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Dual-laser flow cytometry reveals *Synechococcus* pigment type diversity

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ABSTRACT: *Synechococcus* cyanobacteria exhibit a highly diverse composition of photosynthetic pigments. The fluorescence excitation ratio ($Ex_{495:545}$) of the blue light-absorbing photosynthetic pigment phycourobilin (PUB; λ_{max} = 495 nm) to the green light-absorbing photosynthetic pigment phycoerythrobilin (PEB; λ_{max} = 545 nm) differs between the pigment subtypes within *Synechococcus* pigment Type 3, which is predominantly found in marine waters. In the present study, we developed a method to assess the $Ex_{495:545}$ of natural *Synechococcus* subpopulations that were distinguished using a flow cytometer equipped with both blue (488 nm) and green (532 nm) lasers. A significantly positive linear relationship of *Synechococcus* strains was obtained between spectrofluorometry and dual-laser flow cytometry ($y = 0.542x + 0.08$). Using this equation, the $Ex_{495:545}$ values of natural *Synechococcus* subpopulations were enumerated. An $Ex_{495:545}$ below 0.60 only occurred in the coastal areas and co-occurred with other subpopulations with higher $Ex_{495:545}$, ranging from 0.73 to 1.80. The co-occurrence of multiple *Synechococcus* subpopulations with different $Ex_{495:545}$ was also observed along the coastline of Japan. However, in the open ocean, only 1 subpopulation was observed, with $Ex_{495:545}$ above 1.63 at the surface and increasing by depth to 2.13. The application of this method to enumerate $Ex_{495:545}$ will advance the study of pigment type diversity and the light acclimation strategy of *Synechococcus* in marine environments.

KEY WORDS: Pigment type · Ratio of phycourobilin to phycoerythrobilin · Phycobilisomes · Excitation ratio

1. INTRODUCTION

The picocyanobacterial genus *Synechococcus* is one of the most abundant phytoplankton groups in the marine environment, and it is widely distributed across oceans worldwide, from eutrophic coastal waters to the oligotrophic open ocean (Olson et al. 1988, Partensky et al. 1999, Flombaum et al. 2013). This ubiquitous distribution is partly due to the pigment diversity that arises from the vast variations in the composition of phycobilisomes. These consist of allophycocyanin at the core and are surrounded by 6 phycobiliprotein rods that contain phycocyanin (PC), phycoerythrin I (PE I), and phycoerythrin II (PE II) (Waterbury et al. 1986, Six et al. 2007). Each phycobiliprotein rod contains specific chromophores that absorb different light colors. *Synechococcus* is divided into 3 major pigment types based on the phycobiliprotein composition: Type 1, Type 2, and Type 3 (Wood et al. 1985, Six et al. 2007, Humily et al. 2013). Pigment Type 1 only possesses PC that contains phycocyanobilin (PCB) as the sole chromophore that absorbs red light (620 nm). Pigment Type 2 possesses PC and PE I, which contains PCB and phycoerythrobilin (PEB) that absorbs green light (510–550 nm). Lastly, pigment Type 3 possesses PC, PE I, and PE II, which contains PEB and phycourobilin (PUB) that absorbs blue light (440–490 nm) (Ong & Glazer 1991, Six et al. 2007, Humily et al. 2014). Type 3 is further divided into 4 subtypes based on the fluorescence excitation ratio at 495 and 545 nm (hereafter, $Ex_{495:545}$), which is a proxy for the ratio of PUB to PEB: Type 3a

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($Ex_{495:545}$ < 0.6); Type 3b (0.6 ⩽ $Ex_{495:545}$ < 1.6); Type 3c ($Ex_{495:545}$ ⩾ 1.6); and Type 3d/e, which is also known as Type IV chromatic acclimator. Type 3d/e can adjust the $Ex_{495:545}$ depending on the ambient light environment (Palenik 2001, Everroad & Wood 2006, Six et al. 2007). The type with a wide acclimation range (0.6 ⩽ $Ex_{495:545}$ < 1.4) is called Type 3d, and the type with a narrow range (0.6 ⩽ $Ex_{495:545}$ < 0.8) is called Type 3e (Humily et al. 2013, Lovindeer et al. 2021).

This pigment diversity in *Synechococcus* spp. is important to their success under the various light conditions found from estuarine to oceanic waters. Metagenomic analysis of 3 phycobilisome genes, namely *cpcBA*, *mpeBA*, and *mpeW*, has been conducted to study pigment type diversity in the ocean (Grébert et al. 2018). The distribution of the genotypes in oceans worldwide has been reported (Humily et al. 2014, Farrant et al. 2016, Grébert et al. 2018, Zhang et al. 2022), where Type 3a is likely to be distributed in coastal waters, while Type 3c is found in oligotrophic waters. Apart from these molecular biological approaches, the $Ex_{495:545}$ of *Synechococcus* in the natural environment has been evaluated using spectrofluorometry (Lantoine & Neveux 1997, Wood et al. 1999), which revealed that the $Ex_{495:545}$ of natural *Synechococcus* communities continuously varies in both horizontal and vertical directions. This continuous variation in the $Ex_{495:545}$ has been evaluated for bulk communities but remains poorly understood for each subpopulation level. It is well known that there is variation in the $Ex_{495:545}$ even within each pigment type. Furthermore, depending on the ambient light conditions, this value is variable within a chromatic acclimation of Type 3d. Evaluating the $Ex_{495:545}$ for each subpopulation level within natural *Synechococcus* communities is important for a better understanding of niche partitioning among the *Synechococcus* pigment types; however, the methodology to examine them at the subpopulation level has not yet been established.

To enumerate *Synechococcus* cell abundance, flow cytometry at 488 nm with a single blue laser has been used in many studies (Wood et al. 1985, Liu et al. 2014, Zhang et al. 2022). As phycocyanin and phycoerythrin are fluorescence pigments, multiple lasers have been used to separately enumerate the various *Synechococcus* pigment types. For example, Xia et al. (2017) evaluated phycocyanin-rich (PC) and phycoerythrin-rich *Synechococcus* using a flow cytometer equipped with blue and red lasers. Similarly, Thompson & van den Engh (2016) and Gale et al. (2023) enumerated freshwater picocyanobacteria using multi-laser flow cytometry. Particularly for pigment Type 3, a combination of blue and green lasers is a powerful tool to enumerate *Synechococcus* with different ratios. Olson et al. (1988) used a dual-beam flow cytometer that emitted 488 nm of blue light and 515 nm of green light for the first time. In addition to the 488 nm laser, the installation of a 515 nm laser in the flow cytometer allowed each beam to efficiently excite PUB and PEB (Olson et al. 1988). Using this method, Olson et al. (1988) and Katano et al. (2007) demonstrated the co-occurrence of 2 subpopulations of *Synechococcus* with different PUB to PEB ratios in coastal waters. The population with a lower fluorescence excited by blue light was defined as the Low PUB type, and the population with a higher fluorescence excited by blue light was defined as the High PUB type. Katano et al. (2007) first calculated $Ex_{495:545}$ by creating a regression equation (R = 0.878) of the PUB to PEB ratio measured with the dual-beam flow cytometer against the $Ex_{495:545}$ of the *Synechococcus* population using a spectrofluorometer to measure samples in the field. However, at that time, the variety among pigment Type 3 was not fully understood.

In the present study, we accurately evaluated the $Ex_{495:545}$ of *Synechococcus* in the natural environment after discriminating between the subpopulations using a dual-laser flow cytometer with blue and green lasers. To generate a regression equation of the $Ex_{495:545}$ and fluorescence signals of the flow cytometer, cultured strains of pigment Types 3a, 3c, and 3d were analyzed with both a dual-laser flow cytometer and spectrofluorometer. This regression equation was applied to the natural *Synechococcus* subpopulations. We found that more than 3 subpopulations expressing different $Ex_{495:545}$ ratios were frequently detected in coastal waters, as demonstrated by molecular studies (Grébert et al. 2018). This study emphasizes that the pigmentation phenotypes of *Synechococcus* are more diverse than previously reported by Olson et al. (1988) and Katano et al. (2007).

2. MATERIALS AND METHODS

2.1. Sampling

Sampling at Stns SJ1, SJ3′, OC6, and SJ11 in the northwestern Pacific Ocean (Fig. 1, Table 1) was performed aboard the training ship 'Shioji-maru'. Water samples were collected using Niskin bottles attached to a conductivity-temperature-depth (CTD) rosette multi-profiler (SBE9plus, Sea-Bird Electronics) from a depth of 5 m. In addition, vertical water samples were collected from depths of 10–81 m at Stn SJ11

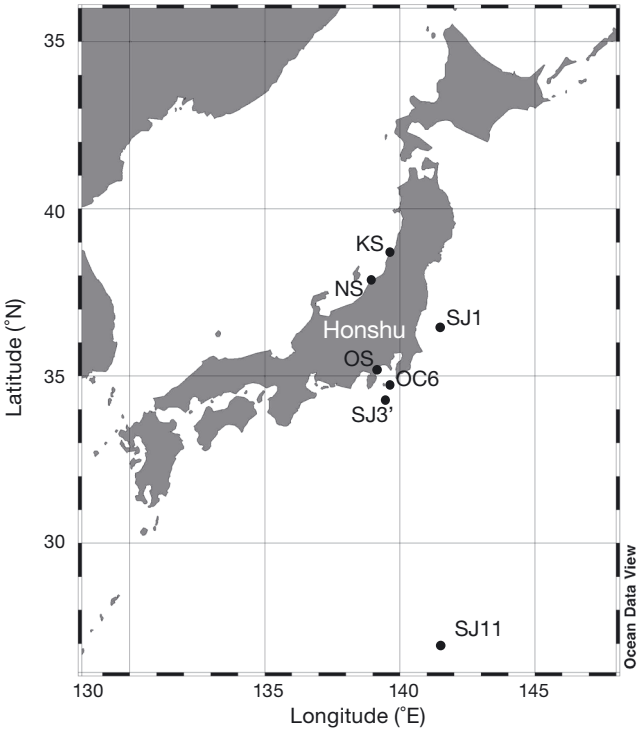

Fig. 1. Sampling stations in the Sea of Japan and the northwestern Pacific Ocean

(Table 1). For flow cytometric analysis, a 1.1 ml water sample was pre-filtered through a 40 µm mesh and preserved with paraformaldehyde (0.2% final concentration). Samples were immediately frozen in liquid nitrogen and kept at −80°C until analysis. At Stns KS, NS, and OS (Fig. 1, Table 1), surface water samples were collected using a sampling bucket, and the samples were kept below 4°C under dark conditions without fixation until analysis, which occurred within 1−3 d from sampling (Table 1).

The water temperature and salinity were measured using the CTD at Stns SJ1, SJ3', O6, and SJ11. At Stns KS and NS, the water temperature was measured with a bar thermometer, and at Stn OS, it was measured with a data logging thermometer (DEFI2-T, JFE-Advantec). Salinity was measured using a refractometer (AS ONE IS/Mill-E) at Stns KS, NS, and OS.

2.2. Flow cytometric analysis and measurement of the PUB to PEB ratio of *Synechococcus*

Synechococcus cells were counted using a flow cytometer (Guava easyCyte BG, Luminex) equipped with blue light (488 nm) and green light (532 nm) lasers at a low flow rate (0.24 µl s^{-1}). As the internal standard, 2.2 µm fluorescent Nile Red beads (Spherotech) were used. Data analyses were performed using Guava InCyte 3.4 software. Three fluorescence intensities — Red-B (red fluorescence, 645−745 nm) and Yel-B (yellow fluorescence, 550−600 nm),

Table 1. Information on the sampling areas and the environmental conditions. Dates are given as d/mo/yr

Stn	Date	Depth (m)	Water temperature (°C)	Salinity	Oceanic region	Climate region	Distance from Honshu, Japan (km)
SJ1	7/08/22	5	24.5	33.4	Northwestern Pacific Ocean	Temperate	72
SJ3'	11/08/22	5	22.6	33.4	Northwestern Pacific Ocean	Temperate	19
OC6	11/06/23	5	22.1	34.3	Northwestern Pacific Ocean	Temperate	77
KS	22/10/22 (24/10/22)[a]	0	21.4	32	Japan Sea	Temperate	0
NS	21/10/22 (24/10/22)[a]	0	21.4	33	Japan Sea	Temperate	0
OS	5/10/22 (6/10/22)[a]	0	24.5	34	Sagami Bay	Temperate	5
SJ11	22/07/22	5	29.1	33.3	Northwestern Pacific Ocean	Sub-tropical	892
		10	28.6	34.7			
		30	23.8	34.7			
		41	22.4	34.7			
		50	22.2	34.7			
		75	21.3	34.8			
		81	21.2	34.8			

[a]Samples collected at Stns KS, NS, and OS were not fixed and were stored at 4°C in the dark until analysis. Dates in parentheses indicate the dates of flow cytometric analysis

both excited at 488 nm of blue light, and Yel-G (yellow fluorescence, 550–600 nm) excited at 532 nm of green light — were recorded for each cell. Red-B and Yel-B fluorescence are indices of the chlorophyll *a* and PUB contents, respectively. Yel-G is an index of the PEB content. The Yel-B to Yel-G ratio was calculated using the mean Yel-B and Yel-G fluorescence of the subpopulation (Fig. 2c) and was used as a proxy for the PUB to PEB ratio. For samples collected from Stns SJ1, SJ3', OC6, and SJ11, PUB and/or PEB fluorescence intensities of *Synechococcus* cells were normalized to those of the fluorescent beads (Fig. 2). Beads were gated in the flow cytogram with Red-B and side scatter as the *y*-axis and *x*-axis, respectively (Fig. 2a). *Synechococcus* cells were gated in the flow cytogram with Red-B and Yel-B as the *y*-axis and *x*-axis, respectively (Fig. 2b). When multiple subpopulations of *Synechococcus* were found in the flow cytogram, Yel-B and Yel-G were used as the *y*-axis and *x*-axis, respectively (Fig. 2c), and each subpopulation and its Yel-B:Yel-G ratio were gated and calculated.

To evaluate the $Ex_{495:545}$ of natural *Synechococcus* subpopulations from the Yel-B:Yel-G ratio, a regression equation was created between the fluorescence ratios evaluated with the spectrofluorometer (FP-8500, JASCO) and flow cytometer by measuring the controlled cultures. The fluorescence excitation spectrum at 580 nm of emission wavelength was measured between 470 and 570 nm with a 1 nm interval and 5 nm bandwidth. $Ex_{495:545}$ was calculated using the fluorescence intensities excited at 495 and 545 nm. Three pigment types of *Synechococcus* strains, namely Type 3a, Type 3c, and Type 3d, isolated from Sagami Bay and the Kuroshio region, were used (Table 2). For the measurement, strains in the exponential growth phase were used. Clones of the strains were non-axenically grown in triplicate at 25°C in PCRS11 medium (Rippka et al. 2000) under a light intensity of 30–40 µmol photons m^{-2} s^{-1} with a 12L:12D cycle. Type 3a and Type 3c strains were incubated under green and blue light (Fig. 3), respectively, using light-emitting diodes (LEDs). Type 3d strains were also incubated under green light to express the $Ex_{495:545}$ of Type 3b (Fig. 3). We did not measure the $Ex_{495:545}$ of Type 3d incubated under blue light, as the $Ex_{495:545}$ overlaps with that of Type 3c (Palenik 2001, Lovindeer et al. 2021).

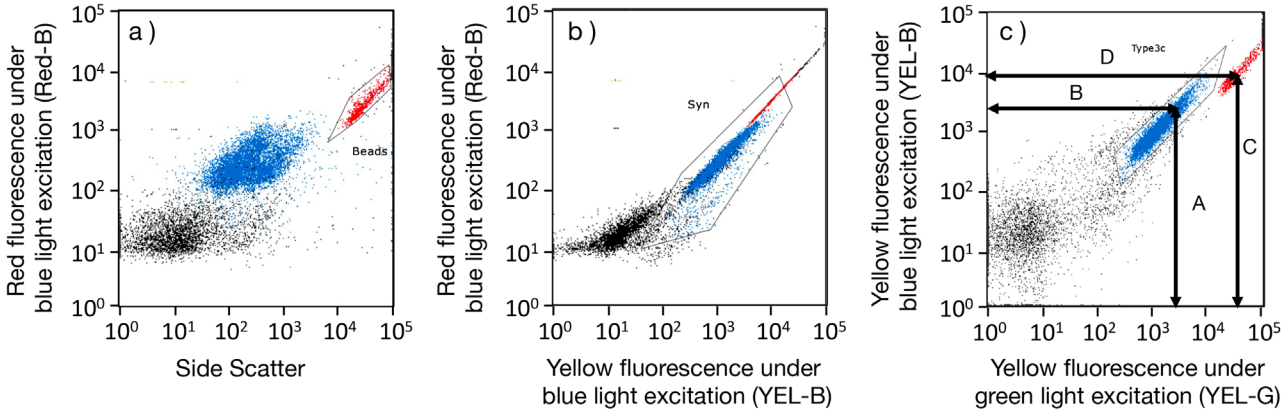

Fig. 2. Gating strategy of the flow cytograms. (a) Red-B and side scatter gating for Nile Red fluorescence beads (red dots). (b) Red-B and Yel-B gating for *Synechococcus* cells (blue dots). (c) Yel-B and Yel-G gating for calculation of Yel-B:Yel-G ratio, (A/C):(B/D), as a proxy of the fluorescence excitation ratio at 495 and 545 nm ($Ex_{495:545}$). Black dots are the debris of *Synechococcus* cells and fluorescent beads

Table 2. *Synechococcus* strains used for creating a linear regression equation and the light conditions during incubation. Dates are given as d/mo/yr. $Ex_{495:545}$: fluorescence excitation ratio at 495 and 545 nm

Strain	Isolation location	Sampling date	$Ex_{495:545}$		Pigment type	Color of LED
			Blue	Green		
Sg1f	Sagami Bay (35.08° N, 139.65° E)	10/05/18	0.43	0.42	3a	Green
Sg1b	Sagami Bay (35.08° N, 139.65° E)	10/05/18	0.4	0.42	3a	Green
Sy1f	Kuroshio region (34.63° N, 140.71° E)	29/05/18	2.11	0.75	3d	Green
Sy1c	Kuroshio region (34.56° N, 141.37° E)	29/05/18	2.36	1.98	3c	Blue
Sy1e	Kuroshio region (34.56° N, 141.37° E)	29/05/18	2.18	2.23	3c	Blue

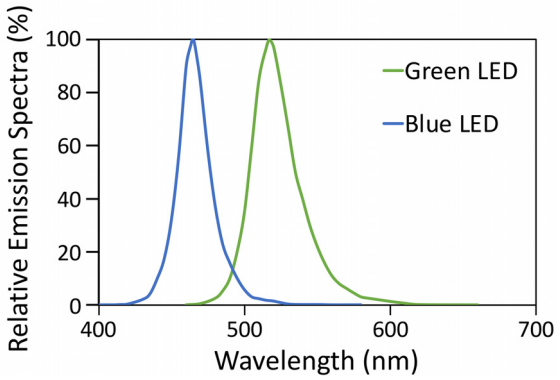

Fig. 3. Emission spectra of the light-emitting diode (LED) lights used for incubation

3. RESULTS

3.1. Linear regression analysis of the PUB:PEB ratio, measured with flow cytometry (Yel-B:Yel-G ratio) against spectrofluorometry ($Ex_{495:545}$)

Synechococcus strains exhibited a variety of excitation spectra and different signals in the flow cytograms of Yel-B and Yel-G (Fig. 4). The $Ex_{495:545}$ and Yel-B:Yel-G ratio of Type 3a strains (Sg1b and Sg1f, n = 6) ranged from 0.35 to 0.39 and 0.48 to 0.58, respectively (Fig. 5). The $Ex_{495:545}$ and Yel-B:Yel-G ratio of Type 3c strains (Sy1c and Sy1e, n = 6) ranged from 1.74 to 1.81 and 3.02 to 3.20, respectively (Fig. 5). The Type 3d strain (Sy1f, n = 3), incubated under green light conditions, exhibited an $Ex_{495:545}$ between 0.68 and 0.70, which was equivalent to the range of Type 3b, and the Yel-B:Yel-G ratio ranged from 1.10 to 1.16 (Fig. 5). A significantly positive linear relationship between the $Ex_{495:545}$ and the Yel-B:Yel-G ratio was obtained by regression analysis ($y = 0.542x + 0.08$, $R^2 = 0.998$, n = 15, $p < 0.001$) (Fig. 5).

3.2. *Synechococcus* abundance enumerated by flow cytometry

The *Synechococcus* cell density at the surface was highest at Stn SJ1, with a total of 1.34×10^5 cells ml^{-1}, and lowest at Stn SJ11, with 0.23×10^4 cells ml^{-1} (Table 3). At Stn SJ11, samples were collected vertically, and the cell density increased slightly with depth ranging from 0.23×10^4 cells ml^{-1} at 5 m to 0.39×10^4 cells ml^{-1} at 81 m, with an exception at 10 m where the density was the lowest (0.18×10^4 cells ml^{-1}) in the water column (Table 3). In the samples collected at the surface along the coastline of Japan — Stns NS, KS, and OS (Fig. 1) — the *Synechococcus* cell

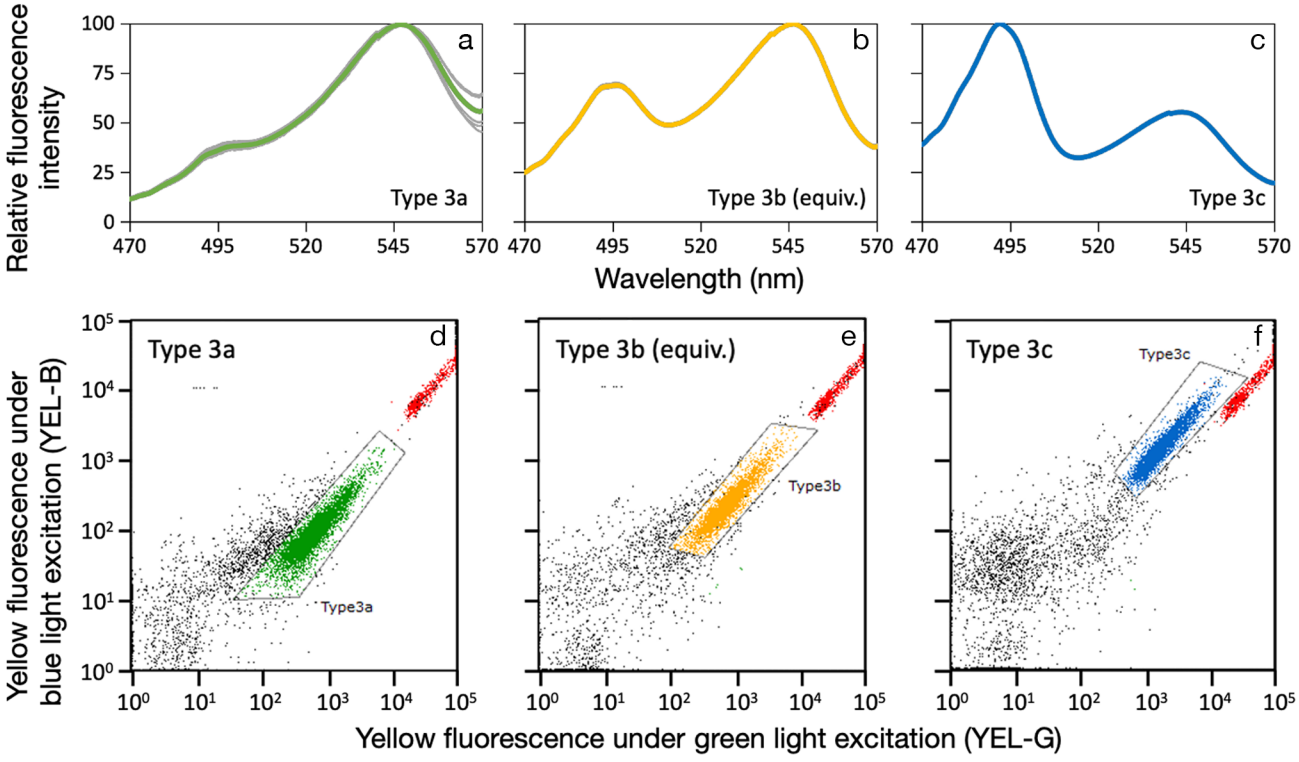

Fig. 4. (a–c) Fluorescence excitation spectra of *Synechococcus* pigment Type 3a, Type 3b (expressed by Type 3d strain), and Type 3c, measured by spectrofluorometry and (d–f) the corresponding flow cytograms. Green lines and dots indicate Type 3a, yellow indicates Type 3b (equiv.), and blue indicates Type 3c. Red dots in panels d–f indicate the Nile Red fluorescence beads

density ranged from 1.42×10^4 to 3.34×10^4 cells ml^{-1} (Table 3). The cell densities of the samples collected at a depth of 5 m at Stns SJ3′ and OC6 were 3.22×10^4 and 5.77×10^4 cells ml^{-1}, respectively (Table 3).

3.3. Natural *Synechococcus* subpopulations detected by dual-laser flow cytometry

Synechococcus from natural seawater was successfully discriminated into multiple subpopulations (Fig. 6). Two subpopulations were detected at Stns SJ3′ and OC6, and at least 3 subpopulations were detected at Stns SJ1, NS, KS, and OS (Fig. 6). Only 1 population was detected at Stn SJ11, the offshore station (Fig. 6).

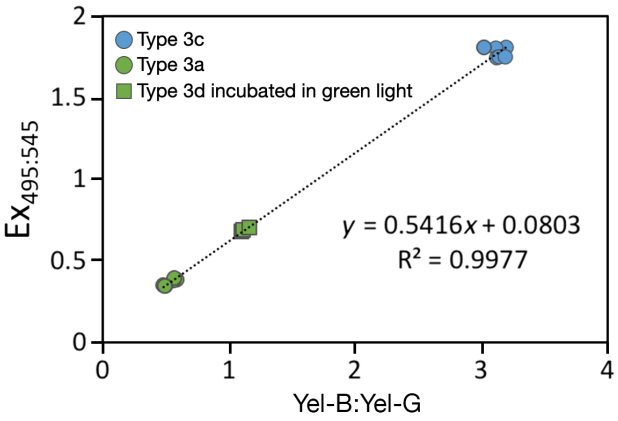

Fig. 5. Correlation between the fluorescence excitation ratio at 495 and 545 nm ($Ex_{495:545}$) and Yel-B:Yel-G ratio of *Synechococcus* cells

3.4. Estimation of the $Ex_{495:545}$ for the natural *Synechococcus* subpopulation by dual-laser flow cytometry

More than 2 subpopulations of *Synechococcus* were frequently detected in the coastal waters of Japan. At Stn SJ1, the $Ex_{495:545}$ values of 3 subpopulations were 1.67, 0.73, and 0.40 (Table 3), which were equivalent to Types 3c, 3b, and 3a, respectively. Two subpopulations occurred at Stns SJ3′ and OC6. The $Ex_{495:545}$ of the subpopulations at Stn SJ3′ (Fig. 6) were 1.54 and 0.37, which corresponded to Type 3b and Type 3a, respectively. Subpopulations at Stn OC6 (Fig. 6) exhibited an $Ex_{495:545}$ of 1.80 and 0.37 (Table 3), corresponding to Type 3c and Type 3a, respectively. The $Ex_{495:545}$ of the single population detected at a depth of 5 m at Stn SJ11 was 1.63 (Table 3), corresponding to Type 3c. Interestingly, the $Ex_{495:545}$ of the *Synechococcus* below 5 m increased with depth from 1.72 to 2.13 (Table 3).

Table 3. Sampling depth, cell concentration of *Synechococcus*, number of subpopulations detected by flow cytometry, the analyzed Yel-B:Yel-G ratio, and the fluorescence excitation ratio at 495 and 545 nm ($Ex_{495:545}$) calculated using the regression equation in Fig. 5. ND: not determined

Stn	Depth (m)	Number of sub-populations	Pigment type	Yel-B:Yel-G	$Ex_{495:545}$	*Synechococcus* cell density (10^4 cells ml^{-1})
SJ1	5	3	3c	2.93	1.67	5.80
			3b	1.20	0.73	3.86
			3a	0.60	0.40	3.70
			Total	–	–	13.36
SJ3′	5	2	3b	2.69	1.54	2.57
			3a	0.53	0.37	0.65
			Total	–	–	3.22
OC6	5	2	3c	3.18	1.80	5.22
			3a	0.54	0.37	0.55
			Total	–	–	5.77
KS	0	3		ND	ND	1.42
NS	0	3		ND	ND	2.99
OS	0	3		ND	ND	3.34
SJ11	5	1	3c	2.87	1.63	0.23
	10			3.03	1.72	0.18
	30			3.28	1.86	0.25
	41			3.45	1.95	0.31
	50			3.44	1.95	0.38
	75			3.74	2.11	0.37
	81			3.78	2.13	0.39

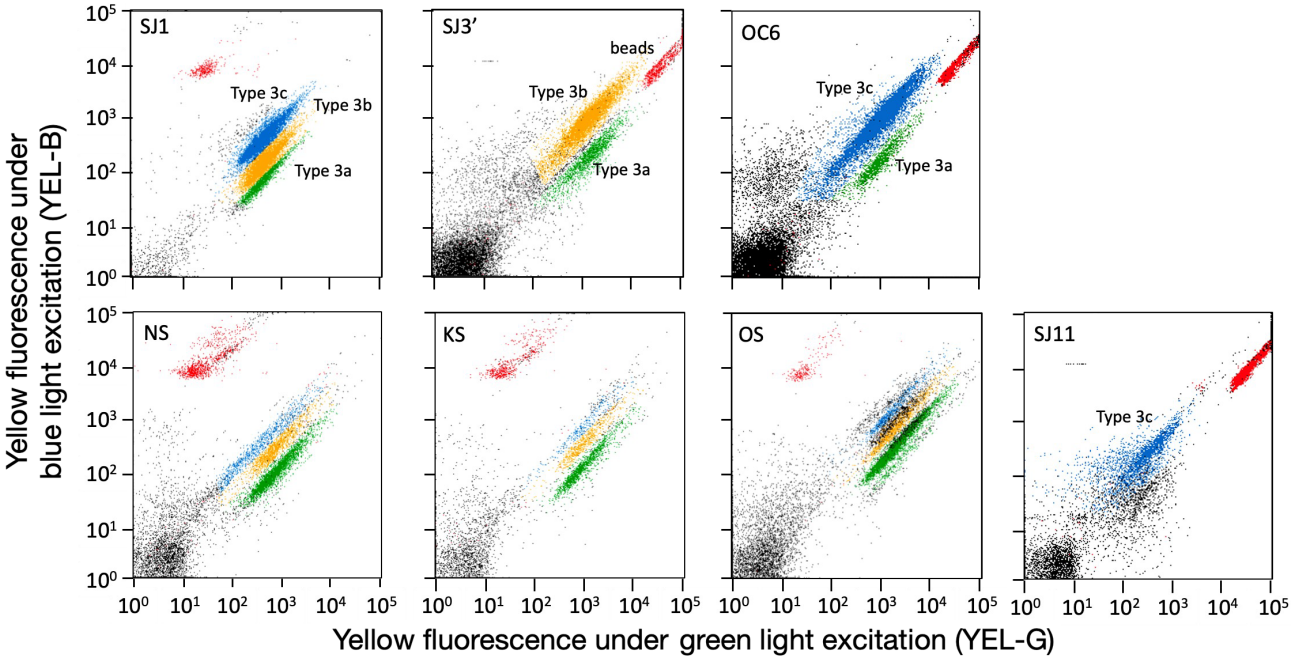

Fig. 6. Flow cytogram of samples collected from Stns SJ1, SJ3′, OC6 at 5 m, SJ11 at 5–81 m and NS, KS, and OS at the surface. Red dots indicate the Nile Red fluorescence beads. Green, yellow, and blue dots indicate Type 3a (equiv.), Type 3b (equiv.), and Type 3c (equiv.), respectively. The fluorescence excitation ratio at 495 and 545 nm ($Ex_{495:545}$) was not evaluated for NS, KS, and OS because appropriate beads were not available for the analysis, thus the coloring in these samples is empirical

4. DISCUSSION

The PUB to PEB ratio ($Ex_{495:545}$), which is used to classify *Synechococcus* into different pigment types (Waterbury et al. 1986, Six et al. 2007), was highly diverse among the natural *Synechococcus* populations (Fig. 6). Differences in the $Ex_{495:545}$ of *Synechococcus* pigment Type 3 may allow them to distribute into various underwater light fields. The discrimination of the subpopulations of *Synechococcus* in natural seawater and the subsequent evaluation of $Ex_{495:545}$ is useful for understanding their ubiquitous distribution.

The discrimination of subpopulations based on the PUB to PEB ratio was first introduced by Olson et al. (1988). However, the subpopulations separated by flow cytometry were not identified by their pigment type; they were only described as Low PUB and High PUB types according to their relative fluorescence properties. In the present study, a regression equation was successfully developed to evaluate the $Ex_{495:545}$ from the fluorescence data. The regression coefficient was significant ($R^2 = 0.998$, $p < 0.001$), allowing us to directly evaluate the $Ex_{495:545}$ of *Synechococcus* subpopulations in their natural environments.

4.1. Horizontal distribution of pigment types

Olson et al. (1988) revealed that Low PUB cells were present only in coastal waters, and the most abundant forms of *Synechococcus* in the open ocean possess a very High PUB, which also extends to greater depths than the Low PUB cells. In the present study, pigment Type 3a, corresponding to the Low PUB type of Olson et al. (1988), was also only detected in samples collected from coastal waters. At Stn SJ11, *Synechococcus* consisted of a single subpopulation of Type 3c, corresponding to the High PUB type. These results are similar to the distribution mentioned in molecular studies (Xia et al. 2017, 2018, Grébert et al. 2018, Zhang et al. 2022), which suggests that the dominant pigment type in the eutrophic coastal waters is Type 3a, while Type 3c is dominant in the oligotrophic open ocean.

Olson et al. (1988) and Katano et al. (2007) reported 2 subpopulations (Low and High PUB types) of *Synechococcus* in coastal waters. In the present study, for the first time, more than 2 subpopulations were detected in 4 samples from coastal waters or the coastline of the Honshu, Japan. From the results of molecular studies, coastal waters contain several *Synechococcus* pigment types, including Type 3a,

Type 3c, and Type 3d, and the recently discovered Type 3f, with a very high content of PUB (Grébert et al. 2018, Zhang et al. 2022). The diversity of the pigment genotypes in coastal waters has been observed using a molecular approach (Xia et al. 2018). However, the phenotypic diversity in pigmentation has not been visualized. At Stn SJ1, multiple subpopulations co-occurred, and their $Ex_{495:545}$ corresponded to those of Type 3a, Type 3c, and/or Type 3b strains. The $Ex_{495:545}$ of Type 3b and Type 3c could also be expressed by the chromatic acclimator of Type 3d. Thus, subpopulations whose $Ex_{495:545}$ correspond to those of Type 3c or Type 3b may have been a mixture of Type 3d acclimating to either blue or green light environments. It would be interesting to further investigate the structure of the *Synechococcus* community in the coastal waters and coastlines and to determine the underlying mechanism of the co-occurrence of different pigment types.

4.2. Increase in the $Ex_{495:545}$ ratio by depth within the range of Type 3c

The $Ex_{495:545}$ of *Synechococcus* cells increased with depth at Stn SJ11 (Table 3). The increase in the PUB to PEB ratio is consistent with the findings of previous studies (Lantoine & Neveux 1997, Wood et al. 1999, Katano et al. 2007). Lantoine & Neveux (1997) and Wood et al. (1999) evaluated $Ex_{495:545}$ of *Synechococcus* using a spectrofluorometer. From their results, the $Ex_{495:545}$ of *Synechococcus* in the eastern Pacific increased from 0.66 at 16 m to 1.08 at 66 m (Wood et al. 1999). Lantoine & Neveux (1997) also reported that the $Ex_{495:545}$ in the mesotrophic area of the Atlantic Ocean was 0.6 in the upper water column but increased to 1.2 below the pycnocline. Katano et al. (2007) calculated the $Ex_{495:545}$ of *Synechococcus* subpopulations using a dual-beam flow cytometer in field samples. The results indicated a slight increase in $Ex_{495:545}$ by depth in the High PUB type between 1.6 and 1.8, which supports our results.

At Stn SJ11, the *Synechococcus* community consisted of only 1 subpopulation, and the $Ex_{495:545}$ increased widely within the range of Type 3c, from 1.60 at 5 m to 2.13 at 81 m. There are 3 possible explanations for this phenomenon. First, the *Synechococcus* community of the water column consisted of subpopulations of Type 3c that have different PUB to PEB ratios for each layer. The $Ex_{495:545}$ varies among *Synechococcus* strains even within the same pigment type. For example, Type 3c strains such as WH8102, CC9605, and Oli31 have $Ex_{495:545}$ ratios of 1.903, 1.999, and 1.774, respectively (Six et al. 2007). Thus, the gradual change in the $Ex_{495:545}$ by depth may have been the niche partitioning within Type 3c *Synechococcus* with the shift in the vertical light regime. This slight difference cannot be detected by metagenomic analysis; therefore, it has never been discussed.

The second possibility is that Type 3d is distributed throughout the water column and expresses different $Ex_{495:545}$ ratios. Many culture experiments exploring the mechanism of chromatic acclimation have been performed under blue or green light (Palenik 2001, Humily et al. 2013, Sanfilippo et al. 2016, Lovindeer et al. 2021, Dufour et al. 2024). Sanfilippo et al. (2016) and Dufour et al. (2024) demonstrated that Type 3d displayed an intermediate $Ex_{495:545}$ under a combination of blue and green lights. These studies support the hypothesis that a single genotype of Type 3d may be distributed in the water column, expressing different $Ex_{495:545}$ to match the blue:green light ratio of the habitat at differing depths. As chromatic-acclimating Type 3d is thought to be the most abundant pigment type of *Synechococcus* spp. in the ocean worldwide, their pigmentation plasticity may be the key process for their ubiquitous distribution (Grébert et al. 2018). The last possibility is that Type 3c and chromatic-acclimating Type 3d may have co-occurred and expressed the same $Ex_{495:545}$.

The present study has revealed the diverse pigmentation of *Synechococcus* in coastal waters using dual-laser flow cytometry. Application of this method with molecular techniques for incubation experiments using natural seawater would provide further information to clarify the mechanism of the chromatic acclimation process in nature.

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