanto Chemical), and Evans blue (EB; Sigma) were dissolved in distilled water as 0.2% and 0.1%, respectively. Both NR and EB were also added to each culture at a one-tenth volume, and incubated for 20 min at a suitable culture temperature for each phytoplankton with light irradiation. A small portion of the cultures was put onto a slide glass, then stained cells were observed under a light microscopy for NR and EB staining, and under a fluorescence microscopy for FDA staining using No. 9 filter set (Axioskop, Zeiss).

Results and Discussion

NR, EB and FDA were applied to live intact cells and formalin-killed (dead) marine phytoplanktons for evaluating whether these dyes were available to determine the cell viability. The results were summarized in Table 1, and an example of the microscopic views of stained *A. catenella* cells were shown in Fig. 1.

FDA was the best dye for determining live intact cells for all species tested in this study (Table 1). Bright green fluorescein, which was derived from FDA degradation by membrane associated or intracellular esterase, was observed from intact cells (Fig. 1. (A)), while there was no obvious green fluorescence from formalin killed cells only with red chlorophyll auto-fluorescence (Fig. 1. (B)). DMSO for dissolving

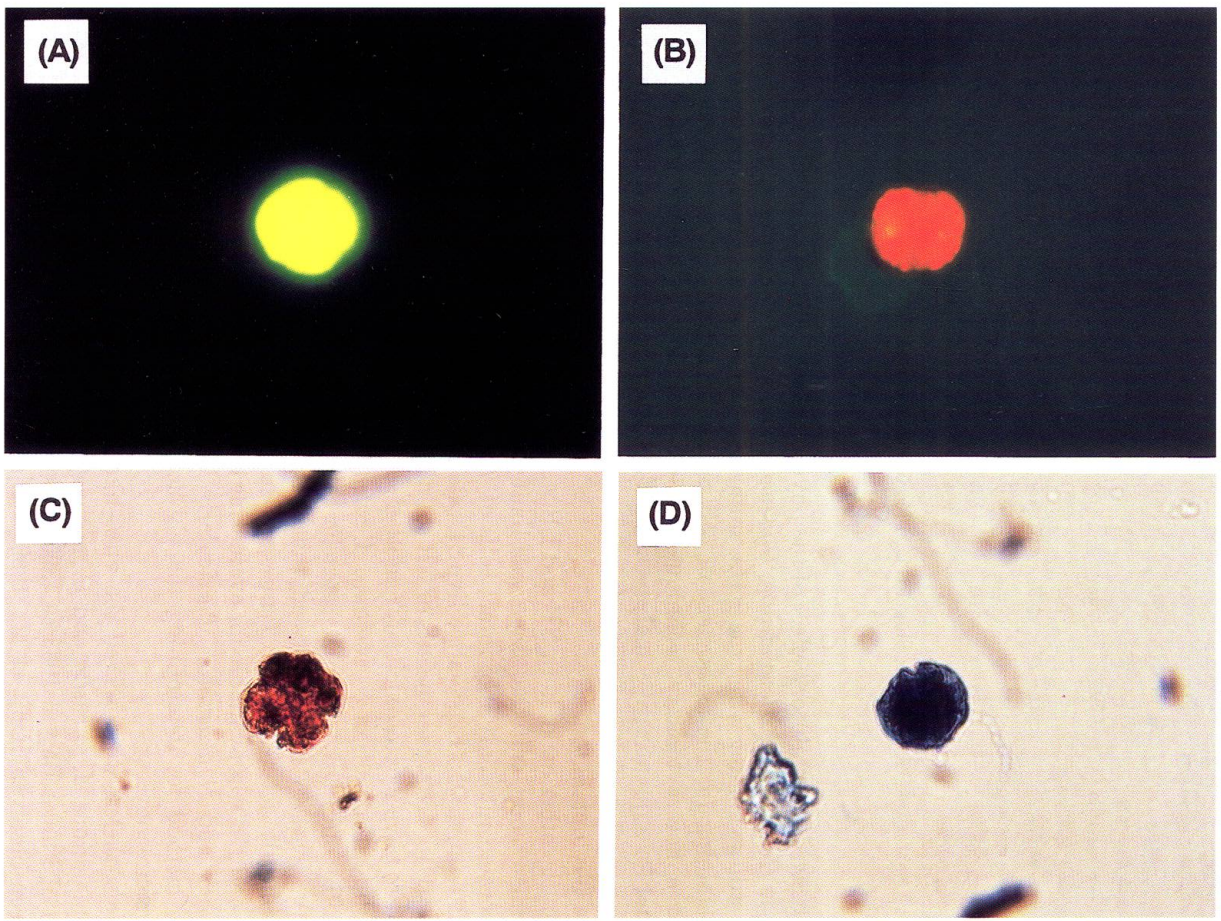

Fig. 1. An example of viable staining of *A. catenella* TN-7 cells by FDA (A and B), neutral red (C), and Evans blue (D). A and C : Healthy live cells were stained ; B and D : Formalin-killed cells were stained.

Table 1. Application of viable staining dyes for marine microalgae

Algae	Strain	NR*1		EB*1		FDA*1	
		Intact	Formalin-killed	Intact	Formalin-killed	Intact	Formalin-killed
Tetraselmis sp.	FK1	+	−	−	−	+	−
Phaeodactylum tricornutum	PD1	ND	ND	−	−	+	−
Chaetoceros sociale	NIES 377	ND	ND	−	+w	+	−
Skeletonema costatum	NIES 323	+	−	−	+	+	−
Alexandrium catenella	TN7	+	−	−	+	+	−
Gymnodinium mikimotoi	34	ND	ND	−	+	+	−
Heterosigma akashiwo	NIES 4	ND	ND	−	+	+	−
Prorocentrum minimum	14	+	ND	−	−	+	−
Prorocentrum micans	13	ND	ND	−	−	+	−
Chattonella marina	NIES 3	ND	ND	−	−	+	−

*1NR; Neutral red, EB; Evans blue, FDA; Fluorescein diacetate, ND: Not determined, w: weak reaction.

FDA showed no inhibitory effect of the phytoplankton cells, some motile species, for example *Heterosigma*, *Alexandrium*, *Gymnodinium*, and *Prorocentrum*, were observed to be still swimming after the FDA staining. Dorsey et al. (1989) reported that FDA staining for the assessment of cell metabolic activity in marine microalgae needed a flow cytometry technique. The use of flow cytometry seemed to be essential for a quantitative analysis using FDA staining, which could be a powerful, sensitive and useful technique for assessment of the physiological condition of marine phytoplankton.

On the other hand, both NR and EB were easily counted the stained cells under light microscopy, but the usage was limited in some species (Table 1). NR was incorporated into only live intact cells of *Tetraselmis* sp., *S. costatum*, *A. catenella*, and *P. minimum*. Especially, live *A. catenella* cells were stained deeply by NR (Fig. 1. (C)). However, results of NR staining were not determined because the dye seemed to have a cytotoxicity for another seven species of phytoplankton (Table 1). Formalin-killed cells of *S. costatum*, *A. catenella*, *G. mikimotoi* and *H. akashiwo* were stained by only EB. Dead *A. catenella* cells were also stained deeply by EB (Fig. 1. (D)). These results suggested that NR was able to apply the assessment of metabolically active cells of *Tetraselmis* sp., *S. costatum*, *A. catenella* and *P. minimum*, and EB was available for evaluating dead cells of *S. costatum*, *A. catenella*, *G. mikimotoi* and *H. akashiwo*. Particularly NR and EB staining were likely to be simple method for determining the physiological conditions of *A. catenella* with light microscopy.

Nevertheless we observed the virus-like growth suppression agents for marine phytoplanktons *A. catenella* and *G. mikimotoi* in seawater samples collected at the mouth of Funka Bay in 1993 to 1995, a strong algicidal activity or cell lysis have not been observed (Onji et al., 1999; Onji et al., 2000). A method to evaluate physiological condition of virus-infected cell is needed. FDA was the best dye for determining cell viability of ten species of marine phytoplankton tested in this study. NR was also available for evaluating live cells of *A. catenella* but not for *G. mikimotoi*, and EB was available for dead cells of both species. We concluded that

it is possible to use FDA, NR and EB for determining physiological condition of virus-infected *A. catenella*, and FDA and EB for *G. mikimotoi*. Furthermore, we suggested that the viable staining using FDA provides us good information about physiological condition of several marine microalgae, especially for investigating environmental pollution, virus infection, and so on.

Acknowledgment

We thank Professor Y. Ishida, Fukuyama University, Dr. Sako, Kyoto University and Akashio Research Institute of Kagawa Prefecture for providing phytoplankton culture strains. We appreciate Dr. D.L. Rowe, the Manchester Metropolitan University, for the critical reading. This study was supported by grants from the Fisheries Ground Preservation Division, Fisheries Agency of the Japanese Government.

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