学位申請論文

リアルタイム PCR を用いる藻類モニタリング法の開発と

琵琶湖・流域河川における溶存有機物質の特性評価に関する研究

Studies on the Development of Real-time PCR Assay for Monitoring Phytoplankton and Characterization of Dissolved Organic Matter in Lake Biwa and the Surrounding Rivers

2017 年 12 月

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CONTENTS

General 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 (Fig. 1) 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 [1-3]. Hence, DOM dynamics and characteristics can affect multiple biogeochemical processes in aquatic environments, including light penetration, pH buffering, oxygen consumption, nutrient availability, and toxicity of pesticides and metals [4]. Many studies have evaluated the characteristics and sources of DOM in Lake Biwa using chemical fractionation methods [1, 5], the natural carbon stable isotope ratio [6, 7], and spectroscopic analyses [1, 8-12].

Okamoto and Hayakawa [13] 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. [14] 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

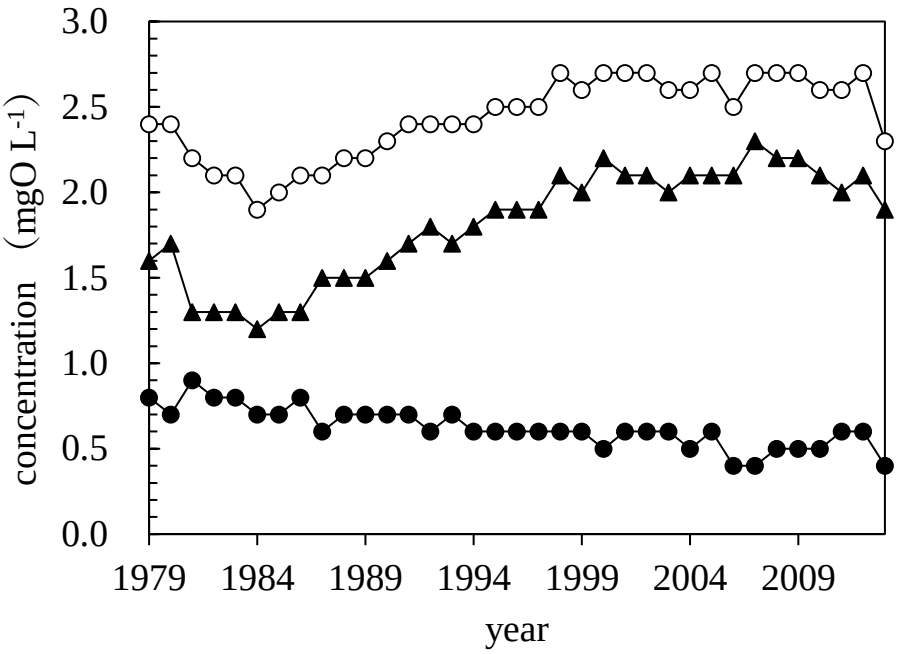

Fig. 1 Annual change in chemical oxygen demand (COD) and biochemical oxygen demand (BOD) in the northern basin of Lake Biwa [3].

○, COD; ●, BOD; ▲, COD-BOD

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 [1, 15-18]. 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 [1, 15-18].

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 [19, 20]. 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 [19-22]. 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 [19-23].

Humic substances (HS), which are major organic constituents of fresh water, marine water, groundwater and soils, interact with heavy metals, affect the movement of hydrophobic organic pollutants in groundwater, absorb the sunlight, and may be precursors of trihalomethane formed during water treatment with chlorine [4, 24]. Humic substances are hydrophobic

organic acids with molecular weights of hundreds to several hundred thousand; they are classified into humic acid (HA) and fulvic acid (FA) based on their solubility in acids and alkalis [25, 26]. More than 90% of HS in Lake Biwa and the surrounding rivers is FAs. Methods of simultaneously determining the concentration and molecular weights of HS in environmental waters by gel chromatography with a fluorescence detector were developed and applied to determining the HS in Lake Biwa and its rivers. The concentrations of aquatic HS in rivers were high in the summer and low in the winter, and the ratio of the higher molecular weight of HS was larger in the warmer season than that in the cooler season [27, 28]. The dynamics of DOM and hydrophobic organic acid (humic substances) in Lake Biwa and its surrounding rivers have been evaluated using chemical fractionation [1, 5, 29]. From the fractionation of DOM into hydrophobic acid (HoA), hydrophobic neutral (HoN) and hydrophilic DOM (Hi) using hydrophobic resin (XAD-7HP, DAX-8), hydrophobic acids in rivers such as the Katsura River, the Kizu River, and the Yodo River were estimated to be 30-60%, which were higher than those (20-25%) in Lake Biwa [1, 5]. Furthermore, the vertical distribution and seasonal changes of DOM and each fraction in Lake Biwa were evaluated, and the increase in the concentrations of DOM, HoA, and Hi during the warm season may be attributed to algal DOM [30]. A three-dimensional excitation-emission matrix (3-DEEM) was used for

algal DOM, resulting in the observation of two fulvic-like fluorescence peaks (Ex/Em=320-350/430-450 nm, 240-260/430-450 nm) and one protein-like fluorescence peak (Ex/Em=280-290/320-330 nm). The fulvic-like fluorophores derived from phytoplankton have fluorescence similar to those of fulvic acid originating in soil but exhibit mainly hydrophilic characteristics, while a protein-like fluorophore has a fluorescence similar to that of amino acid (tryptophan) and exhibits hydrophobic characteristics [15, 16]. However, the method of analyzing humic substances using chemical fractionation is time consuming and cannot be applied to the water samples with low DOC concentrations. Therefore, developing a method for rapidly and sensitively analyzing humic substances in environmental waters is necessary.

Analysis of DOM and fluorophores in Lake Biwa and its surrounding rivers found that positive relationships between the fluorescence intensities of fulvic-like DOM and DOC may be observed in the Katsura River, the Kizu River, and the Yodo River, but not the Uji River [8, 31]. These results indicate that the organic pollution in the lake was a higher contributor not only of humic substances from its watershed, but also of inner products from algae. Supposing that the relative fluorescence intensity (RFI) per DOC, RFI/DOC, of soil FA (Dando FA) is 1, the RFI/DOC values of the surrounding rivers and Lake Biwa were 0.6-0.9 and 0.1-0.3, respectively.

The RFI/DOC values of Lake Biwa were 0.2-0.6 as a standard BiwakoFA (LBFA). These results suggest that the contribution of soil FA to DOM in rivers might be larger than to DOM in Lake Biwa [31].

The fulvic-like fluorophores (Ex/Em = 320-350/430-450 nm, 240-260/430-450 nm) and a protein-like fluorophore (Ex/Em = 280-290/320-330 nm) were always detected in Lake Biwa using a three-dimensional excitation-emission matrix (3-DEEM) [15, 16]. The dynamics of DOM and fluorophores in Lake Biwa were evaluated, and it was found that the fluorescence intensities of fulvic-like fluorophores in surface water during the completely stratified period (July-September) tend to be lower than those during other months and in bottom water, even though DOC concentrations in surface water tend to be higher than those of others due to high primary production. These phenomena might be due to the photolysis of fulvic-like fluorophores in surface water during the stratified period. It has been reported that the fluorescence of DOM in the lake has sources other than epilimnetic primary production [11], and it is degraded by solar radiation in the epilimnion during summer [10]. Furthermore, seasonal changes in the UV absorbance of lake water were different from those of DOC. The clearly positive correlation between UV absorbance and HS was observed in an inner bay (Akanoi); however, the correlation in offshore water was weaker than in the inner bay [1]. Therefore, it is necessary to analyze the behavior and

photoirradiation effects of both allochthonous and autochthonous DOM separately. Many studies of the photochemical degradation of DOM and aquatic HS have been performed [32-36]. However, few studies about the effects of photoirradiation on the characteristics of algal DOM have been conducted.

In the 1st chapter, in order to clarify the characteristics of algal DOM and its contribution to the DOM in Lake Biwa, phytoplankton-monitoring methods were developed 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.

In the 2nd chapter, the method for rapidly and sensitively analyzing

humic substances in environmental waters was investigated because the method of analyzing humic substances using chemical fractionation is time consuming and cannot be applied to the water samples with low concentrations of dissolved organic carbon (DOC). Then, the rapid analysis of humic substances (mainly FA) in waters using three dimensional excitation emission matrix (3-DEEM) and DOC was developed as compared with fractionation analysis using microporous resin (DAX-8), and it was further applied to the dynamics of FA in Lake Biwa and its surrounding rivers. Humic substances in the Yodo rivers as measured by rapid analysis using soil fulvic acid (Dando FA) as a standard were in relatively good agreement with those by fractionation analysis, with the exception of the Uji River, of which waters are affected by Lake Biwa by running through the Seta River. This rapid analysis was also applied to the dynamics of humic substances in the rivers flowing into Lake Biwa. In the case of rapid analysis of humic substances in Lake Biwa, Biwako FA was used as a standard instead of Dando FA.

In the 3rd chapter, the effects of photoirradiation on changes in the fluorescence intensities, DOC concentrations, and molecular weights of DOM in Lake Biwa and its surrounding rivers were evaluated and compared with the results of humic substances and algal DOM. During the completely stratified period (summer), the fluorescence intensities of fulvic-like

fluorophores in the surface water in the northern basin of Lake Biwa decreased and were lower than those in other months and in bottom water. The fluorescence quenching and degradation of high-molecular substances by further solar irradiation were hardly observed in the surface water samples but were significantly observed in bottom water samples. On the other hand, changes in the DOC concentrations in all samples were relatively small with solar irradiation. These results suggest that in the northern basin of Lake Biwa, the susceptibility of fulvic-like fluorophores to degradation by further solar irradiation is dependent on the water depth collected during the stratified period (summer), but the rest of fulvic-like fluorophores might be resistant to further photochemical degradation regardless of the water depth. Furthermore, the effects of the wavelength region on the characteristics of DOM and fluorophores in Lake Biwa and its surrounding rivers were examined by Xe lamp irradiation using two kinds of wavelength cut filters. From these results, it is considered that wavelengths between 290 and 495 nm and below 290 nm might largely affect the characteristics of fulvic-like fluorophores and protein-like fluorophores, respectively.

1. Application of SYBR Green real-time PCR Assay to Monitoring of Phytoplankton during Cultivation and Lake Biwa

1.1 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 [1, 2]. Okamoto and Hayakawa [13] 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. [14] 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 [1, 15-18]. 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 [1, 15-18].

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 [19, 20]. Then, a rapid and versatile real-time PCR (polymerase chain 1 reaction) assay using a small subunit of the ribosomal RNA gene (SSU rDNA) was investigated [19-22]. 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 [19-23].

In this chapter, in order to clarify the characteristics of algal DOM and its contribution to the DOM in Lake Biwa, phytoplankton-monitoring methods were developed 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.

1.2 Materials and methods

1.2.1 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. 1-1) from January 2016 to 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 25μm, φ20cm, Rigo-sha, Japan) and stored at -80℃ until DNA extraction. Their cells were counted under a microscope (IX71N- 22PH-D, Olympus Japan).

1.2.2 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 [15, 16]. *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

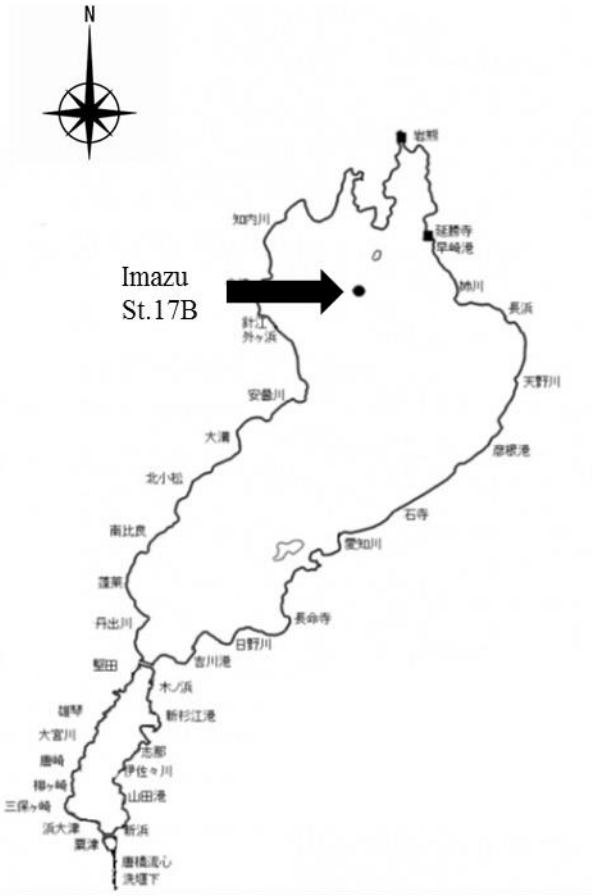

Fig. 1-1 Sampling station (St. 17B) in Lake Biwa

[15, 16].

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.

1.2.3 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.

1.2.4 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.*

Table 1-1 Primer list in this study.

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. dorsidentiferam*	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		

dorsidentiferum, *C. ovata*, and *F. capucina* by reference to Ishikawa's method [37]. The primer sets used in this study are listed in Table 1-1.

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).

1.2.5 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 [15, 16]. Fluorescence readings were normalized by fluorescence intensity (Ex=345 nm/Em=450 nm) of 10 μg L^{-1} quinine sulfate (0.05 1 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) [38] 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.

1.3 Results and discussion

1.3.1 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. 1-2(a)). These three fluorescence maxima were also observed in algal DOM released from four kinds of phytoplankton during the cultivation (Fig. 1-2(c)-(f)), but peak C is not detected in soil humic substances (Fig. 1-2(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. 1-3(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 [39]. 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 depth of 10 m. In January 2017, the DOC

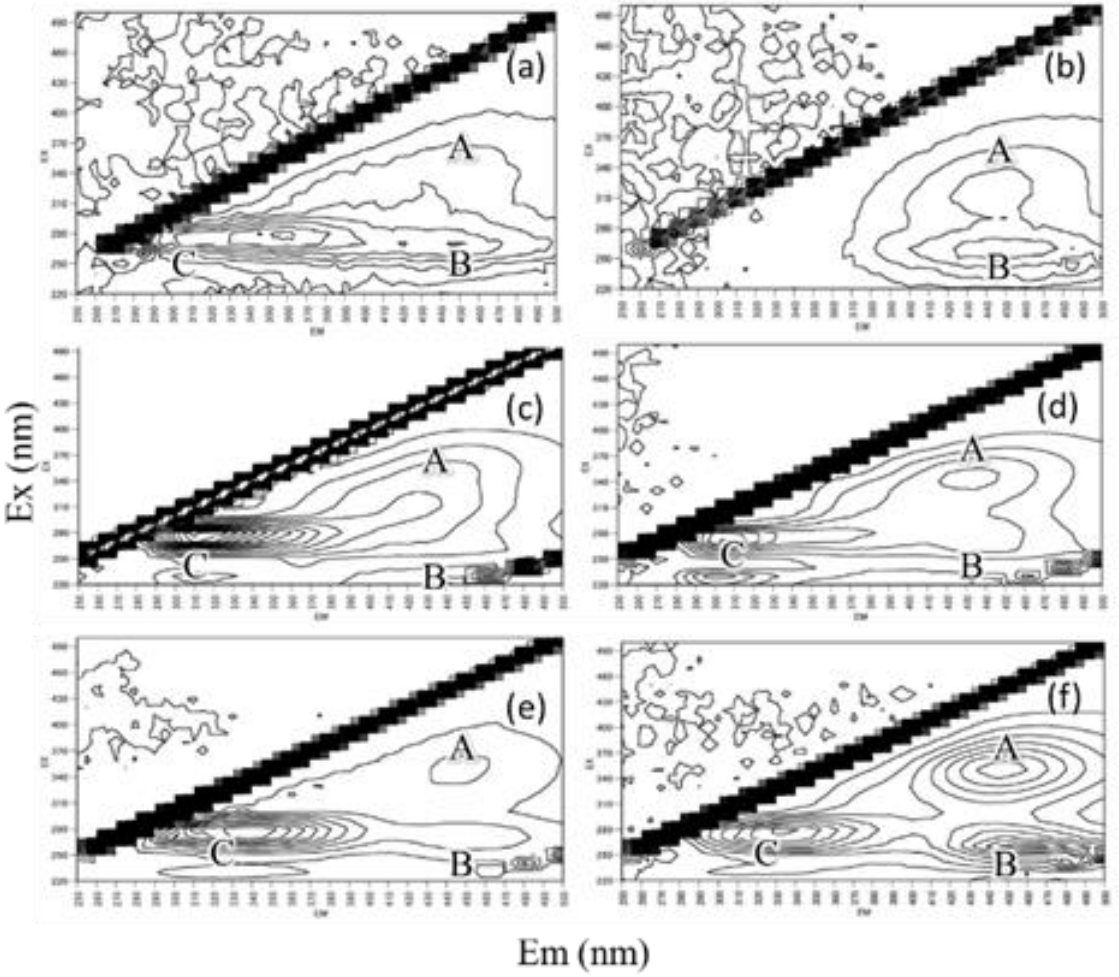

Fig. 1-2 3D-EEM contour plots.

(a) surface water of Lake Biwa (St. 17B, water depth 10m, 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

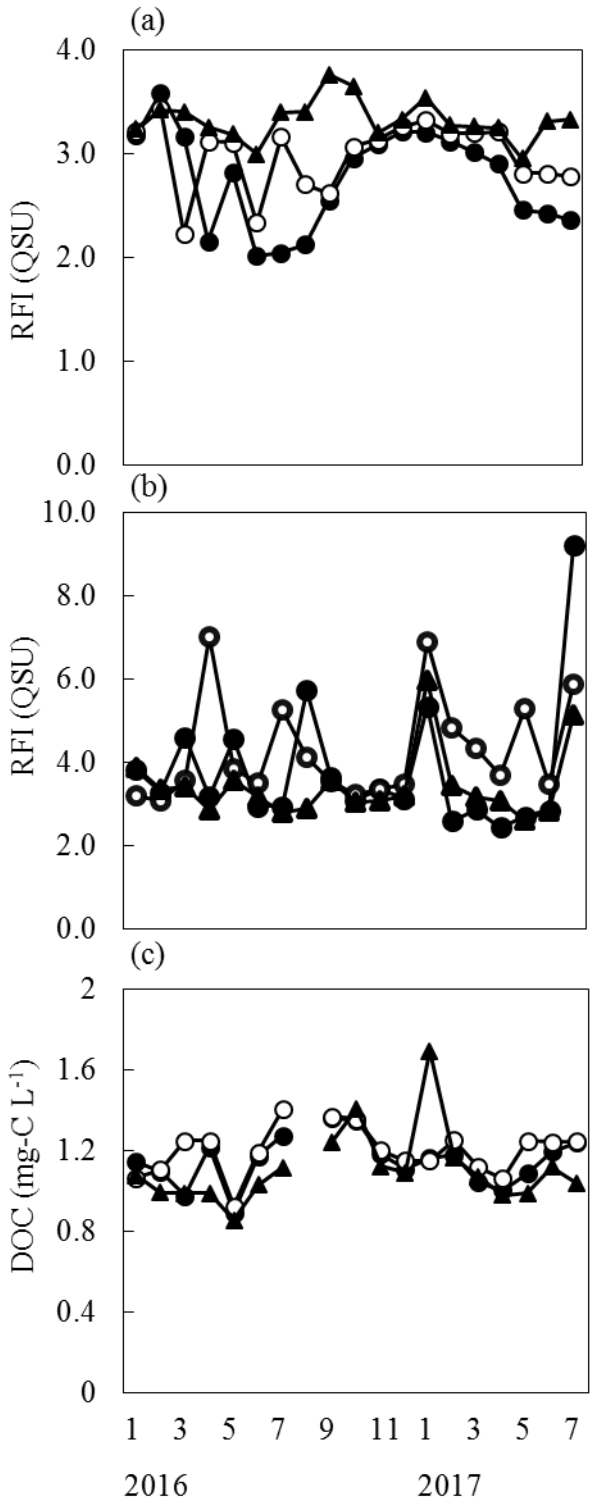

Fig. 1-3 Monthly changes in the fluorescence intensities of peaks A (a) and C (b), and the DOC concentrations (c) in the northern basin of Lake Biwa

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

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. 1-4. 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 [40, 41]. Chlorophyll a and chlorophyll c were reported to be high in June and November 2016 and in August 2016, respectively [3]. 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.

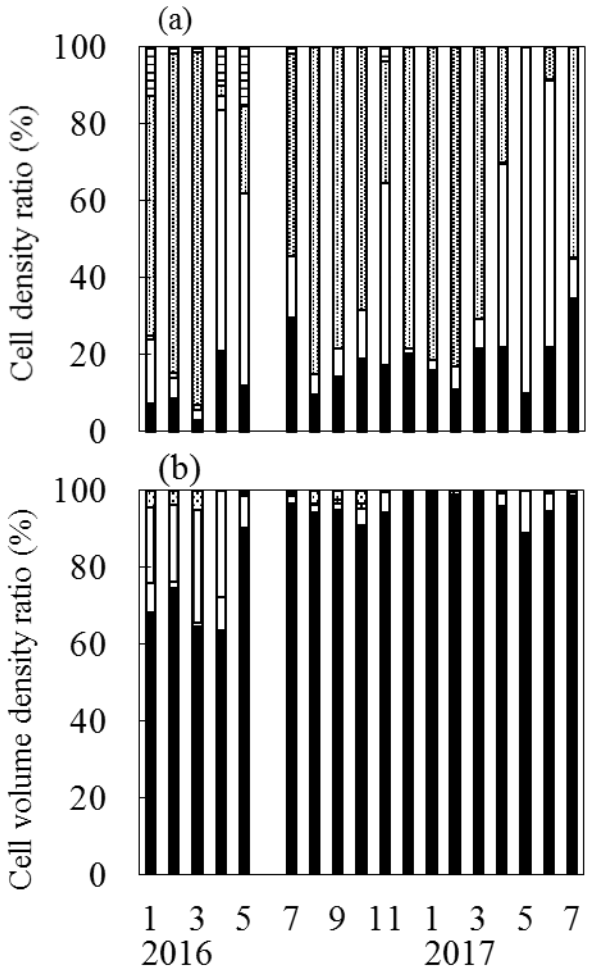

Fig. 1-4 Monthly changes in the ratios of cell density (a) and cell volume density (b) of phytoplankton species in the northern basin of Lake Biwa (St. 17B).

■: Chlorophyceae; □: Bacillariophyceae; ▥: Dinophyceae;

⁘; Cyanobacteria

1.3.2 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 1-1). 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. 1-5.

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 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. These results of *M. aeruginosa* are shown in Fig. 1-5. 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

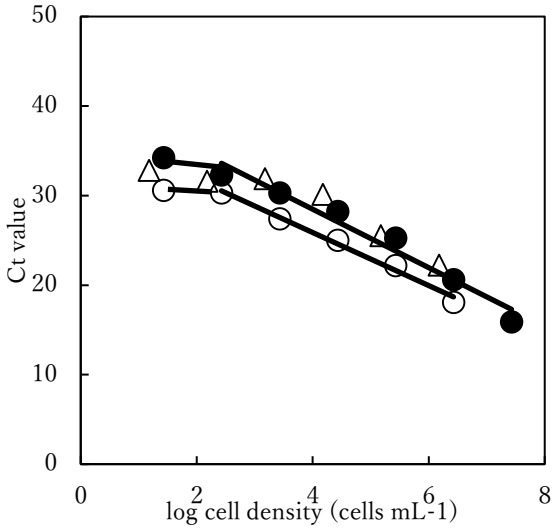

Fig. 1-5 Correlations between the Ct values and the cell densities of *Microcystis aeruginosa*.

dilution with pure water:●, *M. aeruginosa* only; ○, coexistence of *S. dorsidentiferum* and *C. ovata*,

dilution with water sample of Lake Biwa; △, *M.aeruginosa* only

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 melting points and product length of the PCR products are listed in Table 1-2. 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.

Table 1-2 Melting point and length of PCR product.

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

1.3.3 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. 1-6 (a)−(d). From the lag phase to the stationary phase, the cell densities

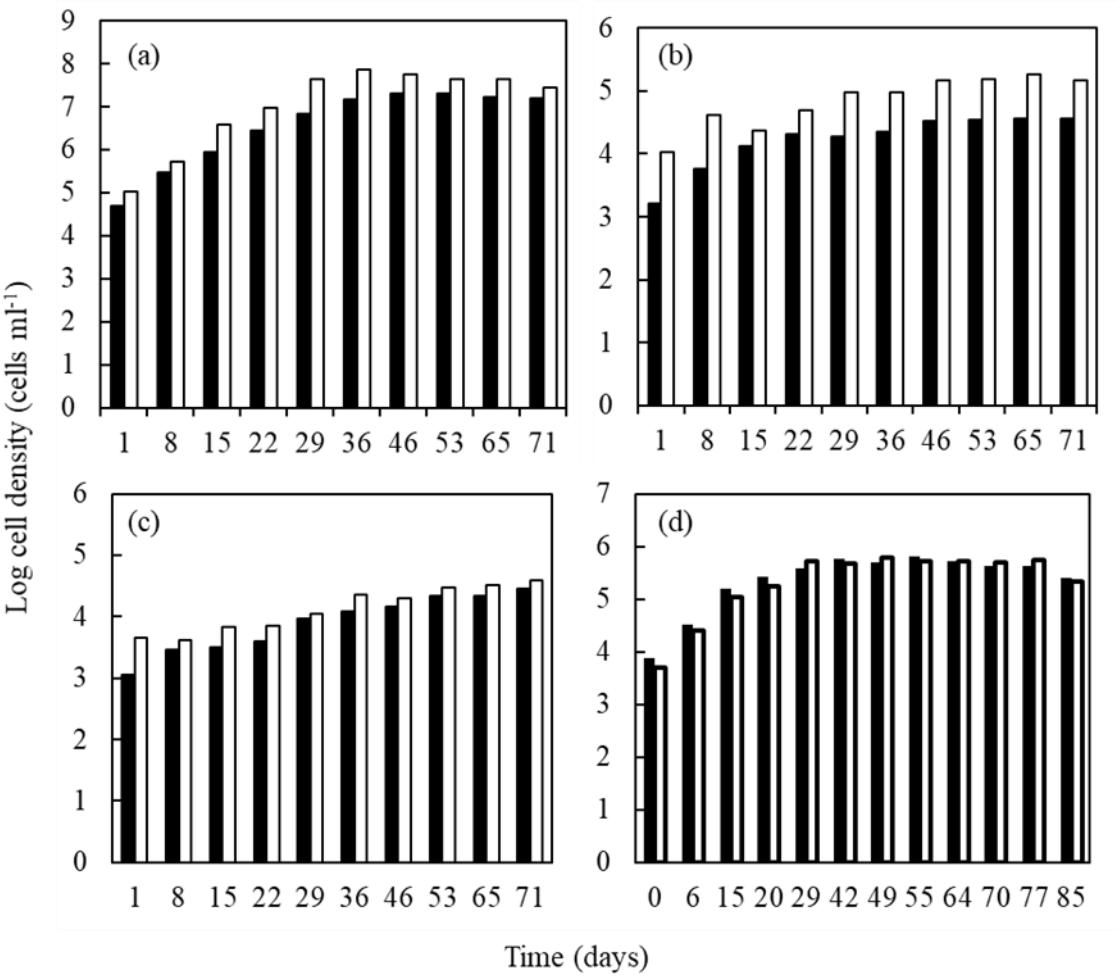

Fig. 1-6 Comparison of the cell densities of four phytoplankton using a real-time PCR assay (open bar) with those obtained by direct counting (close bar) during cultivation.

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

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. 1-7 (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 [16].

1.3.4 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 1-3). The cell density of *Microcystis* spp. by real-time PCR assay was almost four times greater than that by direct

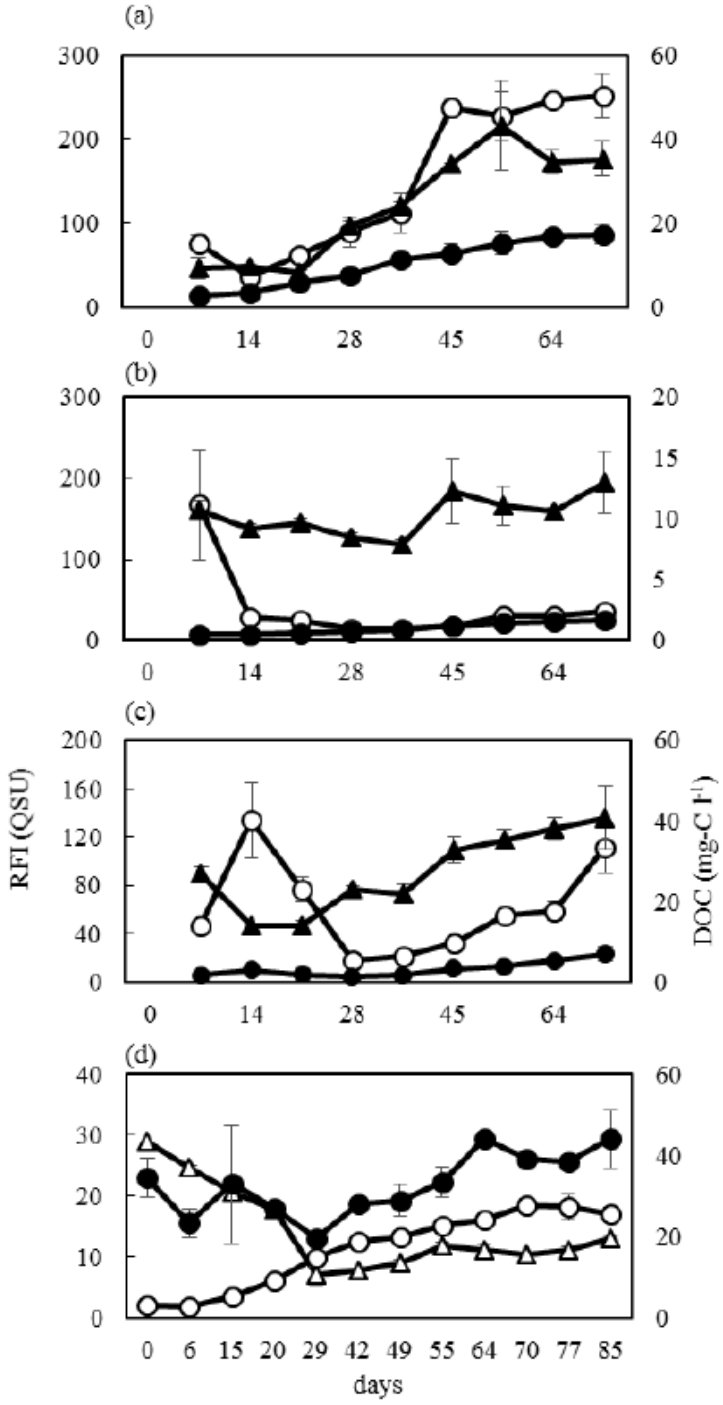

Fig.1-7 Time changes in the fluorescence intensities of peak A and C, and the DOC concentrations of four phytoplankton during cultivation.

●: Peak A, ○: Peak C, ▲: DOC

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

Table 1-3 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.

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

counting. This may be due to counting loss because of the colony formation and small size of *Microcystis* spp. Tomioka et al. [20] 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 [42, 43]. Then, we are under study for the seasonal changes 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.

2. Rapid Analysis and Dynamics of Humic Substances in Lake Biwa and Its Surrounding Rivers

2.1 Introduction

In Lake Biwa, an increase of refractory dissolved organic matter (DOM) has been a problem since 1985. The main origin of the refractory DOM may be soil humic substances and algal DOM [1]. Humic substances (HS), which are major organic constituents of fresh water, marine water, groundwater and soils, interact with heavy metals, affect the movement of hydrophobic organic pollutants in groundwater, absorb the sunlight, and may be precursors of trihalomethane formed during water treatment with chlorine [4, 24]. Humic substances are hydrophobic organic acids with molecular weights of hundreds to several hundred thousand; they are classified into humic acid (HA) and fulvic acid (FA) based on their solubility in acids and alkalis [25, 26]. More than 90% of HS in Lake Biwa and the surrounding rivers is FAs. Methods of simultaneously determining the concentration and molecular weights of HS in environmental waters by gel chromatography with a fluorescence detector were developed and applied to determining the HS in Lake Biwa and its rivers. The concentrations of aquatic HS in rivers were high in the summer and low in the winter, and the ratio of the higher molecular weight of HS was larger in the warmer season than that in the

cooler season [27, 28]. The dynamics of DOM and hydrophobic organic acid (humic substances) in Lake Biwa and its surrounding rivers have been evaluated using chemical fractionation [1, 5, 29]. From the fractionation of DOM into hydrophobic acid (HoA), hydrophobic neutral (HoN) and hydrophilic DOM (Hi) using hydrophobic resin (XAD-7HP, DAX-8), hydrophobic acids in rivers such as the Katsura River, the Kizu River, and the Yodo River were estimated to be 30-60%, which were higher than those (20-25%) in Lake Biwa [1, 5]. Furthermore, the vertical distribution and seasonal changes of DOM and each fraction in Lake Biwa were evaluated, and the increase in the concentrations of DOM, HoA, and Hi during the warm season may be attributed to algal DOM [30]. A three-dimensional excitation-emission matrix (3-DEEM) was used for algal DOM, resulting in the observation of two fulvic-like fluorescence peaks (Ex/Em=320-350/430-450 nm, 240-260/430-450 nm) and one protein-like fluorescence peak (Ex/Em=280-290/320-330 nm). The fulvic-like fluorophores derived from phytoplankton have fluorescence similar to those of fulvic acid originating in soil but exhibit mainly hydrophilic characteristics, while a protein-like fluorophore has a fluorescence similar to that of amino acid (tryptophan) and exhibits hydrophobic characteristics [15, 16]. However, the method of analyzing HS using chemical fractionation is time consuming and cannot be applied to the water samples with low concentrations of dissolved organic

carbon (DOC). Therefore, developing a method for rapidly and sensitively analyzing HS in environmental waters is necessary.

Analysis of DOM and fluorophores in Lake Biwa and its surrounding rivers found that positive relationships between the fluorescence intensities of fulvic-like DOM and DOC may be observed in the Katsura River, the Kizu River, and the Yodo River, but not the Uji River [8, 31]. These results indicate that the organic pollution in the lake was a higher contributor not only of humic substances from its watershed, but also of inner products from algae. Supposing that the relative fluorescence intensity (RFI) per DOC, RFI/DOC, of soil FA (Dando FA) is 1, the RFI/DOC values of the surrounding rivers and Lake Biwa were 0.6-0.9 and 0.1-0.3, respectively. The RFI/DOC values of Lake Biwa were 0.2-0.6 as a standard BiwakoFA (LBFA). These results suggest that the contribution of soil FA to DOM in rivers might be larger than to DOM in Lake Biwa [31].

In this chapter, the rapid analysis of HS(mainly FA) in waters using 3-DEEM and DOC was investigated as compared with fractionation analysis using microporous resin (DAX-8), and it was further applied to the dynamics of FA in Lake Biwa and its surrounding rivers.

2.2 Materials and methods

2.2.1 Reagents and apparatus

Dando FA (Dystric Cambisol, Dando, Aichi, Japan) [38] and Biwako FA (LBFA) isolated from Lake Biwa [44] were supplied by the Japanese Humic Substances Society (JHSS) and used as standards without further purification. A microporous resin (Supelco DAX-8, 40-60 mesh, mean pore size 225 Å) was used for the fractionation analysis after washing [30]. The DAX-8 resin was soaked with 0.1 M sodium hydroxide, washed with pure water, and sequentially purified by Soxhlet extraction with methanol, diethyl ether, acetonitrile, and methanol [5, 29, 30]. All other chemicals were of the best commercial grade. Pure water was prepared using a Millipore Milli-Q water purification system.

A Total Organic Carbon (TOC) meter, Shimadzu TOC-V CSH, was used to determine the DOC concentration. The fluorescence properties of DOM were measured with three-dimensional excitation-emission matrix (3-DEEM) using a Shimadzu RF-5300PC fluorescence spectrophotometer, as previously reported [15, 16]. Fluorescence readings were normalized by fluorescence intensity (Ex=345 nm/Em=450 nm) of 10 μg/l quinine sulfate (0.05 M H_2SO_4 solution) 10 QSU. The values were treated as relative fluorescence intensities (RFI). A Horiba F-51 pH meter was used to measure pH.

2.2.2 Sampling of water samples from Lake Biwa and its surrounding rivers

Water samples were collected from Lake Biwa and its surrounding rivers (Fig. 2-1). The water samples of Lake Biwa were collected monthly at Imazu (St.17B) in the northern basin since 2008 using a Van Dorn water sampler. Samples of river surface water were collected from the Ado River (AD01-08), the Ane River (AN01-04), the Amano River (AM01, 02) and the Uso River (U01, 02) flowing into Lake Biwa, and river water samples were also collected from the Katsura River (11, 13), the Uji River (21), the Kizu River (31, 3H) and the Yodo River (41) [28]. At the stations, river water samples were taken at a depth of 0.5 m and filtered through a membrane filter (0.45 μm, Millipore) as soon as possible to avoid biodegradation.

2.2.3 Fractionation of DOM in Lake Biwa and its surrounding rivers.

The fractionations of DOM in Lake Biwa and its surrounding rivers were carried out according to the method of Nagai et al. [5]. DAX-8 resin was used instead of XAD-7HP. Filtered water samples (200 mL) adjusted to a pH of 8 were passed through a DAX-8 column (50 mm x10 mm i.d.) at a flow rate of 1.5 mL/min to sorb hydrophobic substances after the DOC concentrations were measured (DOC 1). The DOC concentrations of water samples passed through a DAX-8 resin column were measured (DOC 2). The hydrophobic acids were then desorbed by backward–flow elution with 40 mL

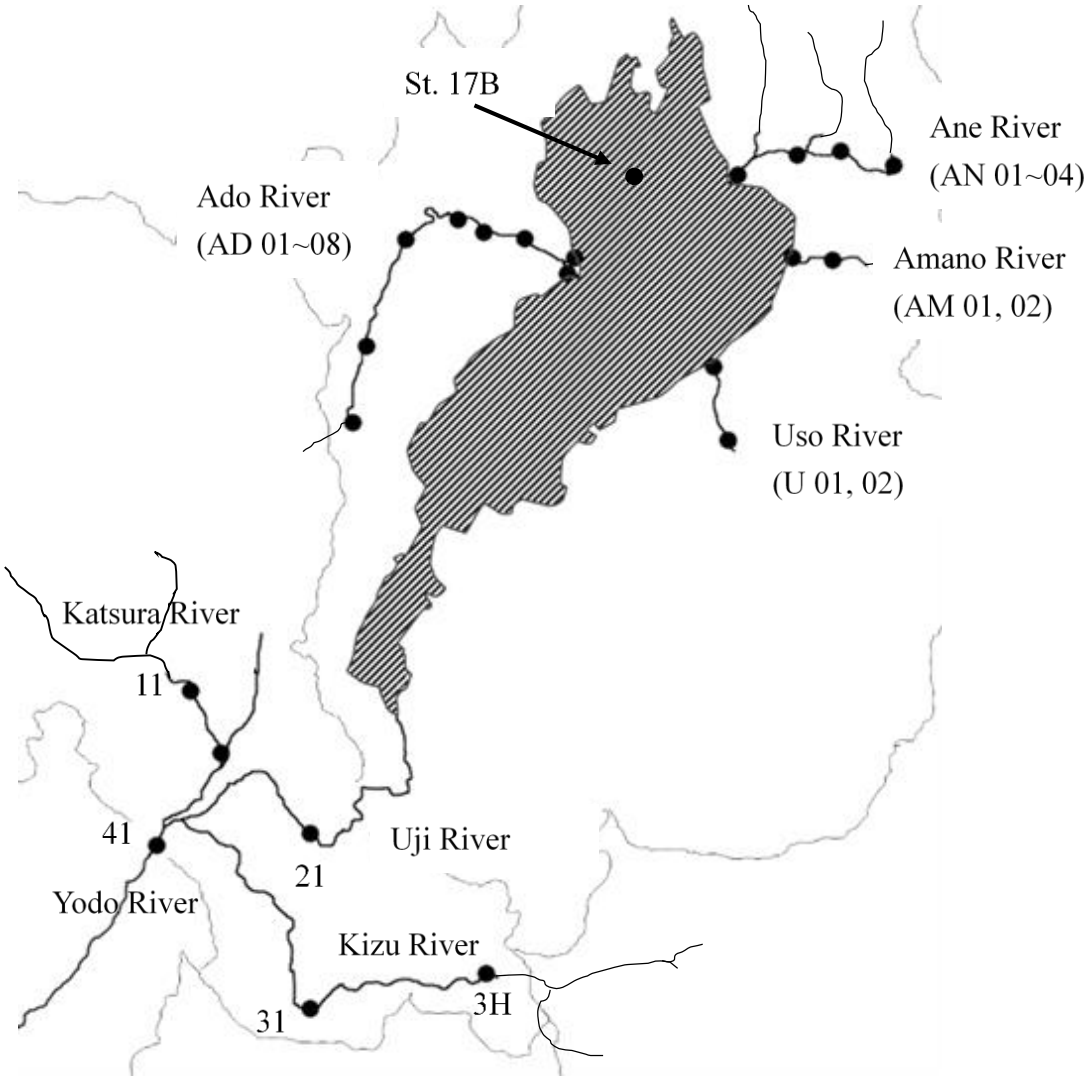

Fig. 2-1 Sampling stations on Lake Biwa and its surrounding rivers

of 0.1 M sodium hydroxide at a flow rate of 0.5 mL/min, and the DOC concentrations were measured (DOC 3). The ratio of hydrophobic bases were lower than 4% [5, 29], so in this study, hydrophobic bases were neglected.

The concentrations of hydrophobic acid (HoA), hydrophobic neutral (HoN), and hydrophilic substances (Hi) were calculated using the following equations:

HoA (hydrophobic acid) = (DOC3-B1) eluent volume/sample volume

HoN (hydrophobic neutral) = DOC1-HoA-Hi

Hi (hydrophilic substance) = DOC2-B2

Here, B1 and B2 are, respectively, blank DOC concentrations of Milli-Q water (pH 2) and 0.1M NaOH passed through a DAX-8 resin.

2.2.4 Rapid analysis of humic substances in environmental water samples using 3-DEEM and DOC measurements.

Dando FA was used as a standard to estimate the FA concentrations in rivers because the soil around the rivers is brown forest soil and the fluorescence intensities of fulvic-like fluorophores (peak A) in rivers were mainly attributed to soil FA such as Dando FA. On the other hand, fluorescence intensities of fulvic-like fluorophores in Lake Biwa were about

1/4-1/2 lower than in the rivers, and the fluorescence intensity of Biwako FA (LBFA) was 1/2 lower than that of Dando FA, so Biwako FA was used as a standard to estimate the FA concentration in Lake Biwa.

2.3 Results and discussion

2.3.1 Vertical distributions in water temperature, DOC concentrations, and fulvic-like fluorophores in the northern basin of Lake Biwa

Vertical distributions in the water temperature, DOC concentrations, and RFI values of fulvic-like fluorophores (peak A) at Imazu (St. 17B) in the northern basin of Lake Biwa are shown in Fig. 2-2. The temperatures of surface waters became higher every May than those of bottom waters. Thermocline was formed at a water depth of 10-20m during June and September, and the temperature of surface waters decreased in October. Then, the temperature difference between the surface and bottom waters was hardly observed in December and January. Changes in the bottom water temperature of Lake Biwa were small(7 to 8℃). In 2015, when the circulation period was from January to April, the temperature of surface water increased to 13.0℃ in May; temperatures were highest between July and October (the stratified period). The temperature of the surface water decreased in October, and a temperature difference between the surface and bottom waters was hardly observed between January and April 2016. The temperature of the

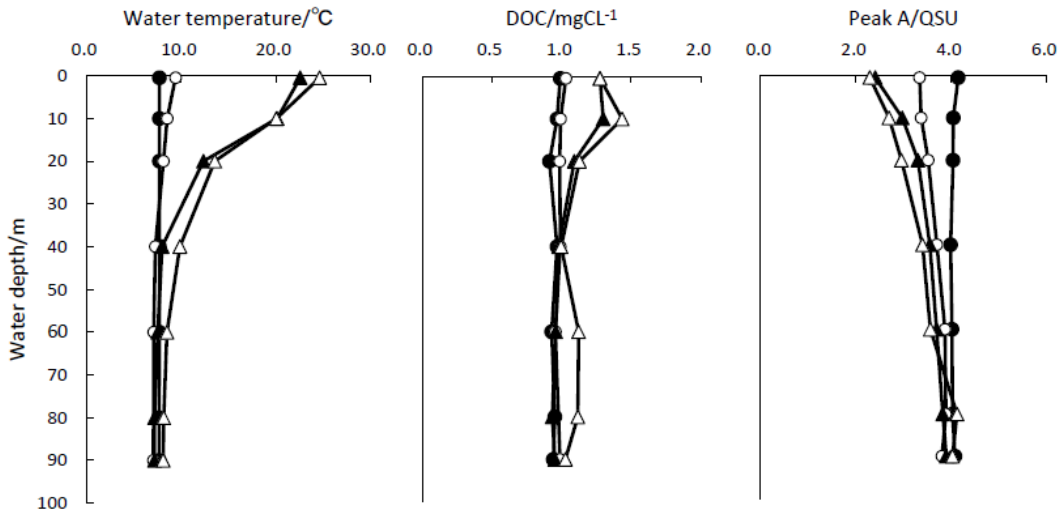

Fig. 2-2 Vertical distributions of water temperature, DOC and fluorescent intensities of fulvic-like fluorophores (peak A) at Imazu (St. 17B) in the northern basin of Lake Biwa in 2015

● February, ○ April, ▲ July, △ October

surface water increased to 14.0℃in May, and the thermocline was formed at a water depth of 10-20m, similar to that in 2015.

The DOC concentration in the northern basin of Lake Biwa (St. 17B) between January and March 2015 was about 1.0mgC/L regardless of water depth, but a high DOC concentration (1.47mgC/L) was observed at a water depth of 80m, where the RFI of protein-like fluorophore (Ex/Em=280/320 nm) also showed a high value (9.55 QSU). As the RFI values of protein-like fluorophores in the northern basin of Lake Biwa were usually 2-4 QSU, and the DOC concentrations and RFI values at other water depths in April 2015 were about 1.0mgC/L and 2-4 QSU, respectively, the high DOC concentration and RFI value of protein-like fluorophore at a water depth of 80m may be due to algal DOM from phytoplankton. DOC concentrations at water depths of 0.5 m and 10 m from July to October 2015 were 1.22-1.36 mgC/L and 1.17-1.44 mgC/L, respectively, and the DOC concentrations at 40m were 0.85-1.00 mgC/L. The DOC concentrations at water depths of 40-90 m under the thermocline (water depth: 10-20 m) tended to be lower than those at water depths of 0.5-10 m and almost constant. DOC concentrations were about 1.0 mg/L, regardless of water depth, from January to April 2016, and those at water depths of 0.5 m and 10 m from July to October 2016 were 1.27-1.39 mgC/L and 1.36-1.40 mgC/L, respectively, while DOC concentrations at water depth less than 40 m were 1.0 mg/L, and their

changes were small. The increase in DOC concentrations at water depths of 0.5-20 m in summer may be due to the bioactivity of phytoplankton.

The RFI values of fulvic-like fluorophores (Ex/Em=340/435nm) in surface waters were lower than those in bottom water during the stratified period and were particularly lower from July to October than in other months; meanwhile, the changes in the values were small during the circulation period. The decrease in the RFI values of fulvic-like fluorophores from July to October may be attributed to fluorescence quenching and the degradation of high-molecular substances by solar irradiation [45].

2.3.2 Seasonal changes in water temperature, DOC, and fulvic-like fluorophore concentration in rivers flowing into Lake Biwa and the Yodo rivers.

The river samples of four Yodo rivers (Katsura, Uji, Kizu and Yodo) and four rivers flowing into Lake Biwa (Ado, Ane, Amano and Uso) were collected in October 2014, and seasonal changes in the behavior of DOM, fulvic-like fluorophores, and so on were measured. The Uji River is affected by Lake Biwa because the water of Lake Biwa flows into the Uji River through the Seta River and the Yanagase impounding dam.

In the Yodo rivers, the DOC concentrations and RFI values of fulvic-like fluorophores were in the following order: Katsura River ≦ Uji River <

Yodo River ≦Kizu River (Table 2-1). The DOC concentrations and RFI values of fulvic-like fluorophores (peak A) were especially high in the Kizu River, and they tended to be high in May and August when the water temperature was high. The concentrations of humic substances (mainly FA) in the Kizu River were about two times higher than those in the Katsura and Uji rivers, and were high in the upper stream of the Kizu River. These results may be due to an increase in water temperature and microbiological activities, which cause the biological conversion of organic matter present in water and soils into humic substances during warmer seasons. Furthermore, high concentrations of FA in the Kizu River might be attributed to the soils around the rivers because, using gel chromatography with a fluorescence detector, the ratio of higher-molecular FA was determined to be high [27, 28], while the DOC concentrations and RFI values of fulvic-like fluorophores in the Uji River were lower than those in the Kizu and Yodo rivers, and seasonal changes were not observed. The results may be influenced by Lake Biwa through the Seta River.

Table 2-1 Analytical results of DOC and fulvic-like fluorophore (peak A) in Yodo rivers (2016)

River	DOC/mgCL^{-1}				peak A/QSU			
	Jan.	May	July	Oct.	Jan.	May	July	Oct.
Katsura	1.26	0.90	1.24	0.88	7.22	7.23	8.72	4.97
Uji	1.65	1.37	1.99	1.62	5.68	5.99	7.69	6.57
Kizu (31)	1.24	2.10	2.29	1.41	11.19	15.26	16.14	13.44
Kizu (3H)	1.50	2.22	2.37	1.67	12.46	17.30	19.84	14.82
Yodo	1.33	1.55	1.92	1.44	8.89	11.22	12.60	8.83

To investigate the effects of the water quality of rivers flowing into Lake Biwa, river samples were collected on October 17, 2014; April 27 and October 26, 2015; and November 21, 2016; and water temperatures, DOC concentrations, and RFI values of fulvic-like fluorophores in these river samples were measured. The DOC concentrations of the Ado River, the Ane River, and the Amano River were 0.11-0.57 mgC/L, 0.23-0.74mgC/L, and 0.34-0.76mgC/L, respectively. The DOC concentrations in these rivers were lower than 1 mgC/L, with the exception of the Uso River, and lower than those in the Yodo rivers (0.53-2.29 mgC/L). Moreover, as the differences in the RFI values of fulvic-like fluorophores and DOC concentrations between the upstream and downstream of the Ado and Ane rivers were small, it is considered that the effects of living drainage and agricultural drainage might be relatively small and the contribution of soil FA was large.

2.3.3 Relationships between DOM and fulvic-like fluorophores in Lake Biwa and its surrounding rivers.

The relationships between the RFI values of fulvic-like fluorophores (peak A) and DOC concentrations in Lake Biwa and its surrounding rivers are shown in Fig. 2-3. Positive correlations between the RFI values of fulvic-like fluorophores (peak A) and DOC concentrations in four rivers flowing into Lake Biwa and Yodo rivers were observed, with the exception of the Uji

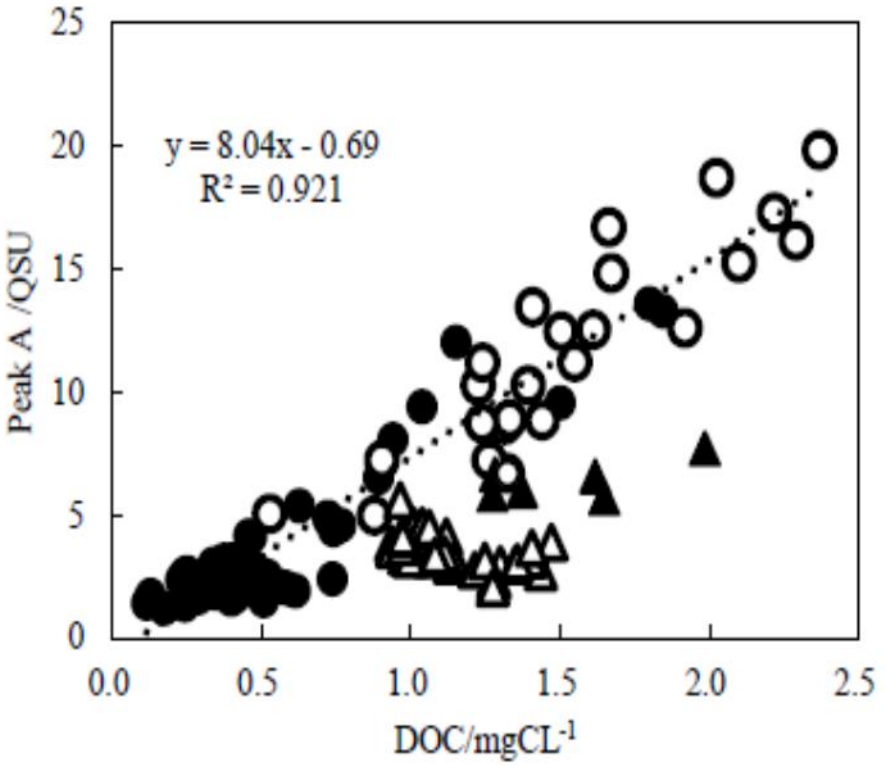

Fig. 2-3 Relationships between the fluorescent intensities of peak A and DOC concentrations in Lake Biwa and its surrounding rivers
● rivers flowing into Lake Biwa, ○ Yodo rivers, ▲ Uji River,
△ Lake Biwa (St. 17B)

River. The RFI/ DOC values of peak A in Lake Biwa were about 1/4-1/3 lower than those in the surrounding rivers, with the exception of the Uji River.

The RFI/DOC value of peak A of Dando FA extracted from brown forest soil was 14.7, which was higher than that of Biwako FA (LBFA) isolated from the water of Lake Biwa using DAX-8, 7.53. The ratios of fluorescence intensities of the Katsura, Kizu, and Yodo rivers were 0.34-0.68, and those of the Uji River were 0.23 - 0.35 when the ratio of Dando FA was 1.0. Meanwhile, the ratios of fluorescence intensities of rivers flowing into Lake Biwa were 0.35 - 0.93, and those of Lake Biwa were 0.12-0.30. The fluorescence intensities of Lake Biwa were 0.24-0.59 as a standard of Biwako FA.

These results suggest that the contribution of soil FA to DOM in the rivers surrounding Lake Biwa might be higher than its contribution to DOM in Lake Biwa.

2.3.4 Rapid analysis of humic substances in rivers by 3-DEEM and DOC measurement

Humic substances (hydrophobic acids) in environmental waters have been measured by gel chromatography with a fluorescence detector [28] and chemical fractionation using hydrophobic resins [5, 29]. However, these

methods are time consuming and cannot be applied to water samples with low DOC concentrations. Then, the rapid analysis of humic substances in waters using a three-dimensional excitation emission matrix (3-DEEM) and DOC was investigated as compared with fractionation analysis. Relationships between concentrations of FA and HoA in Yodo rivers are shown in Fig. 2-4. The FA concentrations were calculated by 3-DEEM and DOC using soil FA (Dando FA) as a standard because the soils around the rivers are brown forest soils. The HoA concentrations were calculated using fractionation analysis. Both results in the Yodo rivers, with the exception of the Uji River, were in good agreement because positive linear correlations were obtained ($y = 1.19x - 0.10$ ($R^2 = 0.884$)).

Analytical results of the concentrations of FA, HoA, Hi, and Total-DOC in the Yodo rivers collected in August and October 2015 and January, May, July, and October 2016 are listed in Table 2-2. The concentrations of hydrophobic acid (HoA) in the Katsura, Kizu, and Yodo rivers were 0.28 - 0.64 mgC/L, 0.70 - 1.20 mgC/L, and 0.59 - 0.86 mgC/L, respectively; the concentrations were high in May and August and low in October and January. Furthermore, the concentrations of hydrophilic substances in the Katsura, Kizu, and Yodo rivers were 0.24-0.83 mgC/L, 0.42-1.00 mgC/L, and 0.53-0.85mgC/L, respectively; the concentrations were high in summer and low in winter, with the exception of the Katsura River (January 2016). The

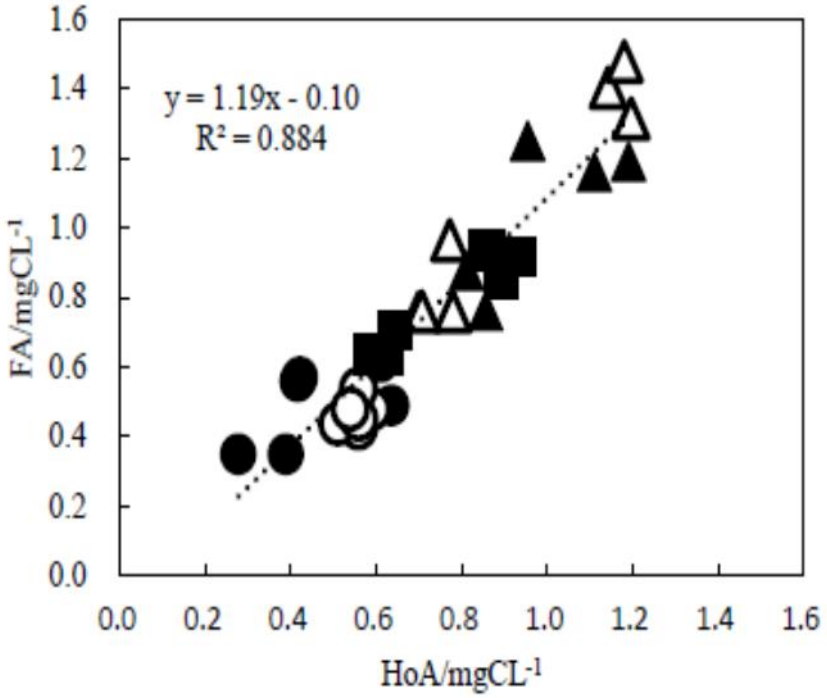

Fig. 2-4 Relationships between the concentrations of FA and HoA in Yodo rivers

● Katsura River, ○ Uji River, ▲ Kizu River (No. 31),
△ Kizu River (No. 3H)

Table 2-2 Analytical results of FA, HoA and Hi in Yodo rivers.

River	FA/mgCL^{-1}	DOC/mgCL^{-1}		
		HoA	Hi	Total
Jan. 2016				
Katsura	0.57	0.42 (33.4)	0.83 (66.0)	1.26
Uji	0.44	0.51 (31.0)	0.77 (46.6)	1.65
Kizu (31)	0.88	0.81 (53.9)	0.61 (40.4)	1.24
Kizu (3H)	0.97	0.77 (51.3)	0.72 (48.1)	1.50
Yodo	0.71	0.64 (48.2)	0.53 (39.7)	1.33
May 2016				
Katsura	0.56	0.42 (59.0)	0.25 (35.7)	0.90
Uji	0.45	0.56 (40.8)	0.61 (44.1)	1.37
Kizu (31)	1.16	1.11 (55.0)	0.80 (39.8)	2.10
Kizu (3H)	1.32	1.20 (53.9)	0.90 (41.9)	2.22
Yodo	0.85	0.89 (57.6)	0.60 (39.0)	1.55
July 2016				
Katsura	0.62	0.61 (48.0)	0.58 (43.7)	1.24
Uji	0.54	0.56 (43.8)	1.19 (47.1)	1.99
Kizu (31)	1.19	1.19 (51.8)	0.84 (36.8)	2.29
Kizu (3H)	1.48	1.18 (49.8)	1.00 (42.1)	2.37
Yodo	0.92	0.93 (48.4)	0.85 (44.4)	1.92
Oct. 2016				
Katsura	0.35	0.39 (44.1)	0.39 (44.2)	0.88
Uji	0.48	0.54 (33.4)	0.99 (61.1)	1.62
Kizu (31)	0.76	0.70 (49.4)	0.58 (41.3)	1.41
Kizu (3H)	0.76	0.71 (42.2)	0.74 (44.0)	1.67
Yodo	0.64	0.59 (40.7)	0.62 (42.7)	1.44

HoA: hydrophobic acid, Hi: hydrophilic DOM.

The values in parentheses are the ratios (%).

concentrations of HoA in the Katsura, Kizu, and Yodo rivers were higher than or similar to the concentrations of Hi. The ratios of HoA in the Yodo rivers were 33.4 - 63.6%. The concentrations of HoA in the Kizu River (31, 3H) and Yodo River (41) were higher (40.3 - 63.6%) than those in the Katsura River (11) (33.4 - 48.0%), which may be due to soil humic substances around the rivers.

On the other hand, the concentrations and ratios of HoA in the Uji River were 0.51 - 0.59 mgC/L and 31.0 - 46.1%, respectively, which were lower than those in Kizu and Yodo rivers. The concentrations of Hi were 0.49-1.19 mgC/L, and no seasonal changes were observed. As the water quality of the Uji River was similar to that of Lake Biwa, the lower ratios of the Uji River may be affected by Lake Biwa because water of Lake Biwa flows into the Uji River through the Seta River and the Yanagase impounding dam.

It is clear from these results that rapid analysis with 3-DEEM and TOC meter using Dando FA from brown forest soil as a standard is useful in determining FA concentrations in rivers without the effect of Lake Biwa.

As it is difficult to determine the DOC concentrations in rivers flowing into Lake Biwa using fractionation analysis because they were low, they were calculated using rapid analysis. Analytical results of the concentrations of DOC and FA in the Ado and Ane rivers are listed in Table 2-3. DOC concentrations of the Ado and Ane rivers were 0.11 - 0.57 mgC/L

Table 2-3 Analytical results of DOC and fulvic acid (FA) in Ado River and Ane River

River	Oct. 2014		Oct. 2015		Apr. 2015		Nov. 2016	
	DOC	FA	DOC	FA	DOC	FA	DOC	FA
Ado River								
AD02	0.34	0.16	0.24	0.06	0.38	0.15	0.51	0.09
AD03	0.25	0.18	0.40	0.08	0.41	0.12	0.57	0.10
AD06	0.26	0.15	0.17	0.05	0.30	0.13	0.51	0.06
AD07	0.27	0.15	0.11	0.07	0.24	0.11	0.49	0.09
AD08	0.24	0.18	0.13	0.09	0.29	0.11	0.52	0.13
Ane River								
AN01	0.40	0.22	0.44	0.12	0.39	0.11	0.74	0.29
AN02	0.48	0.20	0.30	0.11	0.41	0.15	0.58	0.12
AN03	0.46	0.20	0.39	0.15	0.36	0.15	0.50	0.09
AN04	0.29	0.12	0.23	0.14	0.35	0.11	0.61	0.11

unit: mgC L^{-1}

and 0.23-0.74 mgC/L, respectively, so rapid analysis was used to determine the FA concentrations in these rivers. The FA concentrations in the Ado and Ane rivers were 0.05 - 0.18 mgC/L and 0.09 - 0.29 mgC/L, respectively, and the ratios of FA concentrations upstream were higher than those downstream. The ratios of FA upstream in the Ado River (AD08) were about 70%, which were higher than those of other rivers. The results indicate that the high ratios of FA in the upstream may be especially influenced by soil FA.

2.3.5 Rapid analysis of humic substances in Lake Biwa using 3-DEEM and DOC measurements

Analytical results of the concentrations of FA, HoA, Hi and Total-DOC in the northern basin of Lake Biwa (St.17B) using rapid analysis and

fractionation analysis are listed in Table 2-4. Here, Biwako FA was used as a standard instead of Dando FA.

The concentrations of hydrophobic acid (HoA), hydrophilic substances (Hi), and Total-DOC during the circulation period (January 2016) were 0.34-0.41 mgC/L, 0.41-0.43 mgC/L, and 0.92-1.08 mgC/L, respectively, irrespective of water depths. The ratios of HoA and Hi were 35.8-38.8% and 38.7-43.5%, respectively. The HoA concentrations in other months which were 0.28-0.48 mgC/L, tended to be higher at water depths of 0.5-20 m and 80m. At water depths of 0.5-20 m, the concentrations of Hi and Total-DOC, which were 0.67-0.92 mgC/L and 1.12-1.49 mgC/L, respectively, were higher than those at other water depths (ca. 0.5 mgC/L and ca. 1 mg/L). High concentrations of Hi (0.7-0.8 mgC/L) were observed at a water depth of 80m in July and November 2016. As the ratios of HoA and Hi were 28-34% and 51-60%, respectively, the ratio of Hi was higher than the ratio of HoA. These results suggest that high DOC concentrations in surface waters during the stratified period may be due to the production by phytoplankton.

The concentrations of FA and HoA by both methods were in good agreement at water depths of 10-20 m in the northern basin of Lake Biwa (St. 17B) during the stratified period. However, at a water depth of 0.5 m in summer the FA concentrations by rapid analysis were lower than the HoA concentrations by fractionation analysis. The results may be dependent on

Table 2-4 Analytical results of FA, HoA and Hi in Lake Biwa (St. 17B)

Sampling	Water depth/m	FA/mgCL^{-1}	DOC/mgCL^{-1}		
			HoA	Hi	Total
Aug. 2015					
	0.5	0.23	0.43(31.1)	0.84(60.5)	1.39
	10	0.39	0.47(33.4)	0.75(52.7)	1.41
	20	0.44	0.34(34.1)	0.57(56.7)	1.00
	40	0.44	0.32(33.2)	0.50(51.3)	0.97
	80	0.51	0.38(31.7)	0.67(56.2)	1.11
Jan. 2016					
	0.5	0.41	0.38(36.5)	0.42(40.3)	0.92
	10	0.42	0.39(36.7)	0.41(38.7)	1.06
	20	0.39	0.39(36.2)	0.43(39.8)	1.08
	40	0.45	0.34(35.8)	0.41(43.5)	0.94
	80	0.57	0.41(38.8)	0.43(40.6)	1.06
July. 2016					
	0.5	0.25	0.44(34.7)	0.69(53.9)	1.27
	10	0.39	0.43(28.8)	0.92(61.6)	1.49
	20	0.45	0.39(30.8)	0.67(53.1)	1.26
	40	0.41	0.31(30.4)	0.59(58.5)	1.01
	80	0.42	0.46(35.5)	0.72(55.8)	1.29
Oct. 2016					
	0.5	0.37	0.46(33.8)	0.75(55.4)	1.35
	10	0.38	0.48(35.3)	0.75(55.5)	1.36
	20	0.44	0.46(32.8)	0.85(60.6)	1.41
	40	0.45	0.30(30.9)	0.55(56.1)	0.97
	80	0.51	0.30(30.8)	0.53(54.9)	0.97
Nov. 2016					
	0.5	0.38	0.33(28.2)	0.67(56.4)	1.18
	10	0.37	0.37(30.8)	0.70(57.9)	1.20
	20	0.36	0.35(31.2)	0.68(60.2)	1.12
	40	0.38	0.28(30.9)	0.51(56.2)	0.91
	80	0.47	0.34(25.9)	0.78(59.7)	1.31

HoA: hydrophobic acid, Hi: hydrophilic DOM.

The values in parentheses are the ratios (%).

the fluorescence quenching and degradation of fulvic-like fluorophores in the surface water of the northern basin of Lake Biwa by solar irradiation [39]. Meanwhile, FA concentrations at a water depth of 80 m were higher than the HoA concentration which may be due to the elution of humic substances from the sediment [46-48]. Therefore, rapid analysis cannot be easily used to analyze the dynamics of FA in the northern basin of Lake Biwa, however comprehensively evaluating DOC, fluorescence intensities and molecular distributions of DOM and fluorophores in Lake Biwa will be helpful in understanding the effects of solar irradiation and the elution of humic substances from sediment.

2.4 Conclusion

In Lake Biwa, an increase of refractory dissolved organic matter (DOM) has been a problem since 1985. The main origin of the refractory DOM may be soil humic substances and algal DOM. The fractionation of hydrophobic acids (humic substances) with DAX-8 resin is time consuming and cannot be applied to water samples with low DOC concentrations. Then, the rapid analysis of humic substances in waters using three-dimensional excitation emission matrix (3-DEEM) and DOC was investigated, as compared to fractionation analysis, and applied to the dynamics of humic substances in Lake Biwa and its surrounding rivers. Humic substances in the Yodo rivers

as measured by rapid analysis using soil fulvic acid (Dando FA) as a standard were in relatively good agreement with those by fractionation analysis, with the exception of the Uji River, of which waters are affected by Lake Biwa by running through the Seta River. This rapid analysis was also applied to the dynamics of humic substances in the rivers flowing into Lake Biwa. In the case of the rapid analysis of humic substances in Lake Biwa, Biwako FA was used as a standard instead of Dando FA.

3. Effects of Photoirradiation on the Characteristics of Dissolved Organic Matter in Lake Biwa and its Surrounding Rivers

3.1 Introduction

Dissolved organic matter (DOM) is the main component of the organic substances in natural water. Hence, DOM dynamics and characteristics can affect multiple biogeochemical processes in aquatic environments, including light penetration, pH buffering, oxygen consumption, nutrient availability, and toxicity of pesticides and metals [4]. Many studies have evaluated the characteristics and sources of DOM in Lake Biwa using chemical fractionation methods [1, 5], the natural carbon stable isotope ratio [6, 7], and spectroscopic analyses [1, 8, 9, 12].

From our results of the fractionation of DOM in the northern basin of Lake Biwa collected from 2002 to 2004, hydrophobic acids (humic substances: HS), hydrophobic neutral DOM, and hydrophilic DOM were estimated to be about 20–25%, 15–20%, and 55–65%, respectively [1]. The dominant fractions of HS were fulvic acids (FA) [27, 28], and some hydrophobic DOM and hydrophilic DOM may be produced by phytoplankton. The fulvic-like fluorophores (Ex/Em = 320-350/430-450 nm, 240-260/430-450 nm) and a protein-like fluorophore (Ex/Em = 280-290/320-330 nm) were always detected in Lake Biwa using a three-dimensional excitation-emission

matrix (3-DEEM) [15, 16]. The dynamics of DOM and fluorophores in Lake Biwa were evaluated, and it was found that the fluorescence intensities of fulvic-like fluorophores in surface water during the completely stratified period (July-September) tend to be lower than those during other months and in bottom water, even though DOC concentrations in surface water tend to be higher than those of others due to high primary production. These phenomena might be due to the photolysis of fulvic-like fluorophores in surface water during the stratified period. It has been reported that the fluorescence of DOM in the lake has sources other than epilimnetic primary production [11], and it is degraded by solar radiation in the epilimnion during summer [10]. Furthermore, seasonal changes in the UV absorbance of lake water were different from those of DOC. The clearly positive correlation between UV absorbance and HS was observed in an inner bay (Akanoi); however, the correlation in offshore water was weaker than in the inner bay [1]. Therefore, it is necessary to analyze the behavior and photoirradiation effects of both allochthonous and autochthonous DOM separately. Many studies of the photochemical degradation of DOM and aquatic HS have been performed [32-36]. However, few studies about the effects of photoirradiation on the characteristics of algal DOM have been conducted.

In this chapter, the effects of photoirradiation (solar and Xe-lamp

irradiation) on changes in the fluorescence intensities, DOC concentrations, and molecular weights of DOM in Lake Biwa and its surrounding rivers were evaluated and compared with the results of soil humic acid (HA), FA, lake FA, and algal DOM. Furthermore, influences of wavelength regions on the characteristics of DOM were examined by Xe-lamp irradiation, using two kinds of wavelength cut filters.

3.2 Materials and methods

3.2.1 Reagents and apparatus

Aldrich HA (extracted from peat soil: Aldrich Chemicals), Dando HA and FA (Dystric Cambisol, Dando, Aichi, Japan) [38], were used as soil HS. Aldrich HA was purified in accordance with the procedures of a previous paper [27]. Dando HA and FA, and Lake Biwa FA（LBFA)[44] were supplied by the Japan Humic Substances Society (JHSS) and used without further purification.

Three kinds of phytoplankton—*Microcystis aeruginosa* (blue-green algae NIES-109, Lake Yogo, Shiga), *Staurastrum dorsidentiferum* (green algae NIES-665, Lake Biwa, Shiga), and *Cryptomonas ovata* (dark brown whip-hair algae NIES-275, Tsuchiura, Ibaraki)—which were supplied by National Institute for Environmental Studies, were cultivated in accordance with the procedures of the previous paper [15, 16]. These phytoplankton were

selected as the predominant algal species in Lake Biwa [16]. All other chemicals were of the best commercial grade. Pure water was prepared by a Millipore Milli-Q water purification system.

Three incubators—an Iwaki LIB-302, an Iwaki ICB-142L, and an NIK LH-100SP—were used to cultivate and biodegrade plankton. A TOMY BS-305 autoclave was used for sterilization. An Olympus IX71N-22PH-D microscope was used to count the number of algal cells. A TOC meter (Shimadzu TOC-V CSH) was used for the determination of DOC concentration. A Kubota KN-70 centrifuge and a Hitachi Koki Himac CR20G Ⅲ refrigerated centrifuge were used for the fractionation and concentration of algal DOM, respectively. A Horiba F-51 pH meter and a TOA CM-60S EC meter were used for the measurement of pH and electric conductivity in environmental water samples, respectively.

3.2.2 Procedure for the characterization of DOM and humic substances in environmental water samples

Environmental water samples were collected from Lake Biwa and its surrounding rivers (Fig. 3-1). The water samples of Lake Biwa were monthly collected at Imazu in the northern basin (St. 17B, 35°23’41N, 136°07’57E) using a Van Dorn water sampler. Water temperature and other basic parameters were measured by a Hydrolab DS5 water quality instrument.

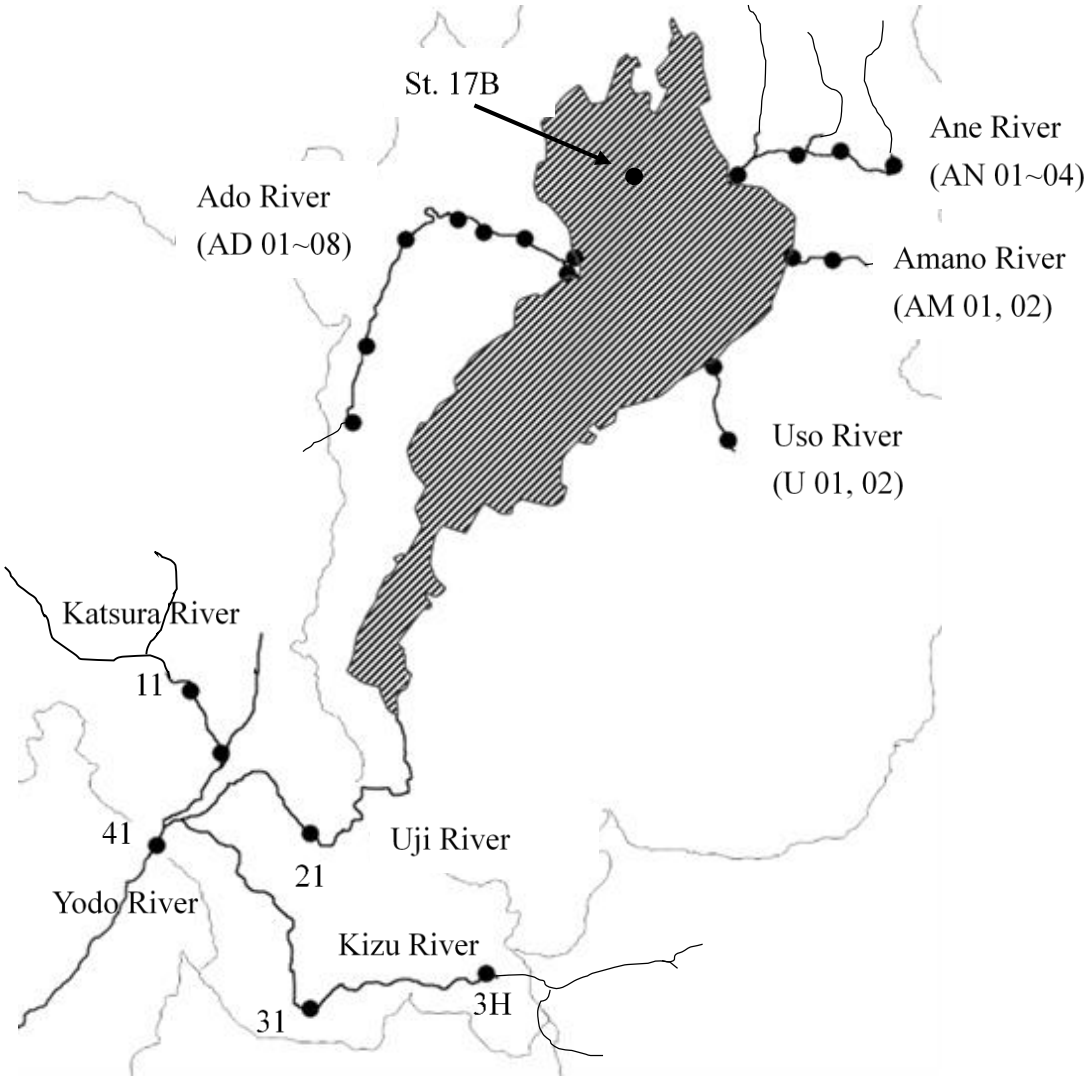

Fig. 1-3 Sampling stations of Lake Biwa and its surrounding rivers

River water samples were collected from the Ane and Ado Rivers flowing into Lake Biwa. River water samples were collected from the Katsura, Uji, Kizu, and Yodo Rivers [27, 28]. In the laboratory, all water samples were filtered through a membrane filter (0.45 μm, Millipore) to avoid biodegradation, 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 substances were analyzed by using gel chromatography with a fluorescence detector that was developed to simultaneously determine the concentration and molecular weight of humic substances [28]. The apparatus used for gel chromatography was a Shimadzu LC-20AD chromatography pump equipped with a Shimadzu RF-20A_{XS} fluorescence detector and a Shimadzu SPD-20AV UV-VIS detector. The test samples (100 μL) were applied to a gel filtration column, Superose HR10/30 (300 x 10 mm i.d.; GE Healthcare), and a Shimadzu C-R7A Chromatopac or a Shimadzu LC solution was used for data analysis. A 0.0l M sodium hydroxide solution was used as an eluent at a flow rate of 0.4 ml min^{-1}. The fluorescence properties of DOM were measured with three-dimensional excitation-emission matrix (3-DEEM), using a Shimadzu RF-5300PC fluorescence spectrophotometer, as previously reported [15, 16]. Fluorescence readings were normalized by fluorescence intensity (Ex=345 nm/Em=450 nm) of 10 μg/l quinine sulfate (0.05 M H_2SO_4 solution) 10 QSU.

The values were treated as relative fluorescence intensity (RFI).

3.2.3 Cultivation of phytoplankton [15, 16]

The three kinds of phytoplankton were cultivated in an improved VT medium as previously reported [15]. *Microcystis aeruginosa* and *S. dorsidentiferum* were grown in one-liter (1 L) flasks at 20℃ and 2000 lux under a 12 h : 12 h light/dark cycle. *Cryptomonas ovata* was grown in a 1 L triangle flask at 15℃ and 2000 lux under the same light/dark cycle.

3.2.4 Photoirradiation of HS, algal DOM, and DOM in environmental water by sun and a Xe lamp

A 40 ml water sample (HS, algal DOM or environmental water) adjusted to pH 8 with NaOH in a 50 ml quartz glass container (60 mm high with a diameter of 40 mm, the quartz glass about 2mm thick) was irradiated inside darkness-controlled chamber at 25℃ using a Ushio Xe lamp (150 W) [49]. The distance between a quartz glass container and the Xe-lamp was fixed to 10 cm. As quartz glass transmits nearly 100% of light in the ultraviolet-visible region [50], quartz glass containers were used for photoirradiation. The solutions (2.0-2.5 mg/L) of HS such as Aldrich HA, Dando HA, Dando FA, and LBFA were prepared. Algal DOM solutions were prepared from the medium of three kinds of phytoplankton by filtration through a membrane

filter (0.45 μm, Millipore). Time changes in the fluorescence properties (3-DEEM) and DOC of these sample solutions were measured after 1, 3 and 5 h by Xe lamp irradiation. Furthermore, the effects of the wavelength region on the characteristics of DOM in these samples were examined by Xe lamp irradiation for 5 h using two kinds of wavelength cut filters, AGC Techno glass UV-29 (cut below 290 nm) [51], or HOYA W-Y 495 (cut below 495 nm) [52].

Time changes in the fluorescence properties (3-DEEM) and DOC concentrations of the same samples in a 50 ml quartz glass container were measured after 1, 3 and 5 h by solar radiation on the roof of the No. 6 building (Kyoto Institute of Technology). The amounts of solar radiation were measured with an illuminometer (Delta OHM PYRA03) during the irradiation experiment.

3.3 Results and discussion

3.3.1 Behavior of DOM and fluorophores in Lake Biwa during stratified and circulation periods

Vertical distributions in the water temperature and concentrations of DOC at Imazu (St. 17B) in Lake Biwa during the completely stratified period (July–September) and the circulation period (January–March) in 2015 and 2016 are shown in Fig. 3-2 (a) and (b), respectively. During the stratified

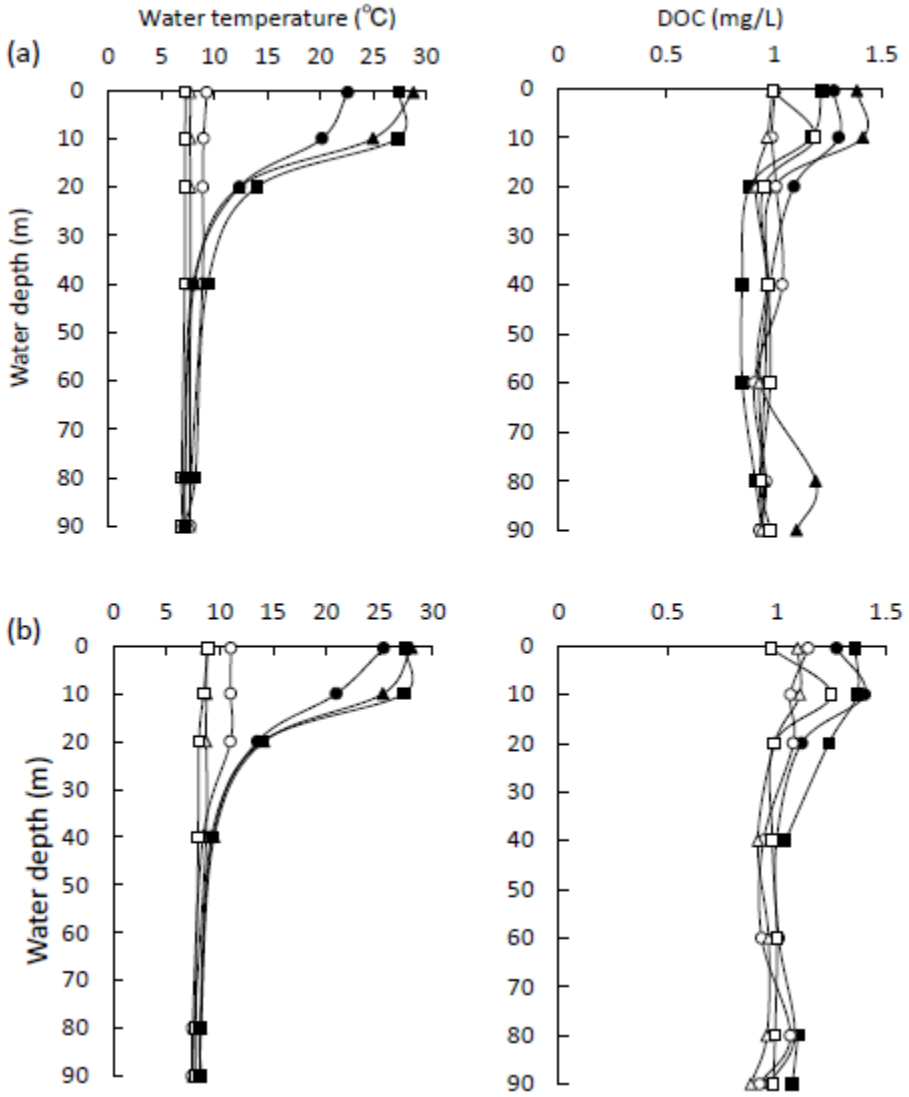

Fig. 3-2 Vertical distributions of water temperature and DOC at Imadzu (St.17B) in the northern basin of Lake Biwa in 2015 (a) and 2016 (b)

circulation period: ○ January, △ February, □ March

stratified period: ● July, ▲ August, ■ September

period, when the thermocline was formed in Lake Biwa, the DOC concentrations at a water depth of 0.5 m were 1.27–1.36 mgC/l and tended to be higher than those at a water depth of 90 m (ca. 1.0 mg/L). The DOC concentrations during the circulation period were about 1.0 mgC/l regardless of the water depth, and their seasonal changes were relatively small.

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 observed by 3-DEEM. As an example, the 3-DEEM contour plot of surface water at St. 17B in Lake Biwa (December 2015) is shown in Fig. 3-3. The vertical distributions in the RFI values of peak A at St. 17B during the circulation period (January–March) and the completely stratified period (July–September) in 2015 and 2016 are shown in Fig. 3-4 (a) and (b), respectively. The RFI values of peak A of surface waters during the stratified periods in 2015 and 2016 were 1.81–2.41 QSU and 2.05–2.55 QSU, respectively, which were lower than those of bottom waters. Furthermore, vertical changes in the gel chromatograms of fulvic-like DOM (peak A, Ex/Em = 340/435 nm) in Lake Biwa collected in February and July, 2016, are shown in Fig. 3-5 (a) and (b), respectively. Four peaks, peak h (RT = 16 min), peak 1 (RT = 29–30 min), peak 2 (RT = 32 min), and peak 3 (RT = 35 min) were detected. It has been reported that the peaks 1 and 2

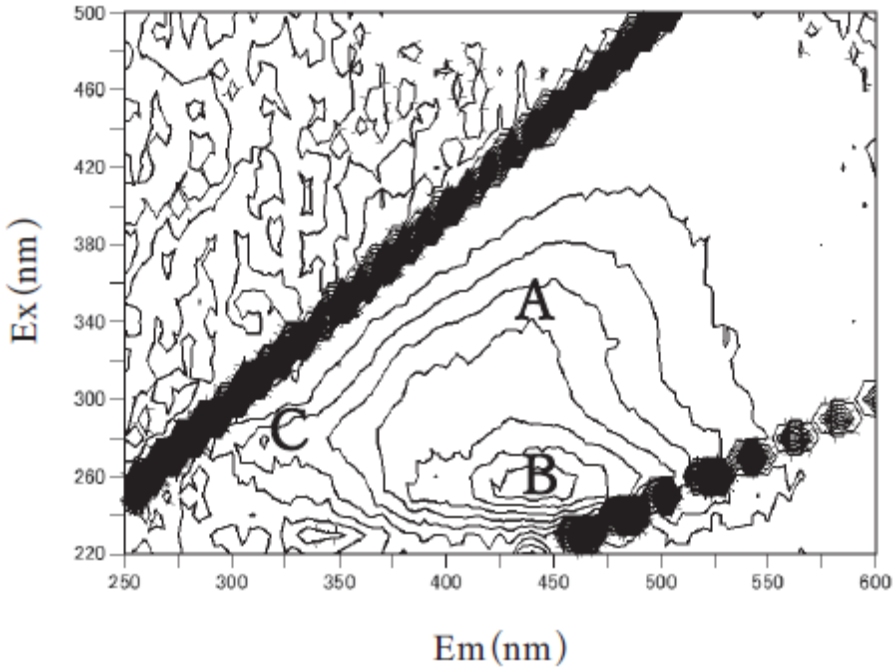

Fig. 3-3 3-DEEM contour plot of surface water at Imadzu (St. 17B) in the northern basin of Lake Biwa (December 2015)

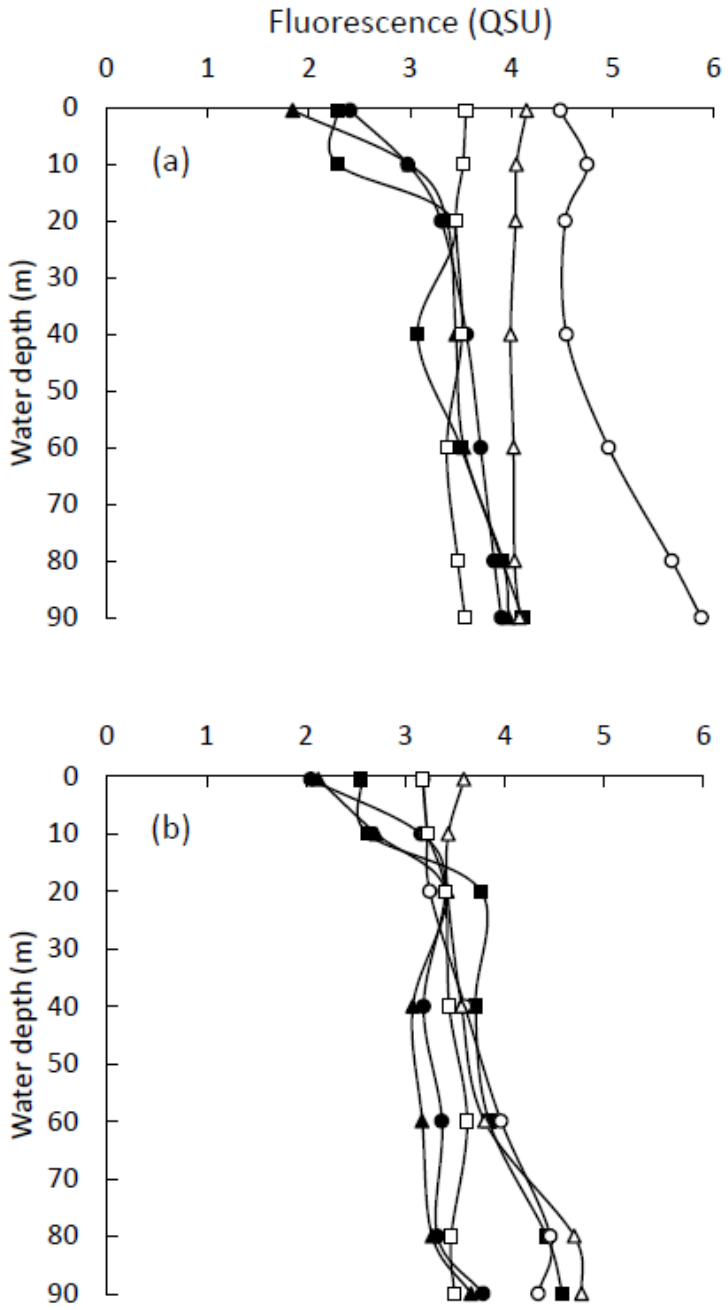

Fig. 3-4 Vertical distributions of the fluorescent intensities of peak A at Imadzu (St. 17B) in the northern basin of Lake Biwa in 2015 (a) and 2016 (b)

circulation period: ○ January, △ February, □ March

stratified period: ● July, ▲ August, ■ September

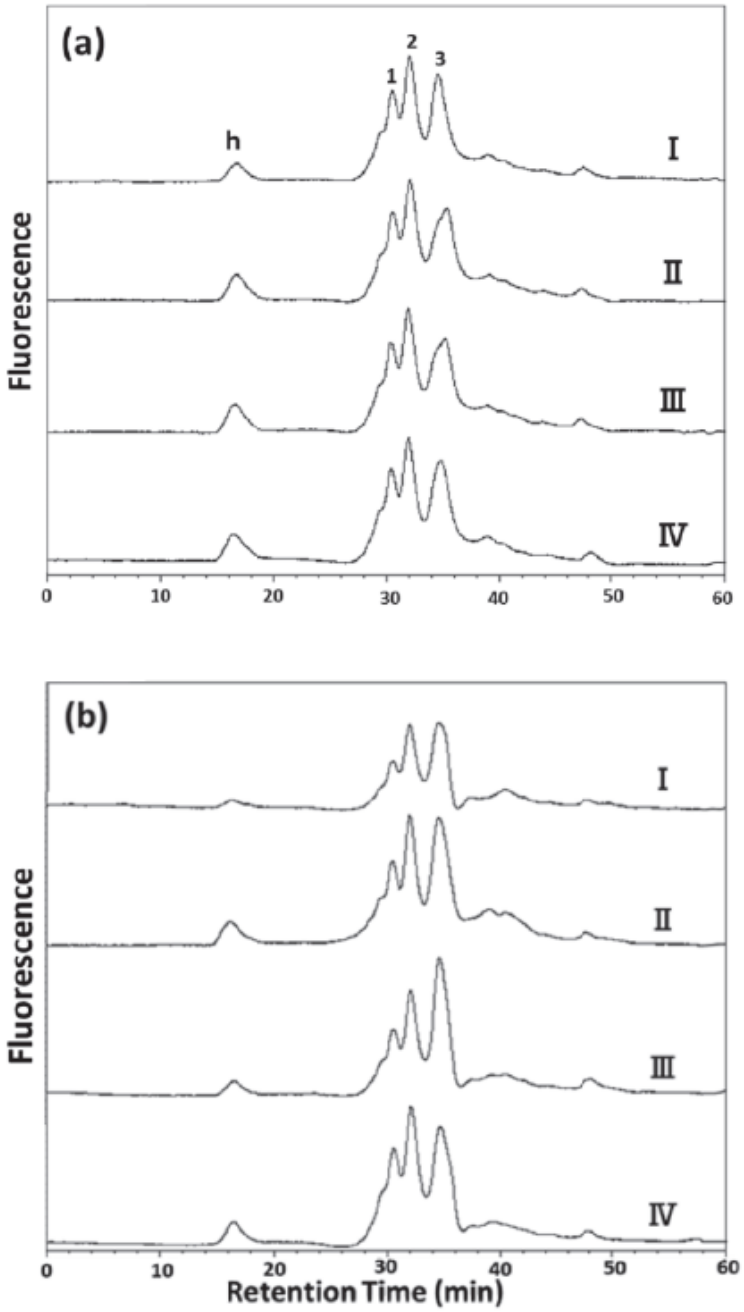

Fig. 3-5 Vertical changes in the gel chlomatograms of fulvic-like DOM (peak A) in Lake Biwa (Ex/Em = 340/435 nm)

(a) February 2016, (b) July 2016

water depth Ⅰ 0.5 m, Ⅱ 10 m, Ⅲ 20 m, Ⅳ 80 m

correspond to the peaks of soil FA, and the peaks 2 and 3 correspond to the peaks of algal DOM and degradation of FA [1, 15, 16]. The fluorescence values (peak area) of these peaks are listed in Table 3-1. The molecular distributions were similar in February regardless of the water depth, while in July, the fluorescence intensities of four peaks at a water depth of 0.5 m were lower than those at other water depths. The fluorescence intensities of peaks 1 and 2 at 80 m were higher than those at 0.5-20 m. It was reported that the RFI and RFI/DOC values of peaks A and B of the bottom waters, which were higher than those of the surface waters during the stratified period, might be due to the elution of HS from the sediment [18]. Then, the dynamic analysis of chemical components in sediment cores and bottom water samples collected at St. 17B in Lake Biwa was carried out under incubation experiments, and the results show that fulvic-like fluorescence DOM in bottom water may be regularly released from lake sediment [48]. On the other hand, the fluorescence quenching and degradation of high-molecular fulvic-like fluorophores in surface water might occur by solar irradiation. Hayakawa et al. reported that 1% attenuation of PAR (photosynthetically active radiation) and ultraviolet radiation (340nm) was at water depth of 16.1 m and 4.6 m in Lake Biwa (St. 17B), respectively [53]. Therefore, changes in the fluorescence properties, DOC concentrations, and molecular weights of HS and algal DOM by light irradiation were

investigated because HS and algal DOM are known to be the principal DOM in Lake Biwa.

Table 3-1 Vertical changes in the fluorescence of peaks h, 1, 2 and 3 in the gel chromatograms of fulvic-like DOM in Lake Biwa (Ex/Em=340/435 nm)

Water depth (m)	Fluorescence (V)			
	peak h (RT=16 min)	peak 1 (RT=29-30 min)	peak 2 (RT=32 min)	peak 3 (RT=35 min)
February 2016				
0.5	17.0	89.1	101.2	125.4
10	28.5	89.2	102.8	136.4
20	29.1	89.3	102.1	139.9
80	29.0	94.5	102.6	136.0
July 2016				
0.5	10.4	58.9	87.7	108.4
10	33.0	116.7	124.7	181.3
20	17.3	81.0	103.9	158.4
80	26.3	131.9	142.4	167.7

3.3.2 Effects of photoirradiation on the characteristics of HS and algal DOM

Two fluorescence maxima were observed in the Aldrich HA and Dando HA by 3-DEEM: one at Ex/Em values of 460/510 nm (peak H1), and the other at Ex/Em values of 260/480 nm (peak H2) [54]. Meanwhile, two fluorescence maxima were observed in the Dando FA and LBFA: one at Ex/Em values of 320/440 nm (peak A), and the other at Ex/Em values of 260/450 nm (peak B). The effects of photoirradiation on the fluorescence properties of humic acids (Aldrich HA, Dando HA) and fulvic acids (Dando FA, LBFA) were examined. Time changes in the fluorescent intensities (I/I_0)

of peaks H1 and H2 of Aldrich HA and Dando HA during photoirradiation by the sun and a Xe-lamp are shown in Fig. 3-6 (a) and (b), respectively. During 5 h of solar radiation, the I/I_0 values of peaks H1 and H2 of Aldrich HA decreased down to 0.376 and 0.609, and those of Dando HA decreased down to 0.459 and 0.685 by solar radiation, respectively. After 5 h of photoirradiation by a Xe-lamp, the I/I_0 values of peaks H1 and H2 of Aldrich HA decreased down to 0.615 and 0.762, and those of Dando HA decreased down to 0.755 and 0.894, respectively. These results suggest that the fluorescence of Aldrich HA and Dando HA may become extinct during photoirradiation, and their fluorescence quenching by solar irradiation may be larger than that by Xe-lamp irradiation.

Time changes in the fluorescence intensity (I/I_0) values of peaks A and B of Dando FA and LBFA during photoirradiation by the sun and a Xe-lamp are shown in Fig. 3-7 (a) and (b), respectively. After 5 h of solar radiation, the I/I_0 values of peaks A and B of Dando FA decreased down to 0.487 and 0.571, and those of LBFA decreased down to 0.511 and 0.563, respectively. After 5 h of Xe-lamp radiation, the I/I_0 values of peaks A and B of Dando FA decreased down to 0.819 and 0.833, and those of LBFA decreased down to 0.743 and 0.747, respectively. The decreasing trends of FA fluorescence during solar irradiation were also larger than those during Xe-lamp irradiation, which matched the HA results. The results indicate that the

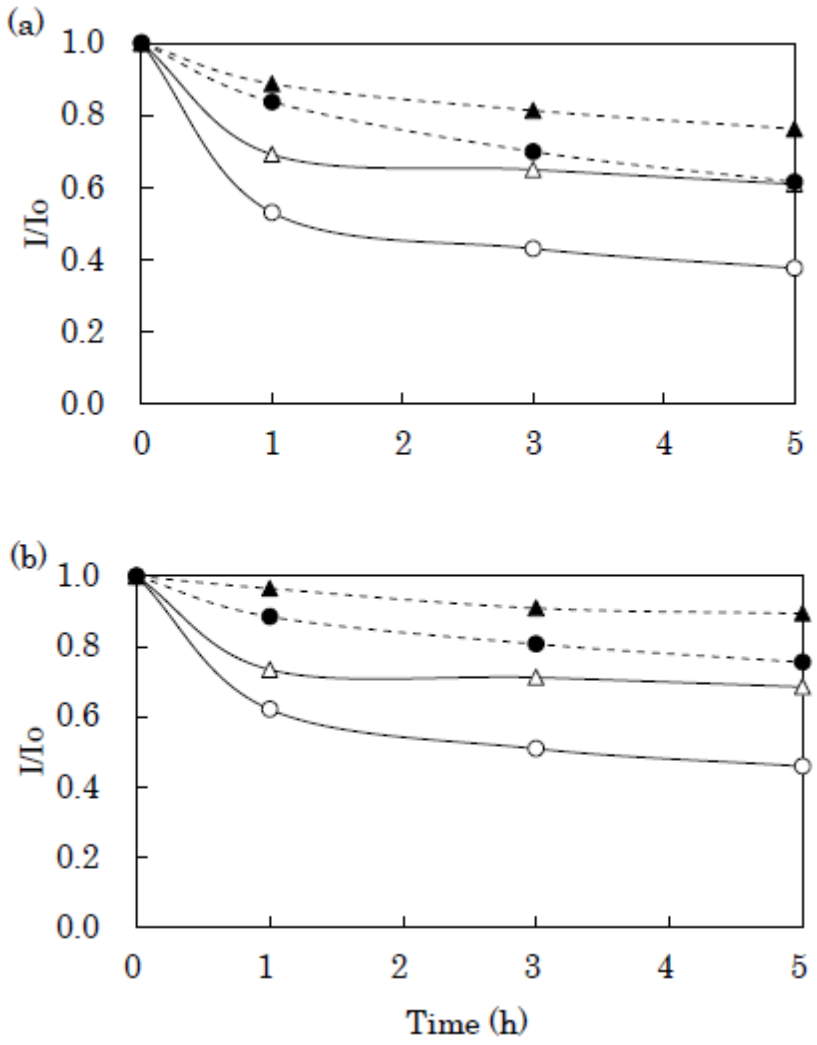

Fig. 3-6 Time changes in the fluorescent intensities of peaks H1 and H2 of Aldrich HA (a) and Dando HA (b) during photirradiation

solar irradiation: ○ peak H1, △ peak H2

Xe-lamp irradiation: ● peak H1, ▲ peak H2

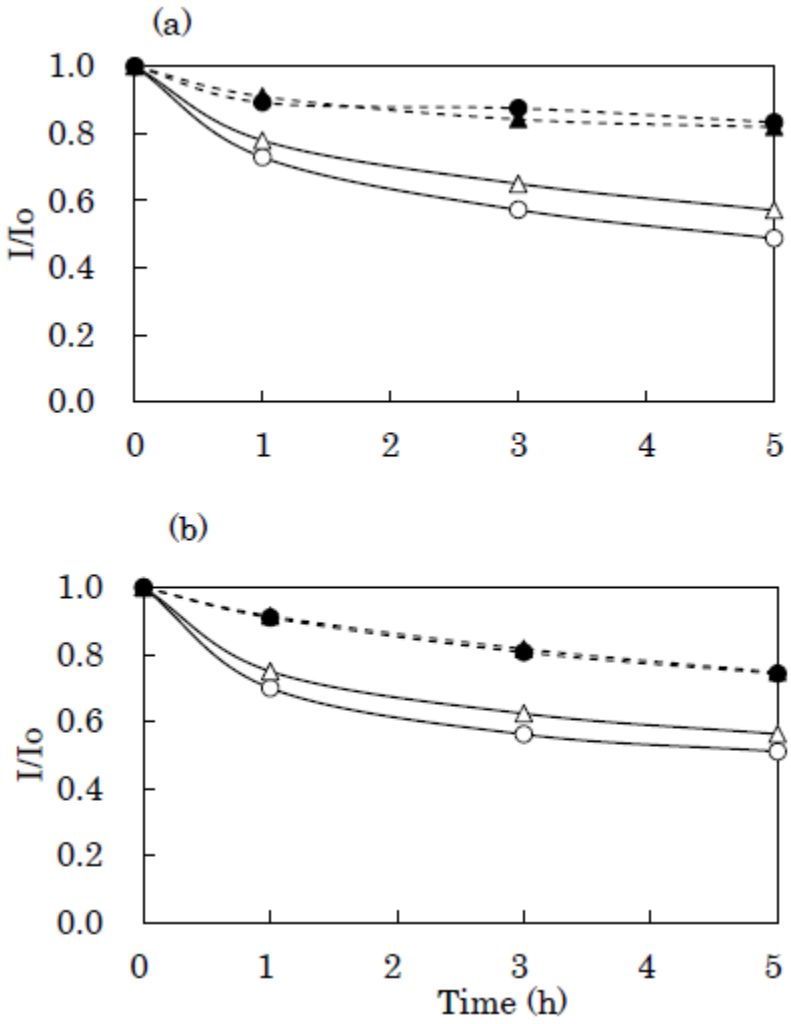

Fig. 3-7 Time changes in the fluorescent intensities of peaks A and B of Dando FA (a) and LBFA (b) during photoirradiation

solar irradiation: ○ peak A, △ peak B

Xe-lamp irradiation: ● peak A, ▲ peak B

fluorescence of HA and FA may become extinct during photoirradiation, and their fluorescence quenching by solar irradiation may be larger than that by Xe-lamp irradiation.

Three fluorescence maxima were observed in the cultivation of three kinds of phytoplankton: two fulvic-like fluorescence peaks (A and B), and a protein-like fluorescence peak (C). Photoirradiation effects by the sun and a Xe-lamp on the time changes in the fluorescent intensities (I/I_0) of peaks A, B, and C of algal DOM from *M. aeruginosa*, *S. dorsidentiferum*, and *C. ovata* were examined, and the results are shown in Fig. 3-8 (a), (b), and (c), respectively. The I/I_0 values of fulvic-like fluorophores in *M. aeruginosa* after 5 h of solar and Xe-lamp irradiation decreased down to 0.249 and 0.289 for peak A and 0.501 and 0.513 for peak B, respectively, while those in *S. dorsidentiferum* decreased down to 0.255 and 0.286 for peak A and 0.463 and 0.488 for peak B, respectively. In the case of *M. aeruginosa* and *S. dorsidentiferum*, the decreasing trends of fluorescence for peak A were larger than those for peak B; however, the difference in the photoirradiation effect (sun and Xe-lamp) on the fluorescence of peaks A and B was confirmed to be small. Meanwhile, in the case of *C. ovata*, the I/I_0 values of peaks A and B after 5 h of solar irradiation decreased down to 0.39 and 0.532, and those after 5 h of Xe-lamp irradiation decreased down to 0.783 and 0.933, respectively. The decreasing fluorescence trends of peaks A and B in *C.*

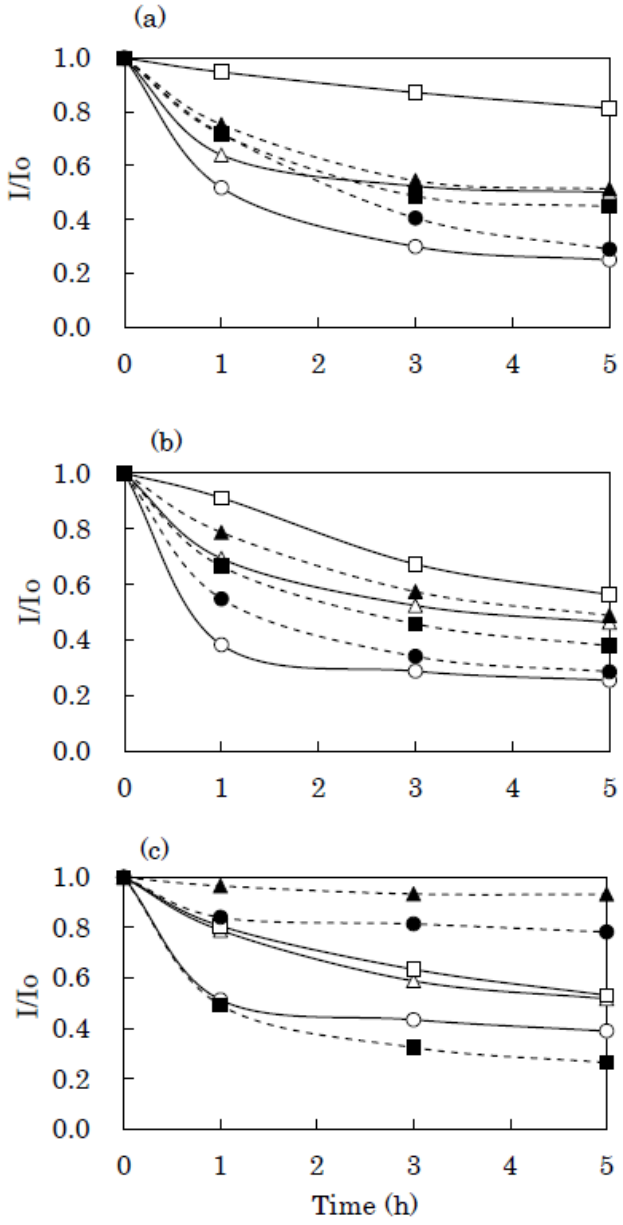

Fig. 3-8 Time changes in the fluorescent intensities of peaks A, B, and C of algal DOM from three kinds of phytoplankton during photoirradiation

(a) *Microcystis aeruginosa*, (b) *Staurastrum dorsidentiferum*,

(c) *Cryptomonas ovata*

solar irradiation: ○ peak A, △ peak B, □ peak C

Xe-lamp irradiation: ● peal A, ▲ peak B, ■ peak C

ovata during solar irradiation were considerably larger than those during Xe-lamp irradiation, which were different from those in *M. aeruginosa* and *S. dorsidentiferum*.

Meanwhile, the I/I_0 values of protein-like fluorophores (peak C) in *M. aeruginosa* after solar and Xe-lamp irradiation for 5 h decreased down to 0.813 and 0.448, respectively, and those of peak C in *S. dorsidentiferum* decreased down to 0.563 and 0.379, respectively. The I/I_0 values of peak C in *C. ovata* after 5 h of solar and Xe-lamp irradiation decreased down to 0.532 and 0.265, respectively. During Xe-lamp irradiation, the fluorescence quenching of protein-like fluorophores (peak C) in *C. ovata* was largest, followed in order by *S. dorsidentiferum* and *M. aeruginosa*, while the fluorescence quenching of fulvic-like fluorophores (peaks A and B) in *C. ovata* was small. These results suggest that the effects of ultraviolet radiation on the fluorescence quenching of protein-like fluorophores (peak C) may be large because the relative intensities (<400 nm) of a Xe-lamp light are larger than those of solar light [49].

Furthermore, the effects of photoirradiation on the DOC concentrations of HS (Dando HA, Dando FA, and LBFA) and algal DOM released from *M. aeruginosa* were investigated, and the results are listed in Table 3-2. The DOC concentrations of these HS after photoirradiation for 5 h decreased by 2–10%, while that of algal DOM released from *M. aeruginosa* decreased by

1–3%. The decrease in the DOC concentrations of these substances by photoirradiation was relatively small.

Table 3-2 Effects of photoirradiation on the DOC concentrations of Dando HA, Dando FA, LBFA, and algal DOM from *M. aeruginosa*

Time (h)	DOC (mg/L)							
	Solar irradiation				Xe-lamp irradiation			
	DHA	DFA	LBFA	M	DHA	DFA	LBFA	M
0	1.09	1.15	1.44	37.9	1.10	1.15	1.45	37.9
1	1.05	1.16	1.40	38.2	1.07	1.15	1.44	38.3
3	1.04	1.12	1.38	37.7	1.08	1.13	1.40	37.3
5	1.07	1.04	1.34	37.5	1.07	1.06	1.37	37.3

DHA: Dando humic acid, DFA: Dando fulvic acid, LBFA: fulvic acid of Lake Biwa
M: algal DOM from *M. aeruginosa* during cultivation for 26 days

Next, the effects of photoirradiation on the molecular weight distributions of HS and algal DOM from were investigated by gel chromatography with a fluorescence detector (Ex/Em = 340/435 nm or Ex/Em = 280/320 nm). The results of Dando FA and Algal DOM from *M. aeruginosa* are shown in Fig. 3-9. In the case of Dando FA, the peak (RT = 29 min) decreased, and the peaks (RT = 30, 32, and 35 min) increased after Xe-lamp radiation. It is believed that low-molecular substances may be produced from high-molecular substances of FA. Furthermore, in the case of algal DOM from *M. aeruginosa*, the peaks (RT = 35, 39 min) of fulvic-like fluorophores (peak A) were larger than the peaks (RT = 29–30, 32 min) observed in soil FA, and all peaks decreased after photoirradiation. Meanwhile, in the case of protein-like fluorophores (peak C), a large peak (RT = 16–17 min) and several peaks (RT = 23–25, 30, 35, 40, and 47 min)

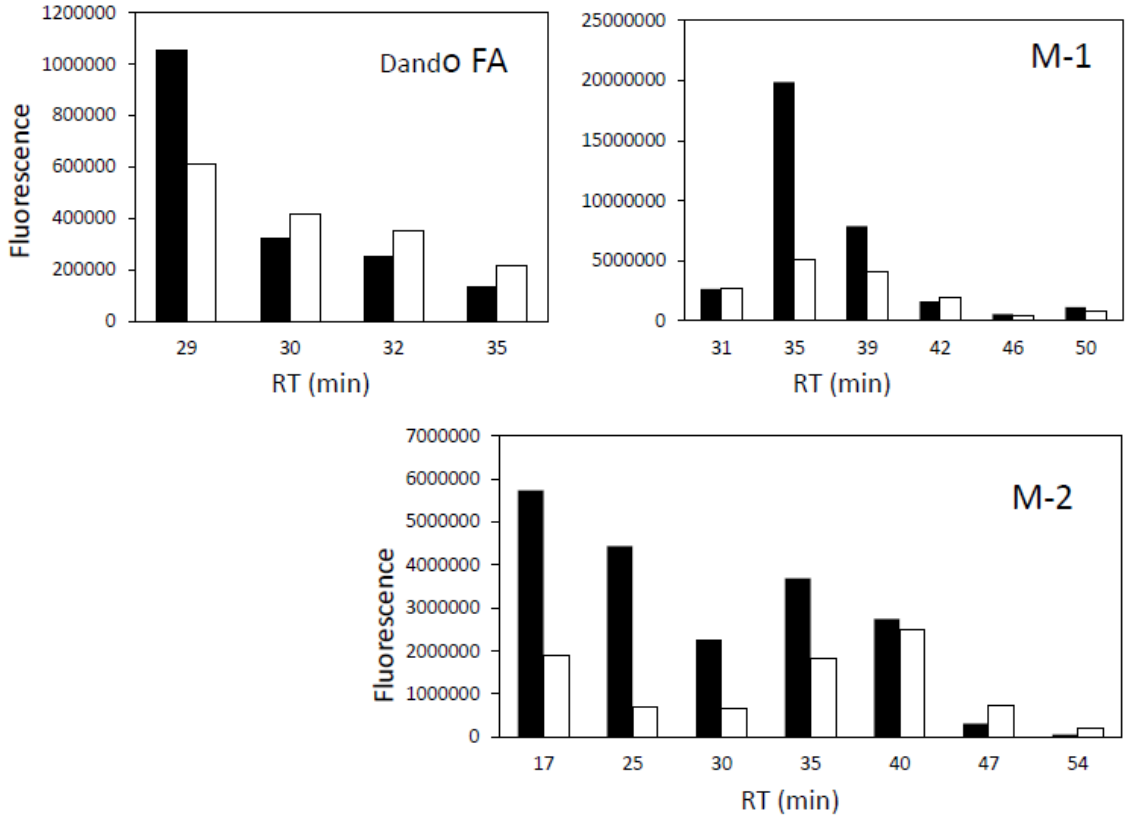

Fig 3-9 Effects of Xe-lamp irradiation on the molecular weight distributions of Dando FA and algal DOM from *M. aeruginosa* (M-1 and M-2) by gel chlomatography with a fluorescence detector

(Ex/Em = 340/435 nm for Dando FA and M-1, Ex/Em = 280/320 nm for M-2)

■ before irradiation, □ after irradiation

were observed, and the peak (RT = 16–17 min) considerably decreased after photoirradiation. These results suggest that light irradiation may influence both fluorescence quenching and the degrading of high-molecular substances of HS and algal DOM.

3.3.3 Effects of photoirradiation on the characteristics of DOM in Lake Biwa and its surrounding rivers

The effects of photoirradiation on the DOC concentrations, fluorescence properties, and molecular weight distributions of DOM in Lake Biwa and its surrounding rivers were investigated. Figure 3-10 shows the monthly changes in the RFI values of fulvic-like fluorophores (peak A) in Lake Biwa (St. 17B) before and after solar irradiation, respectively. The RFI values of peak A at a water depth of 0.5 m during August–October 2015 and June–August 2016 were especially low as compared to those in other months and at a water depth of 80 m.

The RFI values of peak A at 0.5 m during August–October 2015 and June–August 2016 were virtually unchanged (3-12%) after solar irradiation; however, they declined by 30–45% in other months. Meanwhile, the RFI values of peak A at 80 m were larger than those at 0.5 m and declined by 30–50% in all months after solar irradiation. As a result, the values at 80 m were fairly close to those at 0.5 m after solar irradiation. However, the changes in

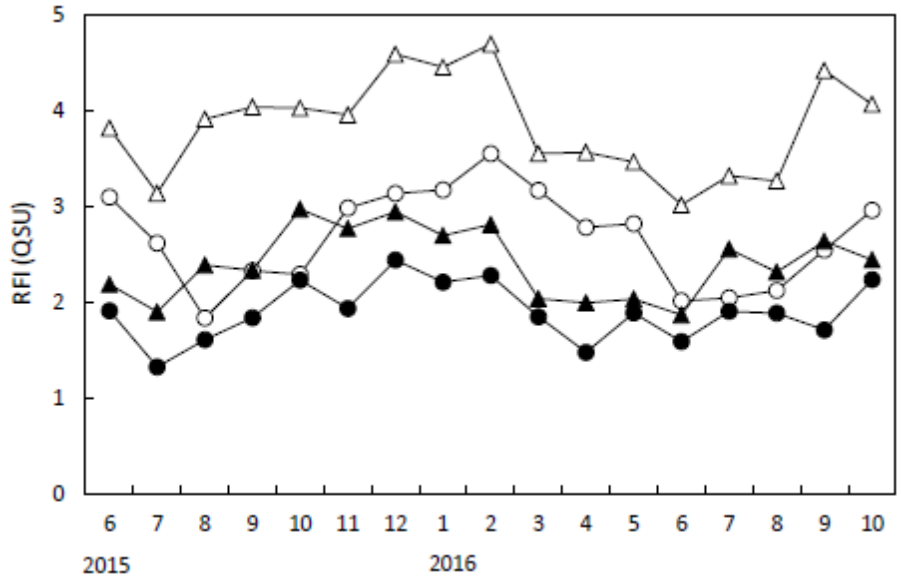

Fig. 3-10 Monthly changes in the RFI values of fluvic-like fluorophore (peak A) in Lake Biwa (St. 17B)

before solar irradiation: water depth ○ 0.5 m, △ 80m

after solar irradiation: water depth ● 0.5 m, ▲ 80 m

the DOC concentrations of water samples of Lake Biwa by further solar irradiation were small (less than 10%) regardless of the water depth and collected month. Skoog et al. also reported that the rest concentration of humic substance fluorescence (Ex/Em = 350/450 nm) in water samples from the Baltic Sea, similar among the sampled sites, are resistant to further photochemical degradation and the DOC concentrations decreased 3-7% [55]. These results suggest that, at the northern basin of Lake Biwa, the susceptibility of fulvic-like fluorophores to degradation by further solar irradiation depends on the water depth collected during the stratified period (summer); however, the rest of fulvic-like fluorophore might be resistant to further photochemical degradation regardless of the water depth.

The RFI values of peak A in the rivers around Lake Biwa during solar irradiation decreased by about 50%, while those during Xe-lamp irradiation decreased by about 20% regardless of the average amount of solar radiation and sampling stations of rivers (Table 3-3). The photoirradiation results of fulvic-like fluorophores in river waters were similar to the results of Dando FA and LBFA (Fig. 3-7), but they were different from the results of peak A of algal DOM released from three kinds of phytoplankton (Fig. 3-8). These results were consistent with the results that 30-60% of DOM in these rivers were fulvic acids by fractionation analysis [5]. On the other hand, the RFI values of peak C in rivers during Xe-lamp irradiation declined by 60-65%,

Table 3-3 Effects of photoirradiation on the fluorescence of peaks A and C in Yodo rivers

River	Irradiation	Peak A (QSU)			Peak C (QSU)		
		I_0	I	I/I_0	I_0	I	I/I_0
Katsura (No. 11)*	solar	7.14	3.34	0.47	7.69	5.87	0.76
Kizu (No. 31)*	solar	16.9	8.41	0.50	7.52	6.48	0.86
Kizu (No. 31)**	solar	16.4	8.54	0.52	8.75	5.70	0.65
Kizu (No. 31)**	Xe	16.4	13.6	0.83	8.75	3.07	0.35
Kizu (No. 3H)*	solar	18.8	9.28	0.49	8.65	5.99	0.69
Kizu (No. 3H)**	solar	20.1	10.2	0.51	9.25	5.52	0.60
Kizu (No. 3H)**	Xe	20.1	15.5	0.77	9.25	3.68	0.40
Yodo (No. 41)*	solar	13.1	6.42	0.49	7.24	5.73	0.79

* collected in August 2015, average amount of solar radiation 609 W/m^2

** collected in August 2016, average amount of solar radiation 419 W/m^2

while those during solar irradiation declined by 14-40% (Table 3-3). The changes in the DOC concentrations in rivers by light irradiation were also small (3-9%). The photodegradation of DOM in river waters in the Lake Biwa watershed has demonstrated that fulvic-like fluorescence DOM is more susceptible to photodegradation than those of protein-like substances and DOC [56]; this is consistent with our results from Lake Biwa. It is believed that the effects of visible radiation on the fluorescence quenching of fulvic-like fluorophores may be large, while the effects of ultra-violet radiation may be larger on protein-like fluorophores.

The effects of the wavelength region on the characteristics of DOM and fluorophores in environmental waters were examined by Xe-lamp irradiation using two kinds of wavelength cut filters and compared with those of soil FA and algal DOM (Fig. 3-11). The I/I_0 values of peak A of soil FA (Dando

FA), algal DOM from *M. aeruginosa* and aquatic DOM in Kizu River (No. 31, October 2016) by Xe-lamp irradiation decreased down to 0.76, 0.47 and

FA), algal DOM from *M. aeruginosa* and aquatic DOM in Kizu River (No. 0.77, respectively, and those by Xe-lamp irradiation with a cut filter (UV-29) decreased down to 0.75, 0.48 and 0.76, respectively. The decrease in the RFI values of peak A by Xe-lamp irradiation with a cut filter (UV-29) was similar to that in the values by Xe-lamp only in the case of soil FA, algal DOM, and aquatic DOM. Moreover, the changes in the RFI values of peak A by Xe-lamp irradiation with a cut filter (W-Y495) became smaller because the I/I_0 values of peak A of Dando FA, algal DOM from *M. aeruginosa* and aquatic DOM in Kizu River by Xe-lamp irradiation with a cut filter (W-Y495) were 0.95, 1.00 and 0.96, respectively, While, the I/I_0 values of peak C of algal DOM from *M. aeruginosa* and aquatic DOM in Kizu River by Xe-lamp irradiation decreased down to 0.25 and 0.34, respectively, and those by Xe-lamp irradiation with a cut filter (UV-29) decreased down to 0.85 and 0.76, respectively. The decrease in the RFI values of peak C in algal DOM and aquatic DOM became smaller when using a cut filter (UV-29). These results indicate that wavelengths between 290 and 495 nm and below 290 nm may largely affect the characteristics of fulvic-like fluorescence DOM and protein-like fluorescence DOM, respectively.

From these results, it is considered that strong solar irradiation in

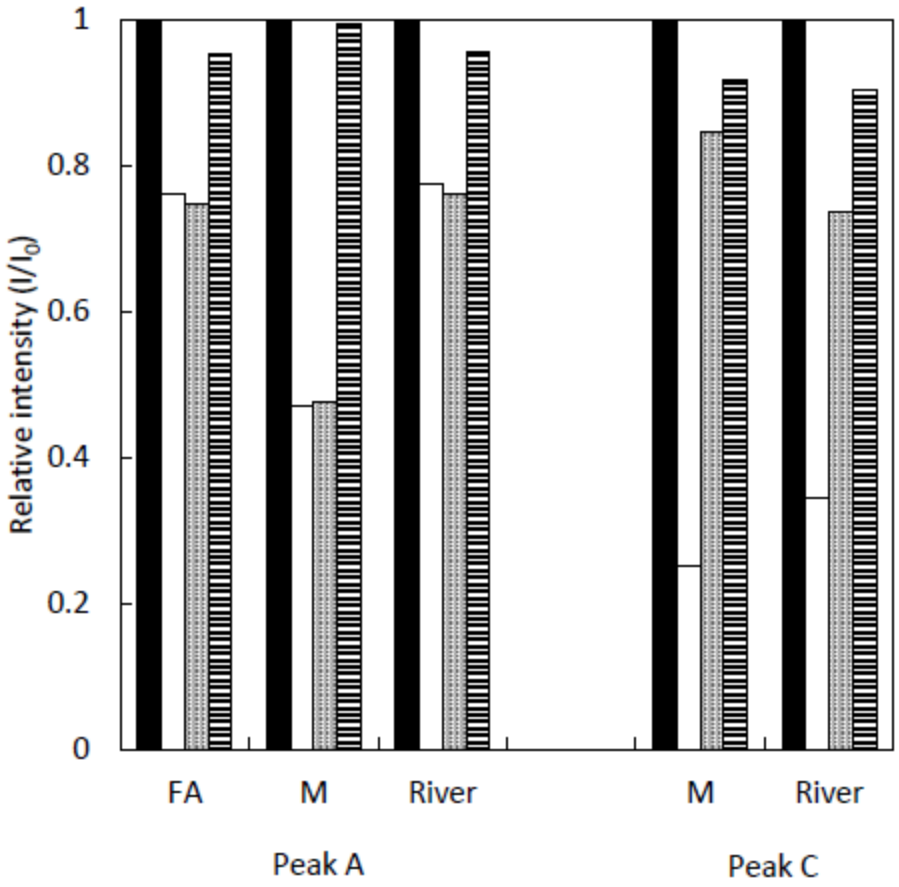

Fig. 3-10 Effects of wavelength regions on the fluorescence intensities of peaks A and C by Xe-lamp irradiation using two kinds of wavelength cut filters

FA: Dando FA, M: algal DOM from *M. aeruginosa*, River: aquatiq DOM in Kizu River (No. 31, October 2016)

■ before irradiation, □ Xe lamp only,

▨ Xe-lamp with UV-29, ▤ Xe-lamp with W-Y 495

summer may influence both the light quenching and degradation of fulvic-like fluorophores in surface water of the northern basin of Lake Biwa. As a result, their molecular distributions may converge to relatively stable conditions.

3.4 Conclusion

The decrease in the fluorescence intensities of HS and algal DOM from three kinds of phytoplankton was observed by both types of photoirradiation (solar and Xe-lamp irradiation), and the fluorescence quenching by solar irradiation of HS and fulvic-like fluorophores released from phytoplankton was larger than that by Xe-lamp irradiation, while the fluorescence quenching by Xe-lamp irradiation of protein-like fluorophores released from phytoplankton was larger than that by solar irradiation. On the other hand, the decrease in the DOC values of these substances by photoirradiation was relatively small.

We clarified by Xe-lamp irradiation experiment using two kinds of wavelength cut filters that fulvic-like fluorophore might be more sensitive to visible light irradiation than protein-like fluorophore, while protein-like fluorophore might be sensitive to UV irradiation. Therefore, the fluorescence quenching and degradation of fulvic-like fluorophores attributed to soil FA and algal DOM in surface water of Lake Biwa might occur by strong solar

irradiation in summer. Furthermore, the rest of fulvic-like fluorophores might be resistant to further photochemical degradation. However, we cannot conclude the effects of photoirradiation on protein-like fluorophores in Lake Biwa because their fluorescence intensities were low and fluctuated by microbiological production in Lake Biwa. Then, further study to clarify this is needed.

General remarks

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. The increase of refractory DOM may be attributed to the contribution of not only humic substances (HS) from soils around the rivers flowing into Lake Biwa but also algal DOM from phytoplankton in the lake.

In the present study, in order to clarify the characteristics of algal DOM and its contribution to the DOM in Lake Biwa, phytoplankton-monitoring methods were developed using SYBR Green real-time PCR with species specific primer sets for each species, and applied to the determination of cell densities of four algal species (*Microcystis aeruginosa*, *Staurastrum dorsidentiferum*, *Cryptomonas ovata*, *Fragilaria capucina*) during cultivation and in environmental samples. Analytical results by real-time PCR assay agreed relatively well with those obtained by microscopy. However, the method developed here cannot be applied 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. Then, it is under study for the seasonal changes 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.

Furthermore, the rapid analysis of HS (mainly fulvic acid (FA)) in environmental waters using three-dimensional excitation-emission matrix (3-DEEM) and DOC was investigated, as compared to fractionation analysis, and applied to the dynamics of HS in Lake Biwa and its surrounding rivers. Humic substances in the Yodo rivers as measured by rapid analysis using soil FA (Dando FA) as a standard were in relatively good agreement with those by fractionation analysis, with the exception of the Uji River, of which waters are affected by Lake Biwa by running through the Seta River. This rapid analysis was also applied to the dynamics of HS in the rivers flowing into Lake Biwa.

In the case of the rapid analysis of HS in Lake Biwa, Biwako FA was used as a standard instead of Dando FA. The concentrations of FA at water depths of 10-20 m in the northern basin of Lake Biwa (St. 17B) by rapid analysis were in relatively good agreement with those by fractionation analysis during the stratified period. However, at a water depth of 0.5 m in

summer the FA concentrations by rapid analysis were lower than those by fractionation analysis. Meanwhile, FA concentrations at a water depth of 80 m were higher than those by fractionation analysis which may be due to the elution of HS from the sediment. Therefore, rapid analysis cannot be easily used to analyze the dynamics of FA in the northern basin of Lake Biwa, however comprehensively evaluating DOC, fluorescence intensities and molecular distributions of DOM and fluorophores in Lake Biwa will be helpful in understanding the effects of solar irradiation and the elution of HS from sediment.

The decrease in the fluorescence intensities of HS and algal DOM from three kinds of phytoplankton was observed by both types of photoirradiation (solar and Xe-lamp irradiation), and the fluorescence quenching by solar irradiation of HS and fulvic-like fluorophores released from phytoplankton was larger than that by Xe-lamp irradiation, while the fluorescence quenching by Xe-lamp irradiation of protein-like fluorophores released from phytoplankton was larger than that by solar irradiation. On the other hand, the decrease in the DOC values of these substances by photoirradiation was relatively small.

It was clarified by Xe-lamp irradiation experiment using two kinds of wavelength cut filters that fulvic-like fluorophore might be more sensitive to visible light irradiation than protein-like fluorophore, while protein-like

fluorophore might be sensitive to UV irradiation. Therefore, the fluorescence quenching and degradation of fulvic-like fluorophores attributed to soil FA and algal DOM in surface water of Lake Biwa might occur by strong solar irradiation in summer. Furthermore, the rest of fulvic-like fluorophores might be resistant to further photochemical degradation. However, the effects of photoirradiation on protein-like fluorophores in Lake Biwa cannot be concluded because their fluorescence intensities were low and fluctuated by microbiological production in Lake Biwa. Then, further study to clarify this is needed.

The present study shows availabilities of new phytoplankton-monitoring methods and rapid analysis of HS to clarify the characteristics and dynamics of algal DOM and HS and their contribution to the DOM in Lake Biwa and its surrounding rivers. The approach employed in this work would contribute to the further understanding of water pollution and improvement of water treatment.

Acknowledgements

This work has been done under the guidance of Professor Etsu Yamada of Kyoto Institute of Technology. The author would like to thank Dr. Yasuro Fuse, Ms. Megumi Ichinose and his laboratory's members of Kyoto Institute of Technology for their kind supports to do the present study.

The author would like to express his sincere appreciation to Professor Hajime Karatani and Professor Junichi Horiuchi of Kyoto Institute of Technology for their useful suggestion to do the present study. The author would like to thank Dr. Satoshi Ichise of Lake Biwa Environmental Research Institute for his kind suggestions to identification and count the phytoplankton. The author would like to thank Professor Masamitsu Yamaguchi and Dr. Hideki Yoshida of Kyoto Institute of Technology for their kind cooperation in using a real-time PCR detection system.

The author would like to thank the staff of Lake Biwa Environmental Research Institute for help and support with sampling.

Finally, the author thanks his family for all supports to do this work.

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