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# Monitoring of Phytoplankton Species in Lake Biwa Using a Real-Time PCR Assay with DNA Extraction from Cells Trapped by Filtering a Lake-Water Sample

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ろ紙法による DNA 抽出とリアルタイム PCR を用いる琵琶湖水中藻類モニタリング

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**Abstract**

It is important to identify algal species in Lake Biwa in order to clarify the influence of inner production by phytoplankton on the refractory dissolved organic matter (DOM). In our previous study, phytoplankton-monitoring methods using a SYBR Green real-time PCR assay with specific primer sets for each species after DNA extraction using a DNeasy Plant Mini Kit (DNeasy method) were applied to the determination of the cell densities of algal species during cultivation and in phytoplankton samples collected using a plankton net. However, this real-time PCR assay cannot be applied to the quantification of phytoplankton in water samples in the northern basin of Lake Biwa because the cell densities of phytoplankton are too low. Thus, in this study, a real-time PCR assay with a DNA extraction from cells trapped by filtering a lake-water sample (filter method) was developed and applied to the determination of vertical distributions and seasonal changes in algal species in the northern basin of Lake Biwa (St. 17B). The results suggest that the increase of DOM and protein-like fluorophores at St. 17B from 2017 to 2018 is associated with the increase of *Fragilaria* spp., and Chlorophyceae such as *Staurastrum* spp. and *Micrasterious* spp.

**Keywords:** Phytoplankton, DNA extraction, Filter method, Real-time PCR, Lake Biwa

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**摘要**

琵琶湖における難分解性溶存有機物質(DOM)への植物プランクトンによる内部生産の影響を明らかにするためには，藻類種の同定が重要である．前報では, DNeasy Plant Mini Kit による DNA 抽出 (DNeasy 法)と種特異的なプライマーによる SYBR Green リアルタイム PCR 法を用いる藻類モニタリング法を，培養期間及びプランクトンネットを用い採取した藻類試料中藻類種の細胞密度定量に適用した．しかし，このリアルタイム PCR 法は，細胞密度の低い琵琶湖北湖の湖水試料の植物プランクトンの定量には適用できない．そこで，本研究では，ろ紙上に捕集した植物プランクトンから DNA 抽出する方法（フィルター法）とリアルタイム PCR 分析を用いる藻類モニタリング法を開発し，琵琶湖北湖(St. 17B)における藻類種の鉛直分布や季節変化などの定量に適用した．その結果は, 2017-2018 年の St. 17B における DOM とタンパク質様蛍光物質の増加が, *Fragilaria* spp. 及び, *Staurastrum* spp. と *Micrasterious* spp.などの緑藻類の増加と関連があることを示している．

**キーワード**: 植物プランクトン, DNA 抽出，フィルター法，リアルタイム PCR，琵琶湖

## Introduction

Lake Biwa is the largest freshwater lake in Japan, and the most important resource in the Kinki region (population 14.0 million). However, refractory dissolved organic matter (DOM) may have gradually accumulated in Lake Biwa (Hayakawa and Okamoto, 2012). It has been reported that the increase in 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 (Imai et al., 1998; Ichise et al., 2013; Aoki et al., 2004, 2008a, b; Ohara et al., 2009). Furthermore, it has been clarified that humic substances might be regularly released from lake sediment (Yamada et al., 2012a; Tsuda, 2017). 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 the lake, because the characteristics of algal DOM differ according to the algal species (Aoki et al., 2008a, b; Yamada et al., 2012a, b).

Recently, real-time polymerase chain reaction (PCR) assay were applied to the identification and quantification of harmful algae to monitor red tide and water bloom (Ishikawa et al., 2005; Kamikawa et al., 2006, 2007; Tomioka, et al. 2008; Yoshida et al., 2003). Therefore, in our previous study (Mizuguchi et al., 2018), phytoplankton-monitoring methods using a SYBR Green real-time PCR assay with specific primer sets for each species after DNA extraction using a DNeasy Plant Mini Kit (DNeasy method) were applied to the determination of the cell densities of algal species during cultivation and in phytoplankton samples collected using a plankton net. However, this method cannot be applied to the quantification of phytoplankton in water samples in the northern basin of Lake Biwa because the cell densities of phytoplankton are too low. The DNA extraction method from filter-collected cells has been reported (Fuhrman et al., 1988; Riemann and Winding, 2001), and Koike et al. (2007) applied a real-time PCR assay combined with this modified extraction method to the monitoring of Symbiodinium in field waters.

In this study, the real-time PCR assay with a DNA extraction from cells trapped by filtering a lake-water sample (filter method) was used instead of the DNeasy method, and the results showed sufficient sensitivity for the quantification of *Microcystis* spp., *Staurastrum* spp., and *Fragilaria* spp. in the water samples of Lake Biwa. Therefore, this method was applied to the determination of vertical distributions and seasonal changes in phytoplankton species in the northern basin of Lake Biwa. Furthermore, the effects of phytoplankton species on the DOM and fluorophores in Lake Biwa were analyzed.

## Materials and methods

### Sample collection and pretreatment of samples until DNA extraction

The water and phytoplankton samples from Lake Biwa were collected at St. 17B in the northern basin (Imazu 35°23’41 N, 136°07’57 E). Lake-water samples were taken at various water depths. Phytoplankton samples (water depth 0–20 m) were collected using a plankton net (mesh 25 μm, φ20 cm; Rigo-sha, Japan). Then 1 L of lake water was passed

through a membrane filter (mixtures of cellulose acetate and cellulose nitrate, pore size: 0.45 μm, φ: 47 mm; Millipore) under a vacuum, and the filters were stored at -20℃ until DNA extraction from cells trapped on the filter. Meanwhile, 1 mL of a phytoplankton sample was filtered through a membrane filter, and the filters were also stored at -20℃. The cells of plankton net samples were counted under a microscope (IX71N-22PH-D, Olympus, Japan).

**Cultivation of phytoplankton and preparation of their standards**

Three kinds of phytoplankton, *Microcystis aeruginosa* (NIES-665, Lake Biwa, Shiga), *Staurastrum dorsidentiferum* (NIES-275, Lake Biwa, Shiga), and *Fragilaria capucina* (NIES-391, Lake Kasumigaura, Ibaraki), were cultivated according to the procedures outlined in previous papers (Aoki et al., 2008a, b; Mizuguchi et al., 2018).

The standards of three phytoplankton were prepared. The cells of each species at the stationary phase were counted using a microscope, and the cell densities of the standards were prepared with the serial dilutions of each cell with Milli-Q water in the ranges of 10 to $10^7$, 10 to $10^5$, and 3.7 to $3.7x10^5$ cells $mL^{-1}$ for *M. aeruginosa*, *S. dorsidentiferum*, and *F. capucina*, respectively. One milliliter of each standard was passed through a membrane filter, and these filters were stored at -20℃ until DNA extraction.

**DNA extraction (filter method)**

Each of the filters with trapped phytoplankton cells was stored at -20℃ for 24 h, thawed at room temperature, and immersed in 5 mL of TE buffer (Tris-HCL 10 mM, EDTA 1 mM, pH 8.0) in a 15 mL centrifuge tube. To make 100 mL of TE buffer, 1 mL of 1 M Tris base (pH 8), 0.2 mL of 0.5 M EDTA solution and 98.8 mL of Milli-Q water were mixed. The tubes were then placed in a heat block (WSC-2620, ATTO, Japan) and heated at 100℃ with an intermittent vortex (for 30 s, three times) for 1 h. Aliquots of 1 mL were taken from the DNA extracts in each 1.5 mL centrifuge tube, and the tubes were stored at -80℃ until real-time PCR assay. Each DNA extraction was performed in triplicate.

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

| Species name | Primer name | Sequences (5'-3') |
|---|---|---|
| *M. aeruginosa* | Micro 2F | ATGAGCAGCCACACTGGGAC |
| | Micro 2R | AGACTTGGCTGACCACCTGC |
| *S. dorsidentiferum* | STAU 1F | GGTCTGCCTACCGGTTGATAC |
| | STAU 1R | GGTCCCGAAGACCAACACAA |
| *F. capucina* | Frag 2F | GGGCCTTTACAGGTCTGGCA |
| | Frag 2R | ACGGCCCATCCACAAATCCA |

**Measurement of phytoplankton using real-time PCR assay**

A real-time PCR assay was performed according to the method in our previous paper (Mizuguchi et al., 2018). The primer sets used here were the same as previous paper (Table 1). A 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 5 μM), 2 μL of the extracted DNA sample, and 8.5 μL of sterilized water. 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). Thermal conditions were as follows: 1 cycle at 95℃ (3 min); 45 cycles of repeated 95℃ (2 s), 58℃ (5 s), and 72℃ (5 s); and a final cycle at 78℃ (1 min). A melting-curve analysis was performed by continuously measuring the fluorescence during heating from 60℃ to 95℃. Threshold cycle (Ct) values were determined using the fit point method (500 relative fluorescence unit) with 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). Sequencing was performed by the MACROGEN Japan Corporation. Computations were partially performed on the NIG supercomputer at the ROIS National Institute of Genetics.

**Characterization of DOM in lake-water samples**

Lake-water samples were passed through a membrane filter (0.45 μm, Millipore), stored in a refrigerator and used in the experiment as soon as possible. Membrane filters were used after washing with 1 M hydrochloric acid and distilled water. Dissolved organic carbon (DOC) was measured using a TOC meter (TOC-$V_{CSH}$, Shimadzu, Japan). The fluorescence properties of DOM were measured with a three-dimensional excitation-emission matrix (3-DEEM), using a Shimadzu RF-5300PC fluorescence spectrophotometer, as previously reported (Aoki, 2008a, b). Fluorescence readings were normalized by fluorescence intensity (Ex=345 nm/Em=450

nm) of 10 μg $L^{-1}$ quinine sulfate (0.05 M $H_2SO_4$ solution) 10 QSU. The values were treated as the relative fluorescence intensity (RFI).

## Results and discussion

### DNA extraction from phytoplankton cells trapped on the filter

A real-time PCR assay with a DNA extraction from cells trapped by filtering a lake-water sample (filter method) was examined instead of using the DNeasy method. Koike et al. (2007) reported that for DNA extraction from filter-collected *Symbiodinium* cells, the filters were immersed in 500 μL of TE buffer and heated in boiling water for 10 min. In this study, the filters with trapped phytoplankton cells were immersed in 5 mL of TE buffer, since this can be done without cutting the filters, and the effects of heating time in boiling water on the DNA extraction were examined. Correlations between the Ct values and logarithmic cell densities of *M. aeruginosa*, *S. dorsidentiferum*, and *F. capucina* were obtained. For *M. aeruginosa* and *F. capucina*, linear calibration curves were obtained for the 10 min heating time. However, in the case of *S. dorsidentiferum*, a linear calibration curve was not obtained for the 10 min heating time but was obtained for the 1 h heating time (Fig. 1). These results suggest that *S. dorsidentiferum* has a strong cell wall composed of cellulose (Nakata, 2012), as compared with *M. aeruginosa*, which has a cell wall composed of peptidoglycan, phospholipid, protein, and lipopoly-saccharide (Stanier et al., 1986). For *M. aeruginosa* and *F. capucina*, linear calibration curves were also obtained for the 1 h heating time. Therefore, DNA extraction using the filter method was carried out at a heating time of 1 h.

### Specificity of primer sets and linearity of real-time PCR

The specificity of designed primer sets was confirmed in the real-time PCR assay with a DNA extraction using the filter method. As the results indicate that the primer sets used in our previous study (Mizuguchi et al., 2018) were also specific for each species, the same primer sets were used here. The real-time PCR assay with DNA extraction via the filter method showed linear correlations between the Ct values and logarithmic cell densities of *M. aeruginosa*, *S. dorsidentiferum*, and *F. capucina* in the ranges of $10^3$–$10^7$ cells {y=-3.06x+42.8 ($r^2$=0.996)}, $10^2$–$10^5$ cells {y=-2.87x+40.6 ($r^2$=0.982)}, and $3.7x10^2$–$3.7x10^5$ cells {y=-3.08x+40.4 ($r^2$=0.992)},

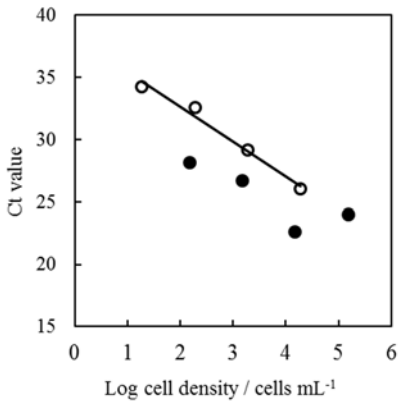


Fig. 1. Correlations between the Ct values and logarithmic cell densities of *S. dorsidentiferum.*
Heating times: ●, 10-minute treatment; ○, 1-hour treatment.

図 1. *S. dorsidentiferum* の Ct 値と細胞密度の関係.
加熱時間: ●, 10 分処理; ○, 1 時間処理.

respectively. The coefficients of variation (%) were lower than 5%. The melting points (Table 2) were similar to those reported in our previous paper (Mizuguchi et al., 2018). The PCR efficiency values are also listed in Table 2.

The amplification curves, melting curves and calibration curves of the target phytoplankton alone were similar to those in the presence of other species. Moreover, similar results were obtained when the cultured cells of phytoplankton were diluted with water samples of Lake Biwa instead of pure water. These results suggest that the effects of the coexistence of other algal species and suspended solid (SS) in lake water were small in this real-time PCR assay. The determination limits of *M. aeruginosa*, *S. dorsidentiferum*, and *F. capucina* in water samples were $10^3$, $10^2$ and $3.7x10^2$ cells $L^{-1}$, respectively. This method was therefore applied to the determination of vertical distributions and seasonal changes in the algal species in the northern basin of Lake Biwa.

Table 2. Melting points and PCR efficiency values of three kinds of phytoplankton.

表 2. 3 種の植物プランクトンの融解点と PCR 効率.

| Species name | Melting point / ℃ | PCR efficiency / % |
|---|---|---|
| *M. aeruginosa* | 86.5~87 | 112 |
| *S. dorsidentiferum* | 85~85.5 | 123 |
| *F. capucina* | 83 | 111 |

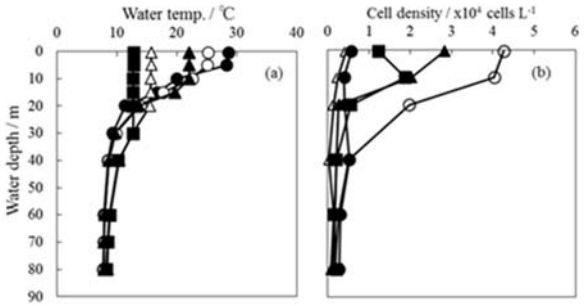


Fig. 2. Vertical distributions of water temperature (a) and cell densities of *Microcystis* spp. (b) at St. 17B in Lake Biwa (2017). ●, August; ○, September; ▲, October; △, November; ■, December.

図 2. 琵琶湖（St. 17B）における水温（a）と *Microcystis* 類の細胞密度（b）の鉛直分布（2017 年）. ●, 8 月; ○, 9 月; ▲, 10 月; △, 11 月; ■, 12 月.

**Vertical distributions of algal species in a water column at St. 17B in Lake Biwa**

The real-time PCR assay with DNA extraction using the filter method was applied to the determination of vertical distributions of the cell densities of algal species in water samples collected at St. 17B in the northern basin of Lake Biwa. The vertical distributions of water temperature and the cell densities of *Microcystis* spp. from August to December in 2017 are shown in Fig. 2. The cell densities of *Microcystis* spp. decreased with the increasing water depth in all months, and those that were relatively high in the surface water in September ($4.3 \times 10^4$ cells $L^{-1}$ at 0.5 m) and October ($2.8 \times 10^4$ cells $L^{-1}$ at 0.5 m) decreased considerably at the thermocline (water depth 10–20 m). From these results, it is considered that sunlight is not sufficient for the photosynthesis of phytoplankton at water depths below the thermocline. It was reported that the euphotic zone of Lake Biwa was from 0 to about 20 m in depth (Tanaka et al., 1999), and 1% attenuation of photosynthetically active radiation (PAR) was at a water depth of 16.1 m in Lake Biwa (St. 17B) (Hayakawa et al., 2008).

Next, the samples from January to August 2017, which were obtained at melting points similar to those of cultured *S. dorsidentiferum*, were analyzed via real-time PCR assay. The cell densities of *Staurastrum* spp. in water samples during the circulation period (January–March) were comparatively low ($< 10^3$ cells $L^{-1}$) except for January 2017 at all water depths and increased in the surface water after April (Fig. 3). The highest cell densities of *Staurastrum* spp. at water depths of 0.5 m and 10 m were $1.3 \times 10^5$ and $1.6 \times 10^5$ cells $L^{-1}$, respectively, in water samples from July 2017. The high cell densities of *Staurastrum* spp. in the surface water from May to July decreased significantly at the thermocline, which were similar to those of *Microcystis* spp. The vertical distributions of *Fragilaria* spp. cell densities in the water samples from June to December 2017 are shown in Fig. 4. The cell densities of *Fragilaria* spp. in the water samples from June also showed the highest values at water depths of 0.5 m and 10 m, $3.1 \times 10^5$ and $5.8 \times 10^5$ cells $L^{-1}$, respectively, and decreased considerably

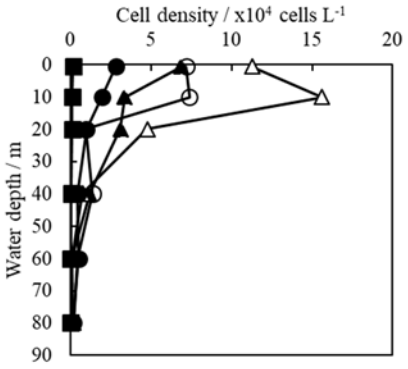


Fig. 3. Vertical distributions of *Staurastrum* spp. at St. 17B in Lake Biwa (2017). ●, April; ○, May; ▲, June; △, July; ■, August.

図 3. 琵琶湖（St. 17B）における *Staurastrum* 類の鉛直分布（2017 年）. ●, 4 月; ○, 5 月; ▲, 6 月; △, 7 月; ■, 8 月.

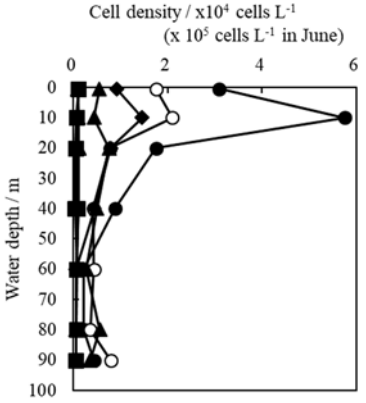


Fig. 4. Vertical distributions of *Fragilaria* spp. at St. 17B in Lake Biwa (2017).●, June; ○, July; ▲, August; △, September; ■, October; □, November; ◆, December.

図 4. 琵琶湖（St. 17B）における *Fragilaria* 類の鉛直分布（2017 年）. ●, 6 月; ○, 7 月; ▲, 8 月; △, 9 月; ■, 10 月; □, 11 月; ◆, 12 月.

at water depths below 20 m. The cell densities of *Fragilaria* spp. in the surface water of August and December also decreased at water depths below 20 m. However, *Fragilaria* spp. of 4000–5000 cells $L^{-1}$ were detected even at water depths below 40 m, in contrast to two other phytoplankton species. This may be due to the high sinking rate of *Fragilaria* spp. (Goda and Ebise, 1977).

**Seasonal changes in algal species in the water column at St. 17B in Lake Biwa**

Monthly changes in the cell densities and the cell volume of three kinds of phytoplankton using a real-time PCR assay (filter method) at a water depth of 10 m in the northern basin of Lake Biwa (St. 17B) in 2017 and 2018 are shown in Fig. 5 (a) and (b), respectively. *Fragilaria* spp. have been started to measure in June 2017 using this method. The cell volume of *Microcystis* spp., *Staurastrum* spp. and *Fragilaria* spp. was calculated to be 110, 32000 and 750 $\mu m^3$ $cell^{-1}$, respectively (Ichise et al., 2013).

The cell densities of *Microcystis* spp. from January to July in 2017 and from February to May in 2018 were lower than $10^3$ cells $L^{-1}$; those from August to December in 2017 and from June to December in 2018 were $3.9x10^3$–$4.0x10^4$ cells $L^{-1}$ and $1.0x10^3$–$2.2x10^3$ cells $L^{-1}$, respectively; and the highest were in September and October 2017. Meanwhile, *Staurastrum* spp. were present during the circulation periods of both years and increased from April to July in 2017 ($1.9x10^4$–$1.6x10^5$ cells $L^{-1}$). However, the cell densities of *Staurastrum* spp. decreased in August and were low from September 2017 to May 2018; those from June to November in 2018 were $2.4x10^4$–$6.1x10^4$ cells $L^{-1}$. Moreover, *Fragilaria* spp. showed the highest cell densities in the water samples from June 2017 ($5.8x10^5$ cells $L^{-1}$) and February 2018 ($1.8x10^6$ cells $L^{-1}$); those were relatively low in summer and autumn. Monthly changes in the cell densities of these phytoplankton at water depths of 0.5 m and 20 m were similar to those at 10 m.

The total cell volume of three kinds of phytoplankton were similar to that of *Staurastrum* spp. because the cell volume of *Staurastrum* spp. is larger than that of other algal species except in June 2017 and February 2018 (Fig. 5 (b)). The cell volume of *Staurastrum* spp. was highest in July 2017 (5.0 $mm^3L^{-1}$) and showed high values in May 2017 and from June to November in 2018. The cell volume of *Fragilaria* spp. was high, 0.43 $mm^3L^{-1}$ in June 2017 and 1.3 $mm^3L^{-1}$ in February 2018. Ichise et al. (2013) reported that the ratios of the cell volume of *Staurastrum* spp., *Fragilaria* spp., and *Microcystis* spp. to the total cell volume of phytoplankton at St. 17B in Lake Biwa from 1980 to 2009 were 43.7%, 2.5% and 1.8%, respectively. Furthermore, they suggest that the large amount of extracellular polysaccharides (EPSs) produced by phytoplankton such as Cyanophyceae and Chlorophyceae could be one of the major sources of organic matter in Lake Biwa.

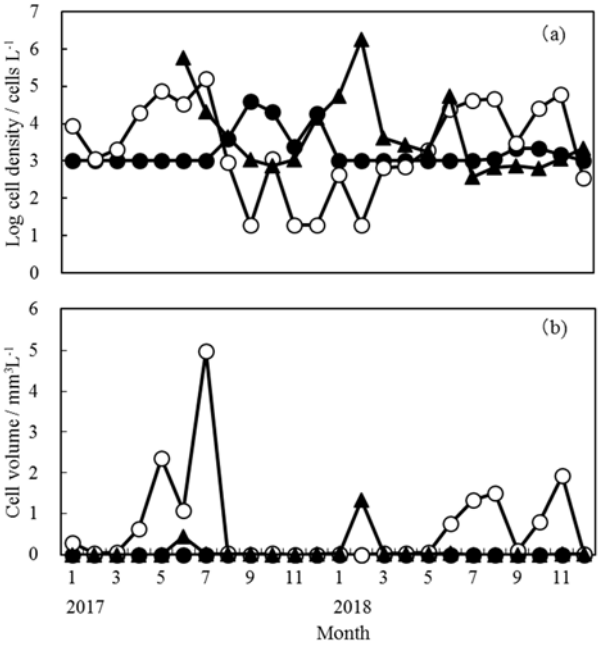


Fig. 5. Monthly changes in the cell densities (a) and cell volumes (b) of three kinds of phytoplankton using a real-time PCR assay (filter method) at St. 17B (water depth 10 m) in Lake Biwa from 2017 to 2018. ●, *Microcystis* spp.; ○, *Staurastrum* spp.; ▲, *Fragilaria* spp.

図 5. 琵琶湖（St. 17B, 水深 10 m）の 2017 年から 2018 年におけるリアルタイム PCR（フィルター法）で求めた 3 種の藻類の細胞密度（a）と細胞容積（b）の月変化. ●, *Microcystis* 類; ○, *Staurastrum* 類; ▲, *Fragilaria* 類.

**Effects of phytoplankton species on the DOM and fluorophores in Lake Biwa**

The concentrations of DOC and fluorophores in water samples from Lake Biwa were simultaneously measured, and the effects of phytoplankton species on the DOM and fluorophores were analyzed. 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 in water samples from Lake Biwa using 3-DEEM. Monthly changes in the DOC concentrations, and the fluorescence intensities of fulvic-like and protein-like fluorophores at water depths of 0.5, 10, 20 and 90 m in the northern basin of Lake Biwa (St. 17B) from 2017 to 2018 are shown in Fig. 6 (a), (b)and (c), respectively. The DOC concentrations in surface waters were high in January 2017, from May to September 2017, and from July to August 2018; they showed a particularly high value, 1.69 mgC $L^{-1}$ at a water depth of 20 m in January 2017. From the results of the fractionation of DOM in Lake Biwa, it was estimated that hydrophobic acids (humic substances) and hydrophilic substances were about 25-40% and around 60%, respectively (Aoki et al., 2004; Nagai et al., 2005). Then it was reported that the increase in the concentrations of DOC and hydrophilic substances in surface waters during warm seasons may be attributed to algal DOM (Ohara et al., 2009). Okamoto et al. (2011) also reported that the sources of refractory DOM depend on both inner production in Lake Biwa (around 70%) and pedogenic DOM from the watershed (around 30%).

Almost all of the RFI values of protein-like fluorophores in surface waters were higher than those in bottom waters, and their variations were larger than those of fulvic-like fluorophores. The RFI values of protein-like fluorophores were especially high (>5 QSU) in January, May, July, and August 2017 and December 2018 at a water depth of 10 m (Fig. 6 (c)). As the RFI values of protein-like fluorophores in the northern basin of Lake Biwa are usually in the range of 2.0-4.0 QSU, those more than 4.0 QSU may be attributed to the production

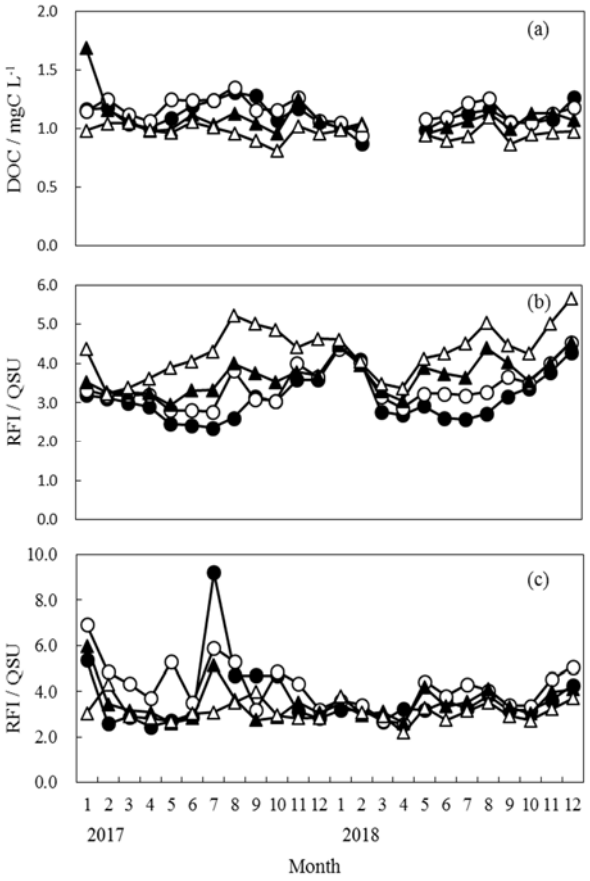


Fig. 6. Monthly changes in the DOC concentrations (a), and the fluorescence intensities of fulvic-like fluorophores (b) and protein-like fluorophores (c) in the northern basin of Lake Biwa (St. 17B).
Water depth: ●, 0.5 m; ○, 10 m; ▲, 20 m; △, 90 m.

図 6. 琵琶湖北湖（St. 17B）における DOC 濃度（a）とフルボ酸様蛍光物質（b）とタンパク質様蛍光物質（c）の蛍光強度の月変化.
水深: ●, 0.5 m; ○, 10 m; ▲, 20 m; △, 90 m.

Table 3. Predominant phytoplankton species in Lake Biwa (St. 17B) at high DOC concentration and RFI values of protein-like fluorophores (>5 QSU) (2016).

表 3. 琵琶湖（St. 17B）において高い DOC 濃度，タンパク質様蛍光物質の RFI 値（>5 QSU）となる場合の植物プランクトンの優占種.

| Sampling | | | Water depth/m | DOC /mgCL$^{-1}$ | Peak C/QSU | Phytoplankton species |
|---|---|---|---|---|---|---|
| Apr. 2016 | 10 | 1.25 | 7.03 | | | *Fragilaria* spp. |
| July 2016 | 10 | 1.40 | 5.28 | | | *Staurastrum* spp. |
| Aug. 2016 | 0.5 | - | 5.74 | | | *Staurastrum* spp. |
| Nov. 2016 | 80 | 0.97* | 5.20 | | | *Micrasterius* spp. |

*: DOC concentrations at water depths of 0.5, 10 and 20 m were 1.35, 1.36 and 1.41 mgC/L, respectively.
-: Not measured

Table 4. Cell densities of three phytoplankton species using a real-time PCR assay(filter method) at high DOC concentration and RFI values of protein-like fluorophores (Peak C) in the northern basin of Lake Biwa (St. 17B, water depth 10 m) .

表 4. 琵琶湖北湖水（St. 17B, 水深 10 m）で DOC 濃度とタンパク質様蛍光物質（ピーク C）の RFI 値が高い場合のリアルタイム PCR（フィルター法）で求めた 3 種の植物プランクトンの細胞密度.

| | 2017 | | | | 2018 |
|---|---|---|---|---|---|
| | Jan.* | May | July | Aug. | Dec.* |
| DOC / mg $L^{-1}$ | 1.15 | 1.25 | 1.24 | 1.35 | 1.18 |
| Peak C / QSU | 6.90 | 5.31 | 5.89 | 5.28 | 5.06 |
| Cell density / $x10^3$ cells $L^{-1}$ | | | | | |
| *Microcystis* spp. | <1.0 | <1.0 | <1.0 | 3.9 | 1.0 |
| *Staurastrum* spp. | 8.9 | 74 | 160 | 0.93 | 0.34 |
| | (0.29) | (2.4) | (5.0) | (0.030) | (0.011) |
| *Fragilaria* spp. | - | - | 21 | 4.4 | 2.2 |
| | | | (0.016) | (0.0033) | (0.0016) |

The values in parenthesis were cell volume ($mm^3L^{-1}$) of phytoplankton species.

*: The cell densities of *Micrasterius* spp. were high. , -: Not measured

by phytoplankton (Aoki et al., 2008a, b; Ohara et al., 2009; Yamada et al., 2012b). When an outbreak of phytoplankton species in Lake Biwa (St. 17B) was observed, a high DOC concentration and RFI value of protein-like fluorophore (>5 QSU) in water sample were obtained in 2016 (Table 3). *Fragilaria* spp., *Staurastrum* spp., and *Micrasterius* spp. occurred as predominant phytoplankton in April 2016, July and August 2016, and November 2016, respectively. The cell densities of *Micrasterias* spp., *Staurastrum* spp. and *Fragilaria* spp. in the plankton net samples using a microscope are shown in Fig. 7. These results suggest that the increase of DOM and protein-like fluorophores in Lake Biwa is associated with an increase in the predominant phytoplankton species. Next, in the case of high RFI values of protein-like fluorophores (>5 QSU) from 2017 to 2018, the DOC concentrations and the cell densities of three algal species using a real-time PCR assay (filter method) at a water depth of 10 m in Lake Biwa are listed in Table 4. In all these months, the DOC concentrations were high (>1.1 mgC $L^{-1}$), and *Staurastrum* spp. were observed. Although the cell densities of *Fragilaria* spp. were not measured in January and May 2017 using a real-time PCR assay (filter method), those in other three months were $2.2x10^3$–$2.1x10^4$ cells $L^{-1}$. The cell densities of *Microcystis* spp. were lower than those of other two

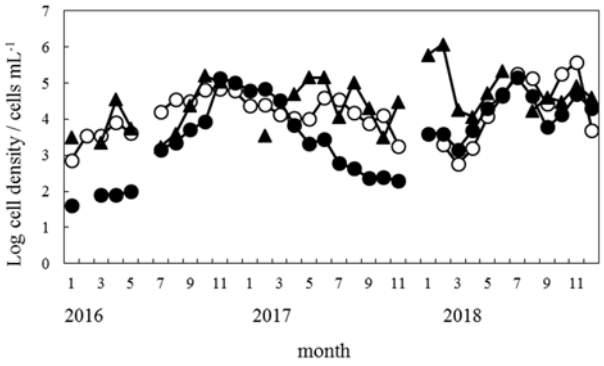


Fig. 7. Annual changes in the cell densities of *Micrasterias* spp., *Staurastrum* spp. and *Fragilaria* spp. in the plankton net samples collected in the northern basin of Lake Biwa (St. 17B) using a microscope.

●, *Micrasterias* spp.; ○, *Staurastrum* spp.; ▲, *Fragilaria* spp.

図 7. 顕微鏡による琵琶湖北湖（St. 17B）で採取したプランクトンネット試料中 *Micrasterias* 類、*Staurastrum* 類及び *Fragilaria* 類の細胞密度の月変化.

●, *Micrasterias* 類；○, *Staurastrum* 類；▲, *Fragilaria* 類.

phytoplankton. In January 2017, the DOC concentration and the RFI of protein-like fluorophore at a water depth of 20 m were also high, 1.69 $mgCL^{-1}$ and 5.98 QSU, respectively, but the cell densities and cell volume of the measured phytoplankton were relatively low. As the unusual occurrence of *Micrasterious* spp. was observed, annual changes in the cell densities of *Micrasterias* spp., *Staurastrum* spp. and *Fragilaria* spp. in the plankton net samples collected at St. 17B using a microscope are shown in Fig. 7. *Fragilaria* spp. were hardly observed in December 2016, January and March 2017. Since high cell densities of *Micrasterious* spp. ($> 10^4$ cells $mL^{-1}$) were observed from November 2016 to March 2017, from May to August 2018 and from October to December 2018, the increase in *Micrasterious* spp. may have affected the increase of DOC and protein-like fluorophores in January 2017 and December 2018. These results suggest that the increase of DOM and protein-like fluorophores in Lake Biwa (St. 17B) from 2017 to 2018 is associated with the increase of *Fragilaria* spp. and Chlorophyceae such as *Staurastrum* spp. and *Micrasterious* spp. However, since the ratios of the cell volume of measured phytoplankton to the total cell volume of phytoplankton in Lake Biwa were lower than 50%, the effects of other phytoplankton on the increase of DOM and protein-like fluorophores in Lake Biwa should be further examined.

## Conclusion

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 the lake, because the characteristics of algal DOM differ according to the algal species. In the previous study, phytoplankton-monitoring methods using a SYBR Green real-time PCR assay with specific primer sets for each species after DNA extraction using a DNeasy Plant Mini Kit (DNeasy method) were developed and applied to the determination of the cell densities of algal species during cultivation and in phytoplankton samples collected using a plankton net. However, this method cannot be applied to the quantification of phytoplankton in water samples in the northern basin of Lake Biwa because the cell densities of phytoplankton are too low. In this study, a real-time PCR assay with a DNA extraction from cells trapped ona filter through the filtration of lake-water sample (filter method) was developed and applied to the determination of vertical distributions and seasonal changes in the algal species in Lake Biwa. Furthermore, the effects of phytoplankton species on the DOM and fluorophores in Lake Biwa were analyzed.

The results of this study show that the a real-time PCR assay with DNA extraction using filter method is an effective method for the quantification of *Microcystis* spp., *Staurastrum* spp., and *Fragilaria* spp. in the water samples from Lake Biwa. However, as other predominant phytoplankton that affect DOM and fluorophores are present in lake water, methods for monitoring of other specific phytoplankton should be developed in the future.

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