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Fluorescent characteristics of dissolved organic matter produced by bloom-forming coastal phytoplankton

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Dynamics and sources of fluorescent dissolved organic matter (FDOM) are important for understanding biogeochemical processes in aquatic ecosystems. This study aimed to analyse direct production of FDOM by marine phytoplankton cultures and reveal fluorescent characteristics of exuded FDOM. Axenic cultures of eight species of bloom-forming marine phytoplankton, including two diatoms; a raphidophyte; two dinoflagellates; a chlorophyte; a cryptophyte and a haptophyte, were incubated in an artificial medium. Excitation emission matrices (EEMs) of FDOM in the culture medium were spectrofluorometrically measured. FDOM production was observed in all species, and fluorescent characteristics of the exudates varied considerably among species. Measured EEMs had peaks at 350/450 nm (excitation/emission) for the diatom *Ditylum brightwellii* and 370/450–470 nm for the raphidophyte *Heterosigma akashiwo* and the chlorophyte *Oltmansiellopsis viridis*, which have previously been regarded as the peaks of terrestrially derived humic-like substances. Direct production of FDOM by marine phytoplankton should be considered in future studies of FDOM dynamics in marine systems. Species-specific features of FDOM might be used for early detection of harmful blooms because this method is simple, rapid and suitable for monitoring.

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KEYWORDS: bloom; excitation emission matrix; fluorescent dissolved organic matter; incubation experiment; phytoplankton; spectrofluorometer

INTRODUCTION

Marine dissolved organic matter (DOM) is one of the major factors affecting the global carbon cycle and is the largest ocean reservoir of reduced carbon (Hansell *et al.*, 2009). DOM also affects various biological processes such as bacterial respiration and microalgal primary production by serving as a substrate for bacterial populations (Findlay and Sinsabaugh, 2003; Findlay *et al.*, 2003), controlling the transport and availability of trace elements (van den Berg *et al.*, 1986; Shiller *et al.*, 2006; Laglera *et al.*, 2007; Laglera and van den Berg, 2009) and absorbing photosynthetically effective sunlight (Sulzberger and Durisch-Kaiser, 2009) and harmful ultraviolet light (Nielsen and Ekelund, 1995; Nielsen *et al.*, 1995). The quality and quantity of DOM are thought to influence these ecological functions of DOM and should be taken into account when evaluating the biogeochemical processes in aquatic ecosystems.

The fluorescent spectroscopic characterization of chromophoric DOM (CDOM), which is a coloured fraction of DOM, is an excellent method for evaluating the source and quality of DOM (Coble, 1996, 2007; Coble *et al.*, 1990, 1993; Stedmon *et al.*, 2003; Jaffé *et al.*, 2008). This method compiles individual fluorescent spectra at each excitation (Ex) wavelength to generate three-dimensional excitation emission matrices (EEMs). EEMs of DOM can be highly variable and are controlled by different physical, chemical and biological processes; therefore, they can have important ecological consequences (Maie *et al.*, 2006; Jaffé *et al.*, 2008). In aquatic systems, fluorescent CDOM (FDOM) is assumed to be derived from biological processes in the system (autochthonous) as well as from the transport of terrestrial organic matter from rivers and the surrounding environment (allochthonous) (Jaffé *et al.*, 2008; Yamashita and Tanoue, 2008). Autochthonous production is thought to be mainly derived from bacterial metabolic by-products (Nieto-Cid *et al.*, 2006; Yamashita and Tanoue, 2008; Shimotori *et al.*, 2009).

It has been well documented that phytoplankton is one of the main sources of organic matter in the sea, because it releases organic compounds such as carbohydrates and polysaccharides (Biddanda and Benner, 1997). Recently, the exudates from marine phytoplankton have also been shown to have fluorescent properties, and they may be a source of marine autochthonous FDOM (Romera-Castillo *et al.*, 2010). In coastal and estuarine environments, various phytoplankton species sometimes form blooms and attain high cell densities. These algal blooms are thought to have substantial impacts on DOM dynamics of a region.

To understand direct FDOM production by marine phytoplankton, it is essential to conduct experiments using axenic cultures. However, the maintenance of axenic cultures is difficult, and very few studies have analysed FDOM production by axenic cultures of marine phytoplankton. Indeed, direct production of FDOM has been tested in only four species in the genera *Chaetoceros*, *Skeletonema*, *Prorocentrum* and *Micromonas* (Romera-Castillo *et al.*, 2010). The optical properties of DOM varied considerably among these four species (Romera-Castillo *et al.*, 2010).

We aimed to evaluate direct production of FDOM by eight major bloom-forming coastal phytoplankton species from diverse taxonomic groups of six classes and to reveal the fluorescent characteristics of the exuded FDOM.

METHOD

Phytoplankton cultures

Axenic cultures of the following species were used in the incubation experiments: the diatoms *Ditylum brightwellii* and *Chaetoceros curvisetus*, the raphidophyte *Heterosigma akashiwo*, the dinoflagellates *Heterocapsa circularisquama* and *Alexandrium catenella*, the chlorophyte *Oltmansiellopsis viridis*, the cryptophyte *Rhodomonas ovalis* and the haptophyte *Pleurochrysis roscoffensis* (Table I). These are commonly found bloom-forming species in the Western Pacific (Omura *et al.*, 2012). A culture of *C. curvisetus* was obtained from sea bottom sediment and made axenic (Ishii, personal communication). Axenic cultures of the other seven species were made by the methods described elsewhere (Imai and Yamaguchi, 1994; Nagai *et al.*, 1998). All the cultures were axenically maintained in the modified IHN medium (Imai *et al.*, 2004). The axenic conditions of each culture were confirmed by DAPI staining and epifluorescence microscopy before the incubation experiments (Imai, 1987).

Incubation experiment

Modified IHN medium (Imai *et al.*, 2004) was prepared using Milli-Q water and dispensed into 50 mL conical glass flasks (30 mL in each) with autoclavable plastic caps (Iwaki, Tokyo, Japan). The culture medium was sterilized by autoclaving at 121°C for 15 min. To prevent

Table I: Coastal phytoplankton species examined

Bacillariophyceae
Ditylum brightwellii (T.West) Grunow, 1885
Chaetoceros curvisetus P.T. Cleve, 1889
Dinoflagellata
Heterocapsa circularisquama Horiguchi, 1995
Alexandrium catenella (Whedon & Kofoid) E.Balech, 1985
Raphidophyceae
Heterosigma akashiwo (Y.Hada) Y.Hada ex Y.Hara & M.Chihara, 1967
Chlorophyceae
Oltmannsiellopsis viridis (P.E.Hargraves & R.L.Steele) M.Chihara & I.Inouye in Chihara, Inouye & Takahata, 1986
Cryptomonadea
Rhodomonas ovalis Nygaard
Prymnesiophyceae
Pleurochrysis roscoffensis (P. Dangeard) J. Fresnel & C. Billard

contamination with organic compounds, the glass flasks were pre-combusted at 450°C for 4 h. Actively growing strains of maintenance cultures were inoculated (600 μL) into each flask in a clean bench. The inoculated cultures were incubated under cool-white fluorescent light at 93–145 μmol photons m^{-2} s^{-1} with 14:10-h light:dark cycle until they reached the stationary phase, which was 6 days for *C. curvisetus*, 14 days for *H. akashiwo*, 27 days for *A. catenella* and 12 days for the other species. Incubation temperature was set at 25°C for *H. circularisquama* and 20°C for the other species, because only the maintenance culture of *H. circularisquama* indicated the growth at 25°C, while the other species showed the growth at 20°C. An autoclaved culture medium without plankton inoculation was also kept in the same conditions as a control. A small amount of each culture was taken to measure the *in vivo* fluorescence using a fluorometer (Model 10-AU 005, Turner Designs, Sunnyvale, CA, USA) at the start, middle and end of the culture experiments. The cell densities of the cultures were determined by microscopic counting at the end of the incubation. All the culture experiments were conducted in triplicate.

Analysis

After reaching the stationary phase, the cultures were filtered into pre-combusted brown glass bottles using radiation-sterilized disposable syringes (Terumo Corp., Tokyo, Japan) and GF/F filters (Whatman, Tokyo, Japan). The glass bottles and glass filters were pre-combusted at 450°C for 4 h. The three-dimensional EEM spectra of the culture filtrates were measured using a spectrofluorometer (Model F-7000, Hitachi High-Technologies, Tokyo, Japan), which was equipped with a 150 W xenon lamp. The corrections of the spectra were performed with Rhodamine B solution, according to the instructions in the instrument operation manual and the method described by Yoshioka *et al.* (Yoshioka *et al.*, 2007). The scanning ranges were 250–400 nm for Ex and 280–480 nm for emission (Em). Fluorescence Intensity was measured at 5-nm intervals for Ex and 1-nm intervals for Em, with a scanning speed of 1200 nm min^{-1}. The bandwidths were 5 nm for both Ex and Em. The EEM spectrum of the control medium was subtracted from each sample EEM spectrum to obtain the net increase in FDOM as a result of the microalgal activities. All the sample data of Fluorescence Intensity were standardized using quinine sulphate units (QSU), where 10 QSU correspond to the Fluorescence Intensity at 350/450 nm of a 10 μg L^{-1} quinine sulphate solution in 0.1 N H_2SO_4. Solutions of quinine sulphate (Nacalai Tesque, Inc., Kyoto, Japan) were measured with each set of samples. The Fluorescence Intensity data of each species were averaged for triplicates.

The bulk dissolved organic carbon (DOC) concentration of the culture filtrate was measured using a Shimadzu TOC-V_{CSH} total organic carbon analyzer (Shimadzu, Kyoto, Japan). The DOC content of each sample was determined using a calibration method based on a potassium hydrogen phthalate standard for each measurement. Each sample was injected five times, and the three values that yielded the minimum standard deviation were used to calculate the average DOC value for a sample. The net increase in DOC was determined by subtracting the control DOC from DOC of each sample.

Calculation of the index

The biomass of the cultures at the final point was estimated using previously reported models and equations (Menden-Deuer and Lessard, 2000; Sun and Liu, 2003) because we did not directly measure the cell biomass. The cell volume was calculated by approximating the complex cell shapes as simple three-dimensional geometric models (Sun and Liu, 2003) based on the cell sizes obtained from microscopic measurements. *Ditylum brightwellii* was approximated as a prism with a triangle-based girdle view shape (30-H), and *C. curvisetus* was approximated as a prism with an elliptic-based girdle view shape (29-H). *Heterosigma akashiwo* and *H. circularisquama* were approximated as a cone + hail sphere shape (9-H) (Sun and Liu, 2003). *Alexandrium catenella* was approximated as an ellipsoid (3-H). *Oltmansiellopsis viridis*, *R. ovalis* and *C. roscoffensis* were approximated as prolate spheroids (2-H) (Sun and Liu, 2003). The calculated cell volumes were converted into cell biomass using the equations described by Menden-Deuer and Lessard (Menden-Deuer and Lessard, 2000). As an indicator of the percentage of extracellular carbon released during total photosynthetic production, the apparent percentage of photosynthetic

Table II: Incubation time, growth rates (μ) during the exponential growth phase, the final cell abundance (C) and biomass (B)

Phytoplakton culture	Time (days)	μ (day^{-1})	C (cells mL^{-1})	B (mg C L^{-1})
Ditylum brightwellii	12	0.76 ± 0.05	11 ± 1 × 10^3	18.8 ± 1.0
Chaetoceros curvisetus	6	0.97 ± 0.07	199 ± 37 × 10^3	5.9 ± 1.1
Heterocapsa circularisquama	12	0.84 ± 0.01	93 ± 22 × 10^3	33.1 ± 7.9
Alexandrium catenella	27	0.48 ± 0.01	22 ± 3 × 10^3	27.6 ± 4.1
Heterosigma akashiwo	14	0.35 ± 0.03	121 ± 59 × 10^3	28.7 ± 13.8
Oltmannsiellopsis viridis	12	0.55 ± 0.06	247 ± 95 × 10^3	18.2 ± 7.0
Rhodomonas ovalis	12	0.40 ± 0.01	253 ± 45 × 10^3	3.5 ± 0.6
Pleurochrysis roscoffensis	12	0.45 ± 0.03	136 ± 18 × 10^3	11.8 ± 1.6

extracellular release (APER) values (Romera-Castillo *et al.*, 2010) were calculated using the following formula:

$$\text{APER}\ (\%) = \frac{\text{DOC}}{(\text{DOC} + \text{Biomass})} \times 100,$$

where DOC and Biomass represent the net increase in DOC in the culture medium (mg C L^{-1}) and the phytoplankton biomass (mg C L^{-1}), respectively.

RESULTS

Growth of the cultures and cell density attained

The cell densities of the stationary phase cultures varied from 11 ± 1 × 10^3 (cells mL^{-1}) for *D. brightwellii* to 253 ± 45 × 10^3 (cells mL^{-1}) for *R. ovalis*. *Heterocapsa circularisquama* produced the highest biomass of 33.1 ± 7.9 mg C L^{-1} among the eight species examined, and *R. ovalis* produced the lowest biomass of 3.5 ± 0.6 mg C L^{-1}. The average growth rate in the exponential growth phase, which was calculated on the basis of the *in vivo* fluorescence values, varied from 0.35 ± 0.03 day^{-1} for *H. akashiwo* to 0.97 ± 0.07 day^{-1} for *C. curvisetus* (Table II). The growth rates of these species in modified IHN medium were previously reported as 0.39–1.27 day^{-1} (Naito *et al.*, 2008), which are comparable with the values obtained in the present study.

DOC and APER

The increased DOC concentrations and APER values are summarized in Table III. Net increase in DOC concentrations ranged from 19.3 ± 5.1 mg C L^{-1} for *H. akashiwo* to 49.4 ± 7.5 mg C L^{-1} for *P. roscoffensis* during incubation periods (Table III). APER values were estimated to be between 46.1% for *A. catenella* and 80.2% for *P. roscoffensis*, with rather wide variations. There were no significant differences in APER values among the eight species (one-way ANOVA, $P = 0.198$).

Table III: Net increase in the dissolved organic carbon (DOC) concentration and the apparent percentage of net photosynthetic extracellular release (APER) for each plankton culture

Phytoplankton culture	DOC (mg C L^{-1})	APER (%)
Ditylum brightwellii	25.1 ± 4.9	55.9 ± 4.5
Chaetoceros curvisetus	26.6 ± 11.3	71.5 ± 16.0
Heterocapsa circularisquama	38.6 ± 14.8	51.0 ± 14.6
Alexandrium catenella	24.1 ± 5.5	46.1 ± 9.4
Heterosigma akashiwo	19.3 ± 5.1	52.4 ± 19.6
Oltmannsiellopsis viridis	27.8 ± 4.5	63.7 ± 7.7
Rhodomonas ovalis	26.4 ± 16.6	72.1 ± 18.6
Pleurochrysis roscoffensis	49.9 ± 7.5	80.2 ± 3.7

APER was calculated as follows: DOC/(DOC + biomass) × 100, where DOC and biomass are the net increases in DOC and biomass during the incubation period, respectively. The data shown are average values ± standard error (SE) ($n = 3$).

Optical properties of DOM exudates from phytoplankton

Figure 1 shows the average EEMs of the net FDOM increases for each triplicate culture filtrate sample. In all the cultures, EEMs had fluorescence peaks in the protein-like and humic-like regions (Table IV, Fig. 1). In the protein-like region, as described by Coble (Coble, 1996) at Ex/Em 275/340 nm (peak T) and Ex/Em 275/310 nm (peak B), the most prominent peaks were at 280/349–357 nm (Ex/Em) for the cultures of *C. curvisetus*, *H. circularisquama* and *A. catenella*. In the corresponding region, *H. akashiwo*, *O. viridis* and *R. ovalis* had slightly shorter Em wavelengths in the range 280/324–337 nm. *Ditylum brightwellii* and *P. roscoffensis* had peaks at 255/316 nm and 250/350 nm respectively, although these peaks were rather uniform (Fig. 1). The protein-like peaks were broad towards the longer Em wavelengths. In particular, *H. circularisquama* had a considerably broader peak toward longer Ex/Em wavelengths in the region and appeared to have an overlapping peak at 290/410 nm (Fig. 1), which corresponded to peak M, i.e. marine humic-like substances, as defined by Coble *et al.* (Coble *et al.*, 1998). Peak M was only observed with *H. circularisquama* in the present study, whereas peak M was

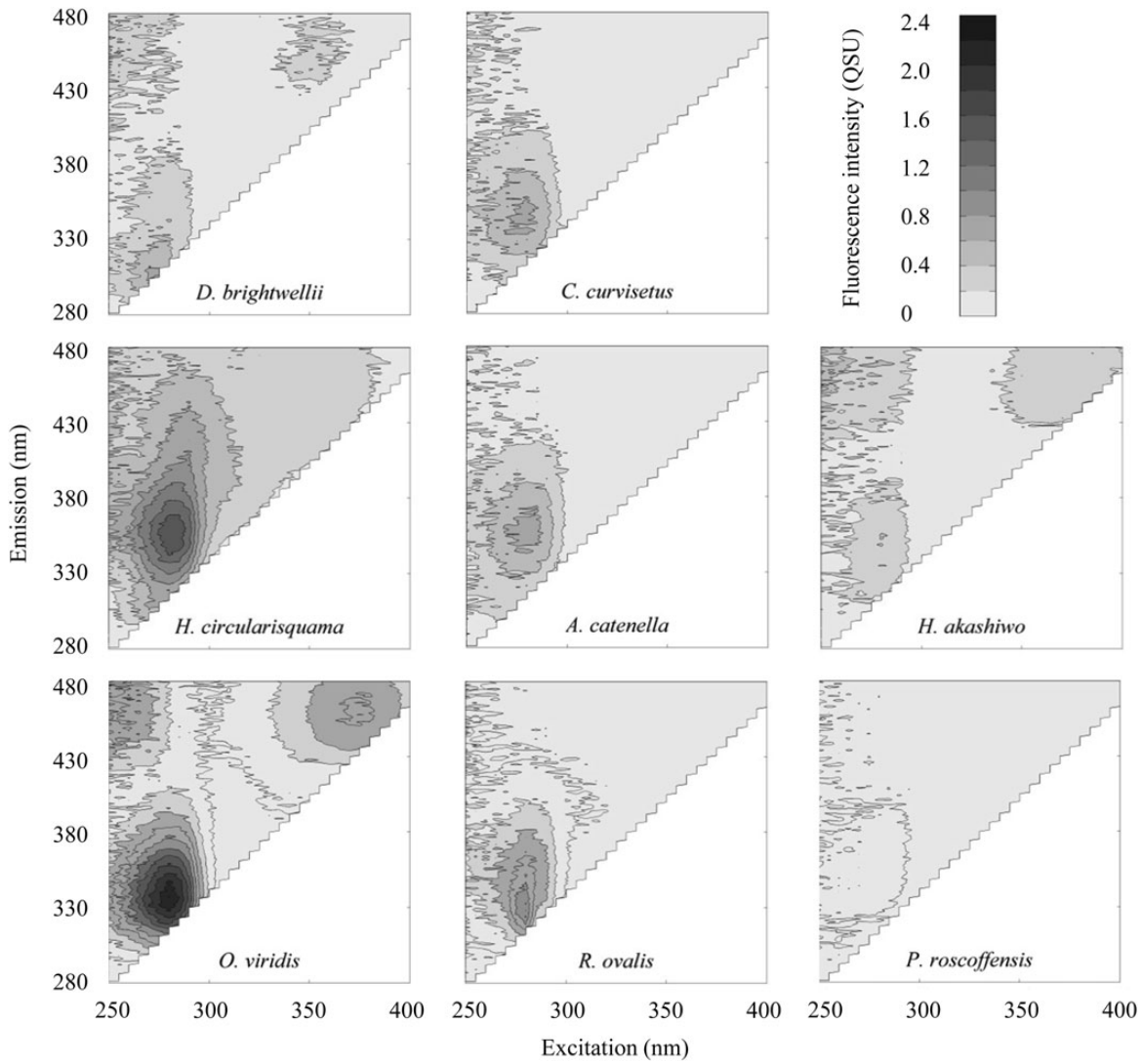

Fig. 1. Average excitation emission matrices of fluorescent dissolved organic matter (FDOM) secreted by each plankton culture.

observed with all the marine phytoplankton axenic cultures examined by Romera-Castillo *et al.* (Romera-Castillo *et al.*, 2010). *Oltmansiellopsis viridis* had the most prominent peak among the eight species in the protein-like fluorescent region, at 280/337 nm, with an intensity of 2.22 ± 0.36 QSU (Fig. 1, Table IV).

Ditylum brightwellii, *H. circularisquama*, *H. akashiwo* and *O. viridis* had the maxima of humic-like fluorescence at 355/454, 340/461, 375/473 and 375/462 nm, respectively (humic-like peak 1 in Table IV, Fig. 1). *Oltmansiellopsis viridis* had the most conspicuous peak in the fluorescent area, with a Fluorescence Intensity of 0.86 ± 0.24 QSU. *Chaetoceros curvisetus*, *A. catenella*, *R. ovalis* and *P. roscoffensis* did not have defined peaks in this area (Fig. 1). Coble (Coble, 1996) referred to the peaks in the region of 350/420−480 nm as peak C and identified them as humic-like DOM components. The peaks observed in the cultures of *D. brightwellii* and *H. circularisquama* were assumed to correspond to peak C. Furthermore, the peaks resembled the peak produced by a *M. pusilla* culture at 348/434 nm and 348/436 nm, as reported by Romera-Castillo *et al.* (Romera-Castillo *et al.*, 2010). *Heterosigma akashiwo* and *O. viridis* had peaks at longer Em wavelengths, i.e. 370/450−470 nm (Ex/Em), and these peaks were very close to the C1 peak reported to be related to terrestrial humic substances by Yamashita *et al.* (Yamashita *et al.*, 2008).

Humic-like peaks were also detected in all the samples at 250−255/446−471 nm (humic-like peak 2), which corresponded to peak A (260/458 nm), as defined by Coble (Coble, 1996). The Fluorescence Intensity of humic-like peak 2 ranged from 0.54 ± 0.04 QSU for *C. curvisetus* to 1.20 ± 0.16 QSU for *O. viridis*.

Table IV: Peak position, averaged Fluorescence Intensity standardized to quinine sulphate unit (QSU) at each peak position and DOC-specific FI of FDOM (QSU L mg C^{-1}) secreted by each species

	Humic-like peak 1				Humic-like peak 2				Protein-like peak			
Phytoplankton culture	Ex (nm)	Em (nm)	FI (QSU)	FI/DOC (QSU L mg C^{-1})	Ex (nm)	Em (nm)	FI (QSU)	FI/DOC (QSU L mg C^{-1})	Ex (nm)	Em (nm)	FI (QSU)	FI/DOC (QSU L mg C^{-1})
Ditylum brightwellii	355	454	0.26 ± 0.02	0.010 ± 0.002	250	464	0.66 ± 0.07	0.026 ± 0.006	255	316	0.58 ± 0.03	0.023 ± 0.005
Chaetoceros curvisetus					250	455	0.54 ± 0.04	0.020 ± 0.009	280	349	0.69 ± 0.04	0.026 ± 0.011
Heterocapsa circularisquama	340	461	0.15 ± 0.01	0.004 ± 0.002	255	441	0.61 ± 0.04	0.016 ± 0.006	280	357	1.60 ± 0.10	0.041 ± 0.016
Alexandrium catenella					250	471	0.55 ± 0.15	0.023 ± 0.008	280	352	0.75 ± 0.07	0.031 ± 0.008
Heterosigma akashiwo	375	473	0.31 ± 0.10	0.016 ± 0.007	250	455	0.90 ± 0.21	0.046 ± 0.016	280	335	0.44 ± 0.19	0.023 ± 0.012
Oltmannsiellopsis viridis	375	462	0.86 ± 0.24	0.031 ± 0.010	250	455	1.20 ± 0.16	0.043 ± 0.009	280	337	2.22 ± 0.36	0.080 ± 0.018
Rhodomonas ovalis					250	446	0.80 ± 0.13	0.030 ± 0.020	280	324	1.24 ± 0.61	0.047 ± 0.038
Pleurochrysis roscoffensis					250	455	0.56 ± 0.11	0.011 ± 0.003	250	350	0.57 ± 0.03	0.011 ± 0.002

Mean value ± SE ($n = 3$).

DOC-specific Fluorescence Intensities of humic like peaks 1 and 2 and protein like peak considerably varied among species (Table IV). The DOC-specific Fluorescence Intensity of humic-like peak 1 was highest for *O. viridis* (Table IV). The DOC-specific Fluorescence Intensity of humic-like peak 2 ranged from 0.011 ± 0.003 QSU L mg C^{-1} for *P. roscoffensis* to 0.046 ± 0.016 QSU L mg C^{-1} for *H. akashiwo* (Table IV). The DOC-specific Fluorescence Intensity of protein-like peak ranged from 0.011 ± 0.002 QSU L mg C^{-1} for *P. roscoffensis* to 0.080 ± 0.018 QSU L mg C^{-1} for *O. viridis* (Table IV). The cell density-specific FDOM production rates were the highest for *D. brightwellii* in terms of both the protein-like and humic-like peaks (Table V). In terms of the biomass-specific FDOM production rates (data not shown), *R. ovalis* had the highest values for both fluorescent peaks.

DISCUSSION

DOC and APER

Sharp *et al.* (Sharp *et al.*, 1977) reported that photosynthetic extracellular release (PER) values obtained using the ^{14}C method reached up to 70%. APER values obtained in the present study were 46–80%, comparable with the previously reported values. In contrast, Lancelot and Billen (Lancelot and Billen, 1985) reported that the PER value using the culture method was 0–20%. Compared with APER values of 10–18% obtained in a previous study using axenic cultures of microalgae (Romera-Castillo *et al.*, 2010), the present study obtained rather high APER values (46–80%). In the present study, the incubation period was 6–14 days, with the exception of 27 days for *A. catenella* (Table II), which was longer than the incubation period (3–6 days) used previously (Romera-Castillo *et al.*, 2010). It was previously reported that PER values of the diatom *Chaetoceros affinis* increased by up to 58% during the phase of decreasing photosynthetic activity because of nutrient depletion (Myklestad *et al.*, 1989). Thus, APER values appear to be affected in nutrient-limited environments (Lancelot and Billen, 1985). The longer incubation period probably resulted in larger release of DOC from the phytoplankton cells, although we did not measure the nutrient concentration. The influence of light and nutrient stress on DOC secretion should be tested in future studies because phytoplankton are likely to experience light and nutrient stresses in natural environments.

Peak assignments and possible functions of the fluorescent DOM

Significant peaks were observed at ~275/340 nm (Ex/Em) in the culture filtrates of all the species. This peak

Table V: DOC (μg C 1000 cells^{-1}) and intensity of each fluorescent component (QSU mL 1000 cells^{-1}), which were standardized on the basis of the cell density at the end of the incubation, secreted by the phytoplankton cultures

Phytoplankton culture	Time (days)	DOC (μg C 1000 cells^{-1})	Humic-like peak 1 (QSU mL 1000 cells^{-1})	Humic-like peak 2 (QSU mL 1000 cells^{-1})	Protein-like peak (QSU mL 1000 cells^{-1})
Ditylum brightwellii	12	2.22 ± 0.45	0.023 ± 0.0024	0.058 ± 0.007	0.051 ± 0.004
Chaetoceros curvisetus	6	0.13 ± 0.06	Not detected	0.003 ± 0.001	0.003 ± 0.001
Heterocapsa circularisquama	12	0.42 ± 0.19	0.0016 ± 0.0004	0.007 ± 0.002	0.017 ± 0.004
Alexandrium catenella	27	1.09 ± 0.29	Not detected	0.025 ± 0.008	0.034 ± 0.006
Heterosigma akashiwo	14	0.16 ± 0.09	0.0026 ± 0.0015	0.007 ± 0.004	0.004 ± 0.002
Oltmannsiellopsis viridis	12	0.11 ± 0.05	0.0035 ± 0.0016	0.005 ± 0.002	0.009 ± 0.004
Rhodomonas ovalis	12	0.10 ± 0.07	Not detected	0.003 ± 0.001	0.005 ± 0.003
Pleurochrysis roscoffensis	12	0.37 ± 0.07	Not detected	0.004 ± 0.001	0.004 ± 0.001

Mean value ± SE ($n = 3$).

was considered to be related to protein-like substances and was previously reported as peak T (Coble, 1996). Romera-Castillo *et al.* (Romera-Castillo *et al.*, 2010) also reported a corresponding peak in cultures of *Chaetoceros*, *Skeletonema*, *Prorocentrum* and *Micromonas*. Phytoplankton are known to release extracellular nitrogenous compounds such as proteins, peptides and amino acids as well as carbohydrates such as polysaccharides (Goldman *et al.*, 1992; Myklestad, 1995). Some of these substances with proteinaceous aromatic structures were probably detected as peak T. Similar peaks were detected in EEM measurements of coastal seawaters (Maie *et al.*, 2007; Yamashita *et al.*, 2008; Para *et al.*, 2010). Yamashita *et al.* (Yamashita *et al.*, 2008) suggested that a tryptophan-like fluorescent peak in a seawater sample from Mikawa Bay, Japan, was derived from relatively fresh long-chain peptides, which were readily degradable. Maie *et al.* (Maie *et al.*, 2007) suggested that the origin of peak T in temperate coastal seawater was a mixture of proteinaceous compounds and the phenolic structures contained in humic substances.

Heterocapsa circularisquama had a peak at 290/410 nm, which was similar to peak M designated by Coble (Coble, 1996). Peak M is found at 290−310/370−410 nm (Ex/Em) and was first reported in seawater collected during a phytoplankton bloom in the Gulf of Maine (Coble, 1996) and thereafter in samples from an upwelling region in the Arabian Sea (Coble *et al.*, 1998). This peak is considered to be related to marine humic-like substances (Coble, 1996). In a recent study, peak M was detected in the culture filtrates of four marine algal species: *Chaetoceros* sp., *S. costatum*, *P. minimum* and *M. pusilla* (Romera-Castillo *et al.*, 2010). These findings are consistent with FDOM produced by *H. circularisquama*, which showed peak M in the present study. The absence of peak M in the cultures of other species examined in the present study may have been possibly due to differences in the metabolic processes among the species, such as different photosynthetic pigments.

Coble (1996, 2007) suggested that terrestrial humic-like materials produce two peaks at 240−260/400−460 nm (Ex/Em) (peak A) and 320−360/420−460 nm (Ex/Em) (peak C). All the species produced peaks at 250−255/446−471 nm (humic-like peak 2), which were very close to peak A. Humic-like peak 2 is also similar to Component 1 (<260/458 nm) in a study that used the EEM-PARAFAC method in Ise Bay, Japan (Yamashita *et al.*, 2008). In other studies, this peak was also reported to be attributable to land-derived components, e.g. Q2 (Cory and Mcknight, 2005) and Component 1 (Stedmon and Markager, 2005).

Ditylum brightwellii had a peak at 350/450 nm (Ex/Em), which corresponded to the region of peak C (Coble, 1996). *Heterosigma akashiwo* and *O. viridis* had peaks at a slightly longer wavelength of 370/450−470 nm (Ex/Em), and these peaks were very close to the peak attributed to terrestrial humic substances (Yoshioka *et al.*, 2007; Yamashita *et al.*, 2008, 2011). These findings suggest that FDOM produced by phytoplankton occasionally has a peak in the region previously assigned to terrestrial humic substances. Thus, we should be cautious when investigating the dynamics and sources of DOM in coastal areas using fluorescent analysis.

It is not known how and why phytoplankton release humic-like substances. Bjørnsen (Bjørnsen, 1988) suggested that DOM exudates are caused by the passive diffusion of metabolic by-products. The low-molecular-weight compounds produced by photosynthetic metabolism and by-products of the decomposition of cellular polymers are assumed to be released extracellularly (Myklestad, 1995). However, it is possible that phytoplankton exude FDOM with ecological functions. Many species of microbial prokaryotes, fungi and some phytoplankton are known to secrete organic iron ligands, known as siderophores, in iron-depleted environments (Naito *et al.*, 2001, 2004; Vraspir and Butler, 2009). Most siderophores appear to have aromatic structures,

although the chemical structures of the siderophores secreted by eukaryotic phytoplankton are not clear at present (Naito *et al.*, 2001; Vraspir and Butler, 2009). Humic substances also have aromatic structures and the capacity for metal complexation. Naito *et al.* (Naito *et al.*, 2001) also suggested that *R. ovalis* secretes siderophores; this species had the highest biomass-specific production of FDOM in the present study. Thus, it is possible that siderophores are involved with the release of humic-like fluorescence by phytoplankton. Therefore, it is necessary to investigate the biological roles of fluorescent exudates in the future.

Fluorescence in natural environments and bloom formation

To evaluate the contributions of phytoplankton FDOM in natural environments, we extrapolated the values obtained in this study to a naturally occurring *H. circularisquama* bloom. The cell density of *H. circularisquama* reached >10 000 cells mL^{-1} during the bloom period in Japanese coastal water (Kamiyama *et al.*, 2001). For this bloom, the Fluorescence Intensity of humic-like peak 1 was estimated to be 1.6×10^{-2} QSU using the data for the Fluorescence Intensity per cell density (Table V). The possible Fluorescence Intensities of humic-like peak 2 and protein-like peak were also estimated in the same manner. Humic-like peak 2 and protein-like peak would have been $\sim 6.60 \times 10^{-2}$ QSU and 1.72×10^{-1} QSU, respectively. These data suggest that it is possible to detect phytoplankton-derived FDOM in the natural aquatic environment, although these are rough estimates and high intensity FDOM in the same region derived from other sources could mask these peaks.

Species-specific peaks are considered to be novel indicators that could facilitate better understanding of the contribution of microalgal activities to FDOM production. For example, Suksomjit *et al.* (Suksomjit *et al.*, 2009) observed significant increase of tyrosine-like, tryptophan-like and humic-like fluorescence, which were centred at 225/305, 280/350 and 230/395 nm, respectively, in coastal seawater during a *Heterosigma akshiwo* and *Chaetoceros* sp. bloom. While axenic cultures of *H. akshiwo* and *C. curvisetus* were indicated to produce the corresponding peaks such as protein-like peak and humic-like peak 2 in our study. The absence of humic-like peak 1 in the natural sea-water was possibly due to the relatively weak Fluorescence Intensity of the peak. Moreover, it was suggested that the fluorescent peaks detected in EEM of an axenic *Micromonas* culture, which were centred at 275/345 and 348/436 nm, were consistent with the peak detected in natural seawater during a *Micromonas* bloom (Romera-Castillo *et al.*, 2010). We observed strong species-specific peaks such as at 375/462 nm for *O. viridis*, which are likely to be identified in the natural environment. It is suggested that the DOC-specific Fluorescence Intensities are also indicators, although we should note that photo-degradability of FDOM and DOC was considered to vary (Mostofa *et al.*, 2007). We should also note that heterotrophic bacteria are known to alter the FDOM property. For instance, they consume peak M to produce peak C (Romera-Castillo *et al.*, 2011). The three-dimensional fluorescence method does not require any special techniques during the pre-treatment procedure; therefore, it is suitable for monitoring. It suggests that early detection of harmful algal blooms is possible using the EEM technique. To test this possibility, we need to monitor time-series of fluorescent EEMs in seawater during the course of a bloom event and compare the optical characteristics of seawater samples and those of the axenic culture filtrates of the harmful phytoplankton species. In the present study, we investigated the fluorescence properties of DOM exuded by eight species of axenic phytoplankton using an artificial medium. Our knowledge about FDOM production by pure cultures of marine phytoplankton is still quite limited. For example, the study does not involve cyanobacterial species, which often form nuisance bloom in coastal areas. Further studies are required to understand the biogeochemical and ecological role of FDOM and its relative abundance in the natural environment.

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