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Application of SYBR Green real-time PCR assay to monitoring of phytoplankton during cultivation and in Lake Biwa

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SYBR Green 系 real-time PCR 法の培養期間及び琵琶湖における藻類モニタリングへの適用

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Abstract

In order to clarify the characteristics of algal dissolved organic matter (DOM) and its contribution to the DOM in Lake Biwa, we developed phytoplankton-monitoring methods using a SYBR Green real-time PCR assay with specific primer sets for each species. The real-time PCR assay was applied to the determination of cell densities of algal species during cultivation and in environmental samples, and analytical results by real-time PCR assay were compared with those by microscopy. The linear relationships between the threshold cycle (Ct) values and cell densities were obtained in the range of $2.7x10^2$-$2.7x10^7$, 8.2x10-$8.2x10^4$, 2.1x10-$2.1x10^4$, and $4.6x10^3$-$4.6x10^5$ cells mL^{-1} for *Microcystis aeruginosa*, *Staurastrum dorsidentiferum*, *Cryptomonas ovata*, and *Fragilaria capucina*, respectively. The PCR efficiency values were 117, 87, 66, and 84% for *M. aeruginosa*, *S. dorsidentiferum*, *C. ovata*, and *F. capucina*, respectively. Furthermore, the effects of the coexistence of other algal species and suspended solids (SS) in lake water were small in the real-time PCR assay.

Keywords: SYBR Green, real-time PCR, phytoplankton, Lake Biwa, dissolved organic matter

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摘要

藻類の溶存有機物質(DOM)の特性と琵琶湖 DOM への寄与を明らかにするため，種特異的なプライマーによる SYBR Green リアルタイム PCR 法を用いる植物プランクトンのモニタリング法を開発した. リアルタイム PCR 法を培養期間及び環境試料の藻類種の細胞密度定量に適用し, 顕微鏡による直接計数法と比較した．threshold cycle (Ct) 値と細胞密度の間には，*M. aeruginosa*，*S. dorsidentiferum*，*C. ovata* 及び *F. capucina* について，それぞれ細胞密度，$2.7x10^2$-$2.7x10^7$，8.2x10-$8.2x10^4$，2.1x10-$2.1x10^4$ 及び $4.6x10^3$-$4.6x10^5$ cells mL^{-1} の範囲で直線性が得られ，この細胞密度範囲での定量性が示された．PCR 効率値は，*M. aeruginosa*，*S. dorsidentiferum*，*C. ovata* 及び *F. capucina* に対し，それぞれ 117, 87, 66 及び 84% であった．さらに，リアルタイム PCR 法では，他の藻類や湖水に含まれる浮遊物質(SS)などの影響は小さかった.

キーワード: SYBR Green，リアルタイム PCR，植物プランクトン，琵琶湖，溶存有機物質

Introduction

Lake Biwa is an irreplaceable water source not only for tap water but also for industry, agriculture, and fishery. As the chemical oxygen demand (COD) in the northern basin of Lake Biwa has increased since 1984 in spite of a decrease in organic loadings from the watershed, refractory dissolved organic matter (DOM), which is not easily decomposed by microorganisms, may have gradually accumulated in Lake Biwa (Aoki et al., 2004; Hayakawa and Okamoto, 2012). Okamoto and Hayakawa (2011) reported that the sources of refractory DOM depend on both inner production in Lake Biwa (77.3%) and pedogenic DOM from the watershed (22.7%). Ichise et al. (2013) analyzed the long-term variation of phytoplankton biovolume and gelatinous sheath (extracellular polysaccharides) volume in Lake Biwa from 1980 to 2009 and reported that the large amount of extracellular polysaccharides produced by phytoplankton could be the major sources of organic matter in Lake Biwa. Furthermore, it is reported that the increase of refractory DOM may be attributed to the contribution of not only humic substances from soils around the rivers flowing into Lake Biwa but also algal DOM from phytoplankton in the lake (Aoki et al., 2004, 2008a, 2008b; Yamada et al., 2012a, 2012b). In order to clarify the influence of inner production by phytoplankton on the refractory DOM in Lake Biwa, it is necessary to identify algal species in Lake Biwa because the characteristics of algal DOM differ according to the algal species (Aoki et al., 2008a, 2008b; Yamada et al., 2012b).

Microscopic analysis is the most popular method for the identification and quantification of phytoplankton species. However, microscopic analysis is unsuitable for accurate, wide-range monitoring because it is difficult to distinguish phytoplankton from each other. Furthermore, phytoplankton in low concentration cannot be detected by microscopy (Kamikawa et al., 2007; Tomioka et al., 2008). Then, a rapid and versatile real-time PCR (polymerase chain reaction) assay using a small subunit of the ribosomal RNA gene (SSU rDNA) was investigated (Ishikawa et al., 2005; Kamikawa et al., 2006; Tomioka et al., 2008; Yoshida et al., 2003). To date, the real-time PCR method has been applied to the identification and quantification of harmful algae to monitor red tide and water bloom (Ishikawa et al., 2005; Kamikawa et al., 2006; Tomioka et al., 2008; Yoshida et al., 2003; Koike et al., 2007).

In this study, in order to clarify the characteristics of algal DOM and its contribution to the DOM in Lake Biwa, we developed phytoplankton-monitoring methods using a real-time PCR assay with specific primer sets for each species. The real-time PCR assay was applied to the determination of cell densities of algal species during cultivation and in environmental samples, and analytical results by real-time PCR assay were compared with those by microscopy.

Materials and methods

Field sample collection

Lake water and phytoplankton samples were collected monthly at Imazu (St. 17B, 35°23’41 N, 136°07’57 E) in the northern basin of Lake Biwa (Fig. 1) from January 2016 to

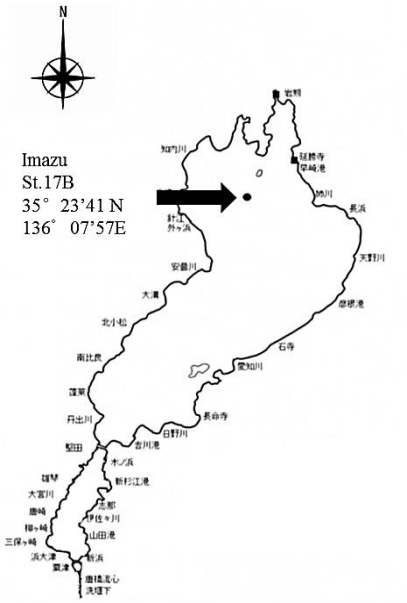

Fig. 1. Sampling station (St. 17B) in Lake Biwa.
図 1. 琵琶湖におけるサンプリング地点(St. 17B).

July 2017. Lake water samples were taken at various water depths. Phytoplankton samples (St. 17B, water depth 0–20 m) were collected using a plankton net (mesh 25 μm, φ20 cm, Rigo-sha, Japan) and stored at -80℃ until DNA extraction. Their cells were counted under a microscope (IX71N-22PH-D, Olympus Japan).

Cultivation of phytoplankton and preparation of their standards

Four kinds of phytoplankton, which were supplied by the National Institute for Environmental Studies, were used. *Microcystis aeruginosa* (NIES-109, Lake Yogo, Shiga), *Staurastrum dorsidentiferum* (NIES-665, Lake Biwa, Shiga), and *Cryptomonas ovata* (NIES-275, Tsuchiura, Ibaraki) were cultivated in an improved VT medium in accordance with the procedures of previous papers (Aoki et al., 2008a, 2008b). *Fragilaria capucina* (NIES-391, Lake Kasumigaura, Ibaraki) was cultivated in 1 L triangle bottles at 15℃ and 2000 lux under a 12h:12h light/dark cycle in an improved VT+Si medium (10 mg of Na_2SiO_3 was added to 100 mL of improved VT medium). These phytoplankton were selected as the predominant algal species in Lake Biwa (Kishimoto 2015, Aoki et al., 2008b).

The standards of four phytoplankton for real-time PCR assay were prepared. The cells of each species at the stationary phase were directly counted using a microscope, and the cell densities of the standards were prepared with the serial dilutions of each cells with Milli-Q water in the ranges of 2.7-2.7x10^7, 8.2-8.2x10^4, 2.1-2.1x10^4, and 4.6x10-4.6x10^5 cells mL^{-1} for *M. aeruginosa*, *S. dorsidentiferum*, *C. ovata*, and *F. capucina*, respectively.

DNA extraction

The cells in about 2 mL of a sample were disrupted at 2500 rpm for 30 s using a Mini-Beadbeater (BioSpec Products, USA). Subsequently, 1 mL of the supernatant was transferred to a 15 mL centrifuge tube and freeze-dried using a freeze dryer (FDU-2200, EYELA, Japan). The lyophilized samples were subjected to the total DNA extraction using a DNeasy Plant Mini Kit (Qiagen, Japan) in accordance with the manufacturer's protocol. The extracted DNA was stored at -20℃ until PCR assay.

Detection of algal cells using real-time PCR

For the identification of algal species, the primer sets used were designed by Primer3Plus based on 16S rDNA of *M. aeruginosa* and 18S rDNA of *S. dorsidentiferum*, *C. ovata*, and *F. capucina* by reference to Ishikawa's method (Ishikawa, 2010). The primer sets used in this study are listed in Table 1.

For PCR amplification and detection, thermal cycling was performed using a real-time PCR detection system (CFX96, Bio-Rad, USA) in a 0.2 mL PCR8-strip tube (Bio-Rad). PCR was carried out in 25 μL volumes comprised of 12.5 μL of SYBR *Premix EX Taq* (Takara Bio Inc., Japan), 1 μL each of the forward and reverse primers (each 50 μM), 2 μL of the extracted DNA sample, and 8.5 μL of pure water. Thermal conditions were as follows: initial heat denaturation at 95℃ for 3 min, 45 cycles of repeated thermal denaturation at 95℃ for 2 s, annealing at 58℃ for 5 s, extension reaction at 78℃ for 5 s, and finally, extension reaction at 78℃ for 1 min. Melting curve analysis was performed by continuous measurement of fluorescence during heating from 60℃ to 95℃. Threshold cycle (Ct) values were determined with the fit point method (500 RFU) by CFX manager ™ software. Each measurement was performed in triplicate. Furthermore, PCR products were analyzed via 1.5% agarose gel electrophoresis (Agarose S for electrophoresis, Nippon Gene, Japan).

Table 1. Primer list in this study.
表 1. 本研究で用いたプライマー.

Class	Species	Primer name	Sequences (5'-3')	Position (5'-3')	Product length (bp)	GenBank Number
Cyanobacteria	*M. aeruginosa*	Micro 2F	ATGAGCAGCCACACTGGGAC	252-271	275	FJ461750
		Micro 2R	AGACTTGGCTGACCACCTGC	507-526		
Chlorophyceae	*S. dorsidentiferum*	STAU 1F	GGTCTGCCTACCGGTTGATAC	610-630	195	LC037445
		STAU 1R	GGTCCCGAAGACCAACACAA	785-804		
Cryptophyceae	*C. ovata*	Crypto 1F	AAGCAGGCTGTTGCTTGAAT	638-657	172	AB240952
		Crypto 1R	TGCTTTCGCACAAGTTCATC	790-809		
Bacillariophyceae	*F. capucina*	Frag 2F	GGGCCTTTACAGGTCTGGCA	426-445	167	LC037435
		Frag 2R	ACGGCCCATCCACAAATCCA	573-592		

Characterization of DOM from Lake Biwa and from four phytoplankton during cultivation

Lake water samples and algal samples during cultivation were filtered through 0.45 μm Millipore filters immediately after collection and analyzed. Dissolved organic carbon (DOC) was measured using a TOC meter (TOC-V_{CSH}, Shimadzu, Japan). The fluorescence properties of DOM were measured with 3D-EEM using a fluorescence spectrophotometer (RF-5300PC, Shimadzu, Japan), as previously reported (Aoki et al., 2008a, 2008b). Fluorescence readings were normalized by fluorescence intensity (Ex=345 nm/Em=450 nm) of 10 μgl^{-1} quinine sulfate (0.05 M H_2SO_4 solution) 10 QSU. The values were treated as relative fluorescence intensity (RFI). Dando fulvic acid (FA) from the A-horizons of brown forest soil (Dystric Cambisol, Dando, Aichi, Japan) (Watanabe et al., 1994) was used as a standard of FA. A Horiba F-51 pH meter and a TOA CM-60S EC meter were used for the pH and electric conductivity, respectively. All other chemicals were of the best commercial grade. Pure water was prepared using a Millipore Milli-Q water purification system.

Results and discussion

Effects of phytoplankton diversity on the characterization of DOM in the northern basin of Lake Biwa

In the water samples of Lake Biwa, two fulvic-like fluorescence peaks, peak A (Ex/Em = 320–350/430–450 nm) and peak B (Ex/Em = 240–260/430–450 nm), and a protein-like fluorescence peak, peak C (Ex/Em = 280–290/320–330 nm), were always observed by 3D-EEM (Fig.

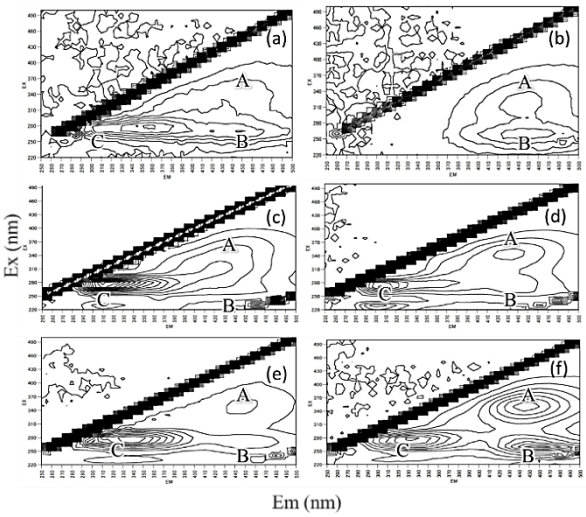

Fig. 2. 3D-EEM contour plots.
(a) surface water of Lake Biwa (St. 17B, water depth10m, April 2016), (b) soil FA (Dando FA), (c) *M. aeruginosa* cultivated during 79days, (d) *S. dorsidentiferum* cultivated during 79days, (e) *C. ovata* cultivated during 71days, (f) *F. capucina* cultivated during 70days.
図 2. 3D-EEM 等高線図.
(a) 琵琶湖表層水, (b) 土壌フルボ酸 (Dando FA), 藻類由来DOM: (c) *M. aeruginosa*, (d) *S. dorsidentiferum*, (e) *C. ovata*, (f) *F. capucina*

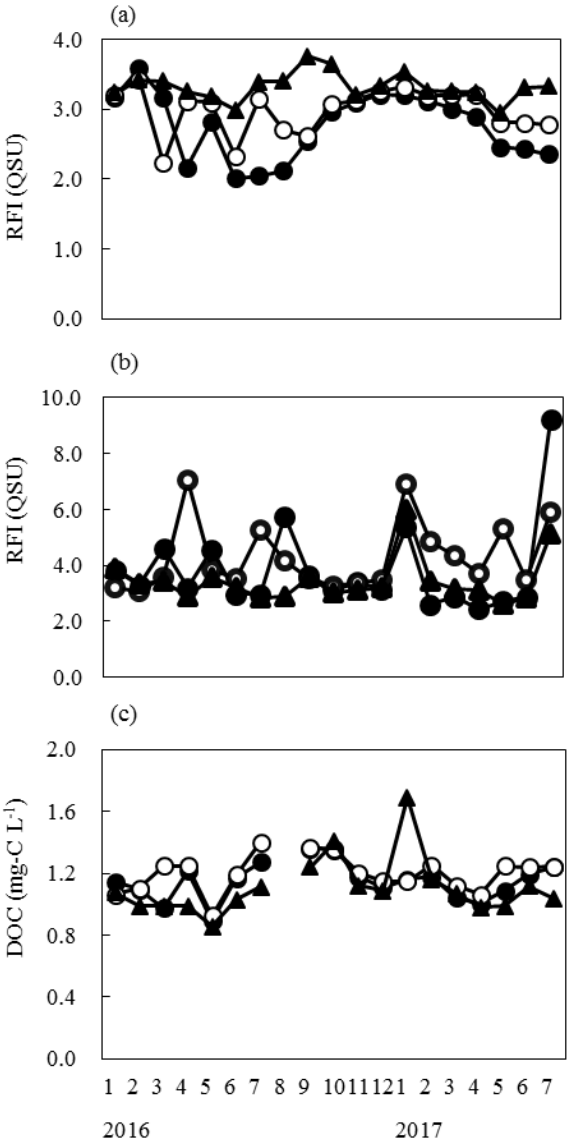

Fig. 3. Monthly changes in the fluorescence intensities of peaks A (a) and C (b), and the DOC concentrations (c) in the northern basin of Lake Biwa (St. 17B).

Water depth: ●, 0.5 m; ○, 10 m; ▲, 20 m

図 3. 琵琶湖北湖 (St.17B) におけるピーク A (a)とピーク C (b) の蛍光強度と DOC 濃度 (c) の月変化.

2(a)). These three fluorescence maxima were also observed in algal DOM released from four kinds of phytoplankton during the cultivation (Fig. 2 (c)-(f)), but peak C is not detected in soil humic substances (Fig. 2 (b)).

The monthly changes in the relative fluorescence intensity (RFI) values of peak A and peak C and the DOC at water depths of 0.5, 10, and 20 m at St. 17B in the northern basin of Lake Biwa are shown in Fig. 3 (a), (b), and (c), respectively.

The RFI values of peak A at water depths of 0.5 m, 10 m, and 20 m were 2.02–3.59 QSU, 2.23–3.43 QSU, and 2.95–3.76 QSU, respectively. The RFI values of peak A (peak B) of surface waters (water depths 0.5 and 10 m) tended to be low during the stratified period. The decrease in the fluorescence of fulvic-like fluorophores in the surface waters of Lake Biwa in the summer may be due to the fluorescence quenching by solar irradiation (Ueda et al., 2016). On the other hand, the RFI values of peak C of surface waters were high, and their variations were larger than those of peaks A and B. The RFI values of peak C were especially high (>5 QSU) in January and July 2017 at water depths of 0.5, 10, and 20 m; high values were also observed in August 2016 at a water depth of 0.5 m and in April and July 2016 and May 2017 at a water

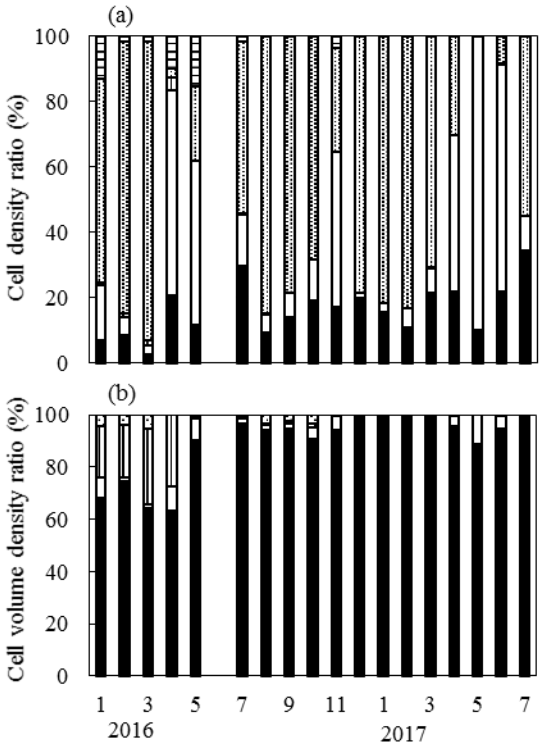

Fig. 4. Monthly changes in the ratios of cell density (a) and cell volume density (b) of phytoplankton species in the northern basin of Lake Biwa (St. 17B).

■: Chlorophyceae; □: Bacillariophyceae;

▥: Dinophyceae; ⊡: Cyanobacteria

図 4. 琵琶湖北湖 (St. 17B) における植物プランクトン種の細胞密度比 (a) と細胞体積比 (b) の月変化.

depth of 10 m. In January 2017, the DOC concentration at a water depth of 20 m was also high (1.69 mg L^{-1}). Monthly changes in the ratios of cell density and cell volume density of phytoplankton species at St. 17B from January 2016 to July 2017 are shown in Fig. 4. The dominant species were bacillariophyceae in April, May, and November 2016 and April–June 2017, and they were cyanobacteria in other months. The ratios of cell volume density of chlorophyceae were 60–70% from January to April 2016, increased to over 90% after May 2016, and were close to 100% in January and July 2017. Furthermore, as the unusual occurrence of *Micrasterious* spp. was observed from November 2016 to April 2017 at St. 17B, the change of algal species may affect the increase of DOC in January 2017 and the protein-like fluorophore (peak C) from January to May 2017. Chlorophyll a is known to be a major chlorophyll of most phytoplankton, such as cyanobacteria and chlorophyceae, and chlorophyll c is a major chlorophyll of the bacillariophyceae species (Jefferey et al., 1997; Kobayashi et al., 2012). Chlorophyll a and chlorophyll c were reported to be high in June and November 2016 and in August 2016, respectively (Shiga Prefecture, 2016).

These results suggest that protein-like fluorescence DOM in Lake Biwa is associated to the production by phytoplankton. Therefore, the monitoring methods of phytoplankton cells in Lake Biwa were examined using real-time PCR assay, and the results were compared with the results by microscopy.

Specificity of primer sets and linearity of real-time PCR

The specificity of designed primer sets was confirmed, and the primer sets were specific for each species in the real-time PCR assay (Table 1). The amplification curves and melting curves of target phytoplankton only were similar to those in the presence of other species. Correlations between the Ct values and the number of cultured cells of *M. aeruginosa* in the absence and presence of *S. dorsidentiferum* and *C. ovata* are shown in Fig. 5. Both calibration curves were similar regardless of the coexistence of other algal species in the case of not only *M. aeruginosa* but also three other species. The linear relationships between the Ct values and cell densities of *M. aeruginosa*, *S. dorsidentiferum*, *C. ovata*, and *F. capucina* were obtained in the range of $2.7x10^2$-$2.7x10^7$ cells mL^{-1} {y = -2.98x + 39.9 (r^2 = 0.957)}, 8.2x10-$8.2x10^4$ cells mL^{-1}{y = -3.69x + 37.7 (r^2 = 0.997)}, 2.1x10-$2.1x10^4$ cells mL^{-1}{y = -4.56x + 40.2 (r^2 = 0.996)}, and $4.6x10^3$-$4.6x10^5$ cells mL^{-1} {y = -3.78x + 45.8 (r^2 = 0.996)}, respectively. The PCR

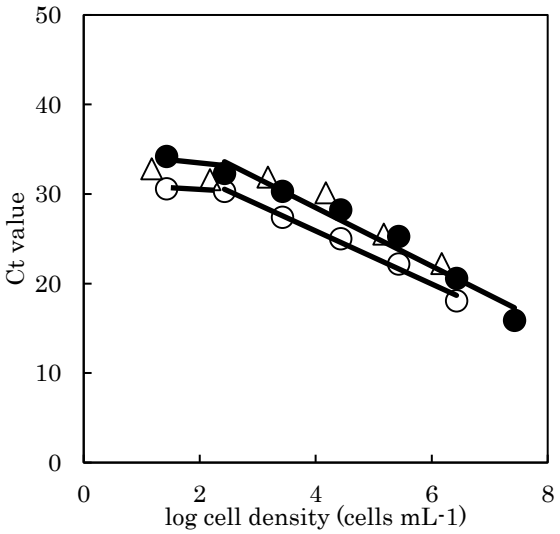

Fig. 5. Correlations between the Ct values and the cell densities of *Microcystis aeruginosa*.
dilution with pure water: ●, *M. aeruginosa* only;
○, coexistence of *S. dorsidentiferum* and *C. ovata*, dilution with water sample of Lake Biwa: △, *M. aeruginosa* only

図 5. *Microcystis aeruginosa* の Ct 値と細胞密度の関係
純水希釈系．●, *M. aeruginosa* 単独;
○, *S. dorsidentiferum* と *C. ovata* が共存する場合,
琵琶湖水希釈系: △, *M. aeruginosa* 単独

efficiency values were 117, 87, 66, and 84% for *M. aeruginosa*, *S. dorsidentiferum*, *C. ovata*, and *F. capucina*, respectively. Moreover, similar results were obtained when the cultured cells of phytoplankton were diluted with water samples of Lake Biwa instead of pure water. The results of *M. aeruginosa* are shown in Fig. 5. From these results, it was found that the effects of the coexistence of other algal species and suspended solid (SS) in lake water are small in the real-time PCR assay. Furthermore, the PCR products of four algal species were analyzed via agarose gel electrophoresis. A specific band was detected from these isolates. The

Table 2. Melting point and length of PCR product.
表 2. PCR 産物の融解点と産物長.

Species	Melting point (℃)	Product length (bp)
M. aeruginosa	86.5	275
S. dorsidentiferum	85	195
C. ovata	81	172
F. capucina	83	167

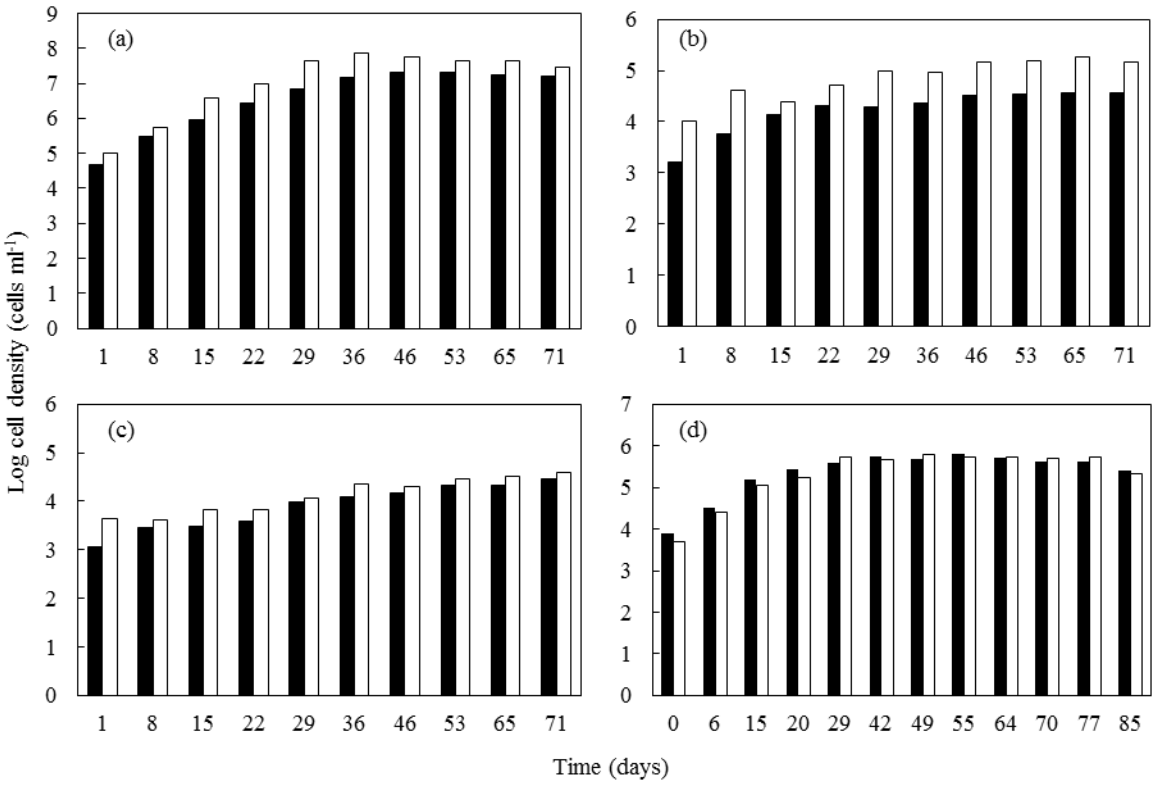

Fig. 6. Comparison of the cell densities of four phytoplankton using a real-time PCR assay (open bar) with those obtained by direct counting (closed bar) during cultivation.

(a) *M. aeruginosa*, (b) *S. dorsidentiferum*, (c) *C. ovata*, (d) *F. capucina*

図 6. 培養期間における 4 種の植物プランクトンの細胞密度の real-time PCR 法（白棒グラフ）と直接計数法（黒棒グラフ）との比較.

melting points and product length of the PCR products are listed in Table 2. These values of target species only were similar to those obtained in the presence of other algal species and in lake water.

These results indicate that the real-time PCR assay may be useful for specific monitoring of phytoplankton.

Time changes in the characterization of algal DOM during the cultivation of four kinds of phytoplankton

Time changes in the characterization of algal DOM during the cultivation of four kinds of phytoplankton were examined. The algal cell densities in various growth phases were determined by direct counting under a microscope and with real-time PCR assay. The growth curves of four kinds of phytoplankton during cultivation using both methods are shown in Fig. 6 (a)–(d). From the lag phase to the stationary phase, the cell densities estimated by real-time PCR assay agreed relatively well with those obtained by direct counting, even though the cell densities of *M. aeruginosa*, *S. dorsidentiferum*, and *C. ovata* by real-time PCR assay were slightly higher than those by direct counting.

Next, the time changes in the RFI values of peak A and peak C and the DOC concentrations of four phytoplankton are shown in Fig. 7 (a)–(d). Peaks A and C and the DOC in *M. aeruginosa* and *C. ovata* became larger as its cell density increased at the stationary phase. The RFI value of peak C and the DOC concentration in *F. capucina* decreased until day 29 of incubation and increased after that, while peak A increased gradually at the stationary phase. The RFI values of peaks A and C and the DOC of *S. dorsidentiferum* were low compared with those in other phytoplankton, which were coincident with the results as reported in our previous paper (Aoki et al., 2008b).

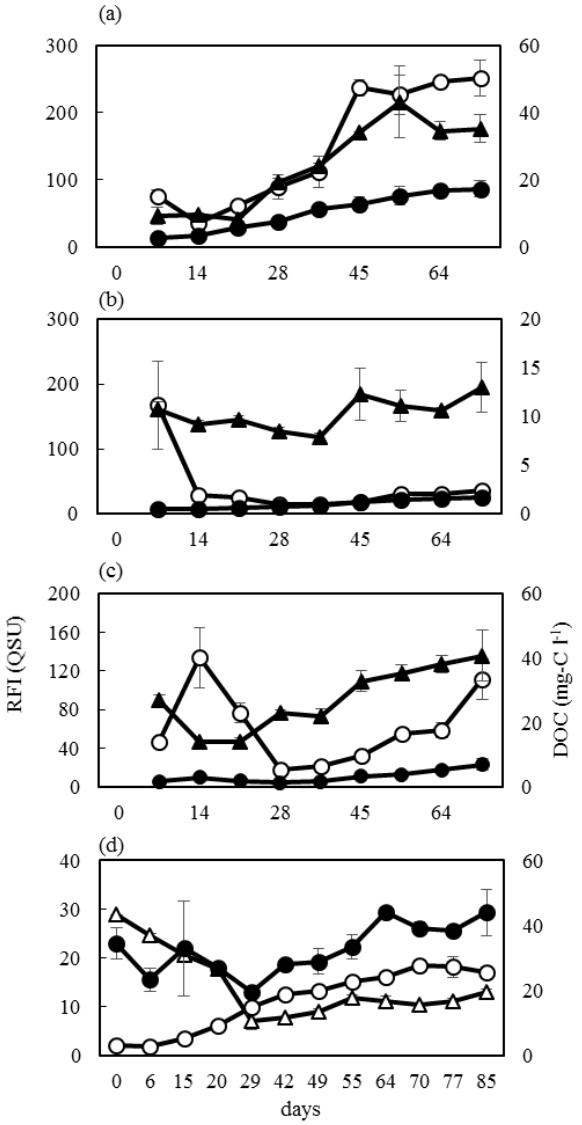

Fig. 7. Time changes in the fluorescence intensities of peaks A and C, and the DOC concentrations of four phytoplankton during cultivation.

●: Peak A; ○: Peak C; ▲: DOC

(a) *M. aeruginosa*, (b) *S. dorsidentiferum*, (c) *C. ovata*, (d) *F. capucina*.

図 7. 培養期間における 4 種の植物プランクトンのピーク A (a)とピーク C (b) の蛍光強度と DOC 濃度 (c) の時間変化.

Application of the real-time PCR assay to environmental samples

The real-time PCR assay was applied to the determination of cell densities of algal species in the plankton net samples collected in October 2015 and January 2016 at St. 17B (water depth 0–20 m) in the northern basin of Lake Biwa, and analytical results by real-time PCR assay were compared with those by direct counting (Table 3). The cell density of *Microcystis* spp. by real-time PCR assay was almost four times greater than that by direct counting. This may be due to counting loss because of the colony formation and small size of *Microcystis* spp. Tomioka et al. (2008) also reported that as cell counting by microscopy is likely to underestimate the abundance of *Microcystis*, real-time PCR is much more appropriate to monitor the abundance of *Microcystis* than is cell counting by microscopy. Meanwhile, the cell density of *Staurastrum* spp. by direct counting was nearly three times greater than that by real-time PCR assay. This is probably attributed to miscounting, since *Xanthidium* spp. are morphologically similar to *Staurastrum* spp. Thus, the difference between the results obtained by real-time PCR and microscopy in Lake Biwa need further careful examination. *Cryptomonas* spp. were hardly detected, and the cell densities of *Fragilaria* spp. were similar according to both methods.

However, the method developed here cannot apply to the quantification of phytoplankton in water samples at St. 17B in the northern basin of Lake Biwa because the cell densities of phytoplankton are too low. Then, the real-time PCR assay with a DNA extraction from trapped cells onto a filter through the filtration of lake water sample was examined, and the results showed sufficient sensitivity for the quantification of *Microcystis* spp., *Staurastrum* spp. and *Fragilaria* spp. in the water samples of Lake Biwa (Fujii et al., 2017a, Fujii et al., 2017b). Then, we are under study for the seasonal changes

Table 3. Analytical results of cell densities of algal species in the plankton net samples collected at St. 17B in the northern basin of Lake Biwa by real-time PCR assay and by direct counting.

表 3. real-time PCR と直接計数法による琵琶湖北湖（St. 17B）で採取したプランクトンネット試料中藻類の細胞密度の分析結果.

Species name	Direct counting (cells mL^{-1})	real-time PCR (cells mL^{-1})
Microcystis spp.※	26080	111100
Staurastrum spp. ※	1600	547
Cryptomonas spp. ※	0	84
Fragilaria spp. ※※	3120	3498

Sampling: ※October 2015, ※※ January 2016

in the phytoplankton species in Lake Biwa using this real-time PCR assay, and the effects of phytoplankton on the DOM in Lake Biwa are further analyzed.

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