Geochemical Journal, Vol. 52, pp. e15 to e20, 2018 doi:10.2343/geochemj.2.0528

EXPRESS LETTER

Algal-derived 24-ethylcholesta-5,22-dien-3β-ol (stigmasterol) is frequently found in high-molecular-weight dissolved organic matter (HMW-DOM) during summer in freshwater and brackish lakes

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(*Received December 13, 2017; Accepted April 30, 2018; Online published June 12, 2018*)

Source and formation of high-molecular-weight dissolved organic matter (HMW-DOM) and its interaction with other organic components such as particulate organic matter (POM) and low-molecular-weight DOM (LMW-DOM) have been poorly understood so far. Molecular and isotopic compositions of HMW-DOM are generally far different from those of POM and LMW-DOM. In this study, we further investigate large proportions of 24-ethylcholesta-5,22-dien-3β-ol (stigmasterol) in the HMW-DOM (but not in POM and LMW-DOM) during summer in three types of lakes: freshwater Lakes Kizaki and Suwa and a brackish-water Lake Suigetsu, in Japan. Moreover, from the carbon and hydrogen isotope analysis, we reveal autochthonous algae (perhaps some minor, specific phytoplankton) as a major source of the abundant stigmasterol in the lakes. These results suggest that HMW-DOM in summer is derived from autochthonous phytoplankton rather than allochthonous terrestrial plants and that HMW-DOM likely has few interaction (e.g., exchange of components) with POM and DOM in the lakes.

Keywords: HMW-DOM, stigmasterol, carbon and hydrogen isotopic compositions, freshwater and brackish lakes

INTRODUCTION

Dissolved organic matter (DOM) represents a large reservoir of organic carbon in the hydrosphere and makes up a key component of the global carbon cycle (e.g., Mostofa *et al*., 2013; Nebbioso and Piccolo, 2013; Repeta, 2015; Hansell and Carlson, 2015). Moreover, DOM is generally derived from multiple sources and affected by complicated transformation and degradation reactions in aquatic environments (e.g., Benner *et al*., 1992; McCarthy *et al*., 1998; McCallister *et al*., 2006a, b). Therefore, the study of DOM including the identification of the biological sources and evaluation of biogeochemical fates is still challenging. Among size fractions of DOM, high-molecular-weight DOM (HMW-DOM; >1 kDa) particularly represents a unique organic carbon pool, which is accompanied by considerable difference in the molecular and stable isotopic compositions from other organic components such as particulate organic matter (POM) and low-molecular-weight DOM (LMW-DOM; <1 kDa) (e.g., Benner *et al*., 1997; Ziegler and Fogel, 2003; Yoshiyama *et al*., 2003; McCallister *et al*., 2006a, b; Repeta, 2015). Source and formation of HMW-DOM and its interaction with POM and LMW-DOM (e.g., transfer and exchange of components, how link with the other pools) thus have been particularly poorly understood so far.

Against this considerable difference in the molecular and stable isotopic compositions of HMW-DOM from the other pools, the molecular distribution of sterols, which are ubiquitous organic compounds and characteristically distributed in different taxa of eukaryotes (e.g., Volkman, 2003), have been used in the tracing biological sources and formation processes of HMW-DOM in aquatic environments (e.g., Yoshiyama *et al*., 2003; McCallister *et al*., 2006b). For instance, Yoshiyama *et al*. (2003) found an unusually high concentration of 24-ethylcholesta-5,22-dien-3β-ol (stigmasterol) in HMW-DOM collected from the five lakes, Nakatsuna, Kizaki, Suwa, Misumaike, and Shirakomaike, in Japan. McCallister *et al*. (2006b) also found such abundant stigmasterol in HMW-DOM collected from York River estuary, in USA. Thus, it is assumed that the further analysis (e.g., of molar and molar-isotopic compositions) of sterols in HMW-DOM in fresh-

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water environments will provide a key for better understanding of the biological sources and formation of HMW-DOM and its interaction with other organic carbon pools in environments.

Here, we further investigated the molar composition of sterols (Supplementary Fig. S1) in the HMW-DOM throughout a year together with that of phytoplankton and sediments in three types of lakes: freshwater lakes Kizaki and Suwa and a brackish water lake Suigetsu, in Japan (Supplementary Fig. S2). Moreover, for the samples from Lake Kizaki, we determined stable carbon and hydrogen isotopic compositions of the sterols, in order to identify the biological sources of sterols associated with the HMW-DOM in these lakes. Lake Kizaki was chosen as a representative lake, where has long been studied so far in diverse fields (e.g., Yoshioka *et al*., 1988; Park and Hayashi, 1993; Yoshiyama *et al*., 2003; Ito, 2013).

Materials and Methods

Samples were collected from Lake Kizaki (36°33′30″ N, 137°50′15″ E), a mesotrophic subalpine freshwater lake (altitude: 764 m, surface area: 1.4 km^2, maximum depth: 29.5 m), in April to December 2012, from Lake Suwa (36°2′57″ N, 137°5′7″ E), a eutrophic freshwater lake (759 m, 13.3 km^2, 7.2 m), in July 2013, and from Lake Suigetsu (35°35′15″ N, 135°52′53″ E), a eutrophic brackish water lake (0 m, 4.3 km^2, and 34 m), in August 2012 (cf., geological location in Fig. S2). Lake water was taken from the center of lakes at the depth just below the thermocline to minimize the effects of biological activity in the surface layer and of contamination of resuspended particles from sediments, and immediately filtrated with two plankton-nets (100 and 40 μm) and a glass fiber filter (Toyo Co. Ltd., GB100R, 30 cm × 30 cm). The filtered samples were thus defined as phytoplankton (100 μm >> 40 μm), POM (40 μm >> 0.6 μm), and DOM (<0.6 μm), and were lyophilized immediately. The HMW-DOM was isolated from the final filtrate by a tangential flow ultrafiltration (TFF) using a Pellicon Lab Casette (Millipore Co. Ltd.) equipped with a PLAC (Molecular Cut-off: MCO = 1 kDa) filter. Principally 100 L of lake water was concentrated to 1 L by TFF and subsequently concentrated to about 200 mL by using a conventional stirring ultrafiltration. Apparatus used was a UHP-76 (Advantec Co. Ltd.) equipped with Amicon YM1 (MCO = 1 kDa, Millipore Co. Ltd.) filter. Finally the concentrate was lyophilized. The HMW-DOM was obtained as a pale brown-colored puffy powder. Surface sediments (0–10 cm depth) were collected from the center of the lake with a gravity corer in June 2008.

The phytoplankton, HMW-DOM and Lake Kizaki sediment were saponified with 1 M KOH in methanol/water (95/5, w/w) in a sealed ampoule. The total DOM was obtained by 1L of filtered water saponified with the 0.1 M KOH under reflux. The neutral lipids were extracted from the alkaline medium with *n*-hexane/diethylether (9/1, v/v). The lipids in POM collected on the GF filter were extracted with dichloromethane/methanol (6/4, v/v) by sonication, dried and then saponified with the 0.1 M KOH. The alcohol fraction was purified by a silica gel column chromatography according to the procedure in Fukushima *et al*. (2006), by eluting with *n*-hexane/diethyl ether (1/1, v/v) after elution of aliphatic and aromatic hydrocarbons with *n*-hexane/benzene (5/1, v/v), and subsequently trimethylsilylated with *N*,*O*-bistrimethylsilyltrifluoro acetamide (BSTFA). Sterols were identified by retention time and mass spectra recorded on a gas chromatograph-mass spectrometer (GC-MS: an HP 6970 GC coupled to an HP 5973 mass selective detector) (Fig. 1). Quantification was performed on a GC-flame ionization detector (FID) using an HP 5890 series II GC, comparing the peak area with that of an internal standard, 16-hydroxyhexadecanoic acid methyl ester, spiked just before trimethylsilylation.

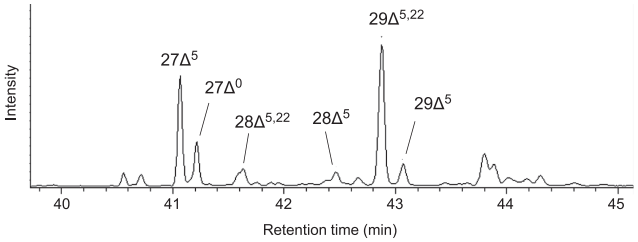

Fig. 1. A representative total ion chromatogram (TIC) on GC-MS analysis (sterols in HMW-DOM fraction for August, Lake Kizaki). Abbreviations: cholest-5-en-3β-ol (27Δ^5), cholestan-3β-ol (27Δ^0), 24-Methylcholest-5,22-dien-3β-ol (28$\Delta^{5,22}$), 24-Methylcholest-5-en-3β-ol (28Δ^5), 24-Ethylcholest-5,22-dien-3β-ol (29$\Delta^{5,22}$), 24-Ethylcholest-5-en-3β-ol (29Δ^5).

In order to determine stable isotope ratios, the alcohol fraction isolated from the HMW-DOM in June, October, and December and surface sediment of Lake Kizaki were acetylated with acetic anhydride/pyridine (1/1, v/v). Purification of acetylated sterols was made by urea adduction. The sterols were further separated into three fractions: stanols, Δ^5 sterols, and other sterols (e.g., $\Delta^{5,22}$ and $\Delta^{5,24}$ sterols) using a silver nitrate (10 wt%) impregnated-silica gel column chromatography, according to the procedure described in Chikaraishi *et al*. (2005). The carbon and hydrogen isotopic compositions were determined by a GC-isotope ratio mass spectrometer (GC-IRMS) using an Agilent 6890N GC coupled to a Thermo Fisher Scientific Delta^{plus} XP IRMS. The contribution of carbon and hydrogen incorporated during derivatizations were corrected by an isotopic mass balance calculation described in Chikaraishi *et al*. (2004).

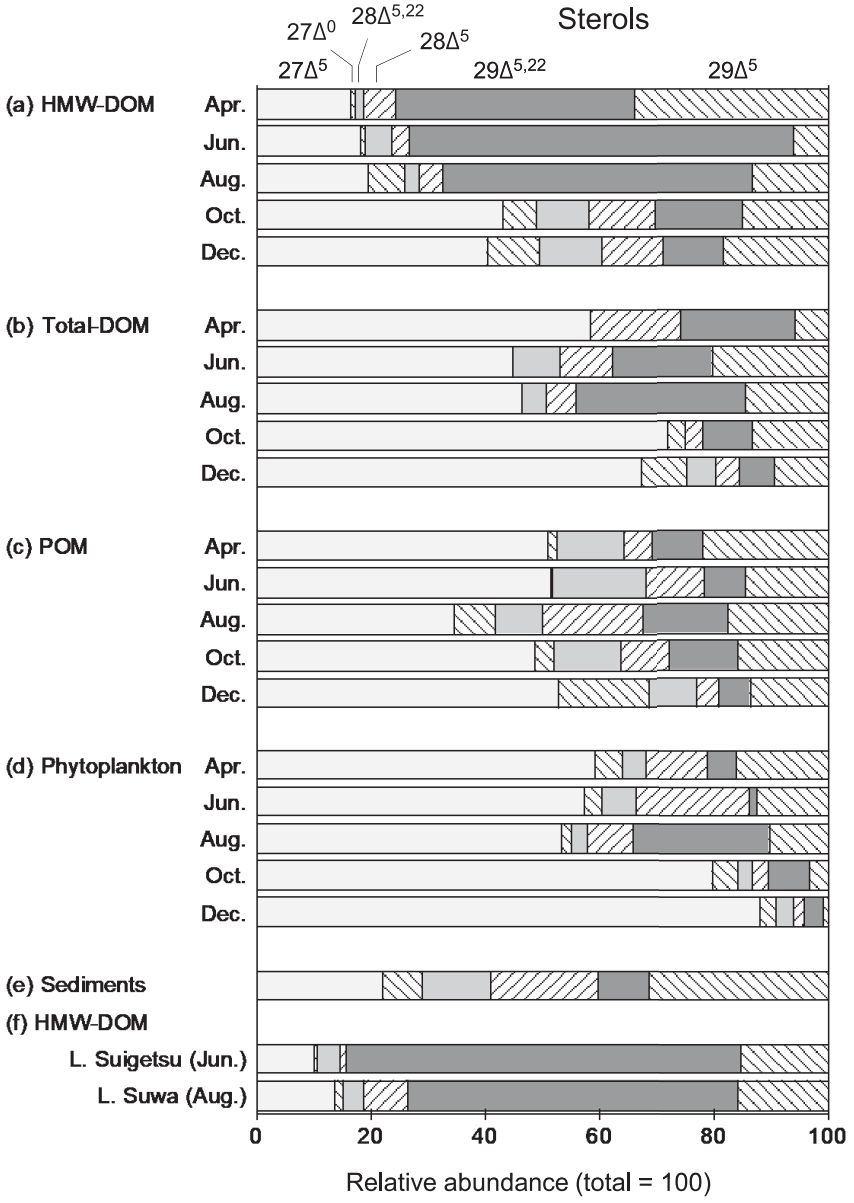

Fig. 2. Relative abundance of sterols in (a) HMW-DOM, (b) total-DOM, (c) POM, (d) phytoplankton and (e) sediments from Lake Kizaki, and in (f) HMW-DOM from lakes Suigetsu and Suwa (total = 100). Abbreviations are given in Fig. 1.

RESULTS

In Lake Kizaki, cholest-5-en-3β-ol (cholesterol, 27Δ^5) is the most dominant sterol in the total-DOM, POM, and phytoplankton (45–85, 34–59, and 53–88 wt% of total sterols, respectively) fractions, whereas the sedimentary sterol is predominated by 24-ethylcholest-5-en-3β-ol (β-sitosterol, 29Δ^5) (31 wt% of total sterols) (Fig. 2). On the contrary, HMW-DOM is uniquely characterized by a considerably high abundance and a large proportion of stigmasterol (29$\Delta^{5,22}$), particularly in May (0.58 μg/L, 65 wt% of total sterols), June (0.76 μg/L, 67 wt% of total sterols), and July (0.86 μg/L, 66 wt% of total sterols). Figure 1(f) also illustrates the predominance of stigmasterol in summer HMW-DOM isolated from Lakes Suwa (0.35 μg/L, 58 wt% of total sterols) and Suigetsu (0.24 μg/L, 69 wt% of total sterols). The predominance of stigmasterol in HMW-DOM fractions thus are commonly found in this and previous studies (e.g., Yoshiyama *et al.*, 2003; McCallister *et al.*, 2006b), which potentially implies that the high abundance of stigmasterol in HMW-DOM is universal, irrespective to the lake environments.

The stable isotope analysis reveals a large variation in the δ^{13}C value between –32.6 and –16.9‰ and in the

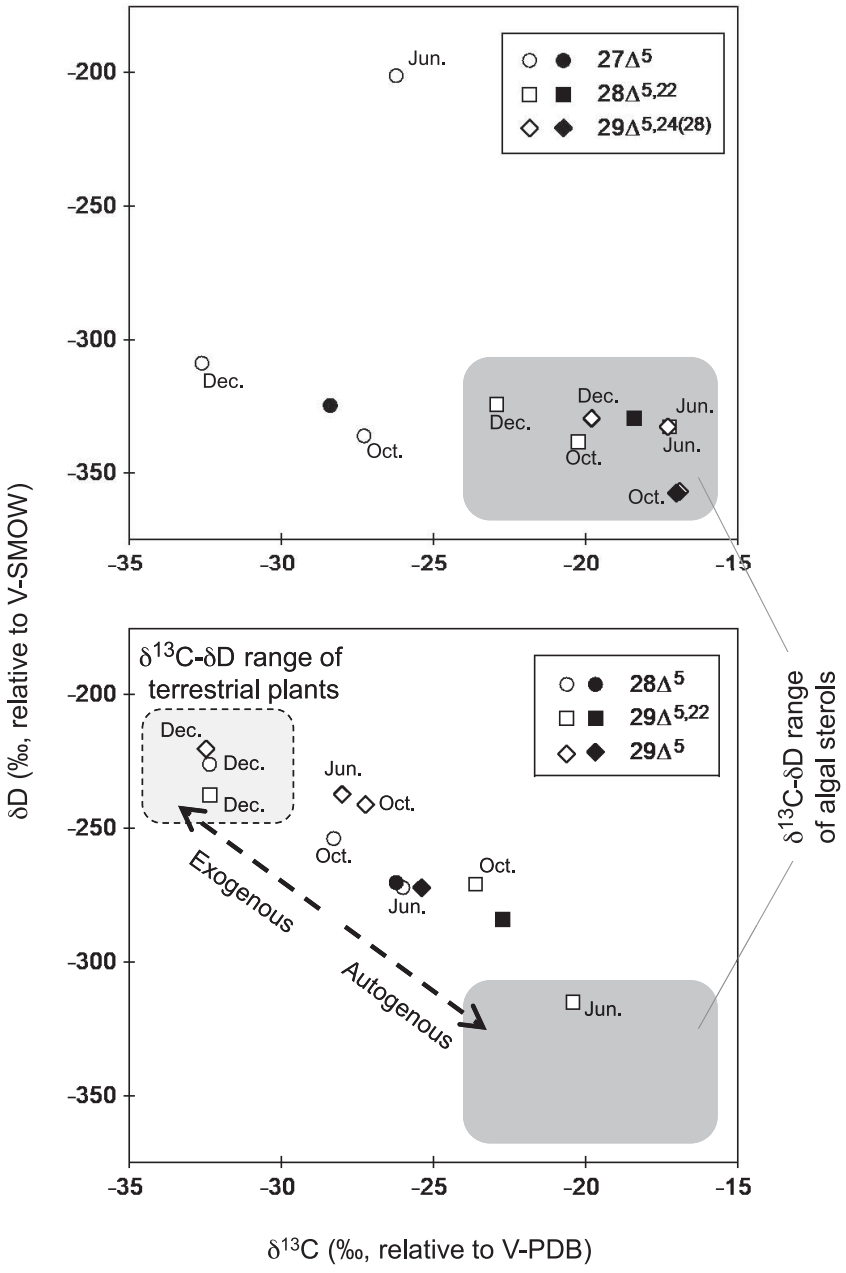

Fig. 3. $\delta^{13}C$-δD cross plots of sterols in HMW-DOM (open symbols) and surface sediments (closed symbols) from Lake Kizaki, with possible $\delta^{13}C$-δD srange of sterols for the potential biological sources. Abbreviations are given in Fig. 1.

δD value between –358 and –201‰ for these sterols (Fig. 3). Among the great diversity in the isotopic compositions, cholesterol is scattered in the δ^{13}C and δD plot (from –32.6 to –26.2‰ for δ^{13}C and from –336 to –201‰ for δD). 24-ethylcholesta-5,22-dien-3β-ol (brassicasterol, 28$\Delta^{5,22}$) and 24-ethylcholesta-5,24(28)*E*-dien-3β-ol (fucosterol, 29$\Delta^{5,24(28)}$) fall within a relatively narrow region, where they are enriched in ^{13}C (between –22.9 and –16.9‰ for δ^{13}C) and depleted in D (between –357 and –325‰ for δD). On the other hand, 24-ethylcholest-5-en-3β-ol (campesterol, 28Δ^{5}), stigmasterol, and β-sitosterol tend to be plotted on the mixing line between the ^{13}C-enriched with D-depleted zone and the ^{13}C-depleted with D-enriched zone. The plots of the three sterols thus shift on the line from the former zone in June toward the latter zone in December.

Discussion

Sources of sterols

Sterols are widely distributed in eukaryotic cell membranes and the structure is variable depending on their biological sources (e.g., Volkman, 2003): among dominant sterols for HMW-DOM and sediments found in this study, it is thought that cholesterol is a widespread sterol found in many types of eukaryotes including algae and animals, but hardly found in terrestrial plants; brassicasterol and fucosterol are representative algae-derived sterols restricted sharply to algae including phytoplankton but not to terrestrial plants; and campesterol, stigmasterol, and β-sitosterol are dominantly found in terrestrial plants but also frequently found in phytoplankton (e.g., Volkman *et al*., 1998). These latter three sterols, however, show isotopic diversity depending on their biological sources: for instance, in the temperate regions, sterols in terrestrial C3 plants (approximately, from –35 to –30‰ for δ^{13}C, from –250 to –200‰ for δD) are relatively depleted in ^{13}C and enriched in D compared to those in algae (approximately, from –15 to –25‰ for δ^{13}C, from –300 to –350‰ for δD) (e.g., Chikaraishi *et al*., 2004; Chikaraishi, 2006). Thus, biological sources of these multi-source sterols (i.e., campesterol, stigmasterol, and β-sitosterol) can be identified by carbon (δ^{13}C) and hydrogen (δD) isotope analysis of them (Chikaraishi *et al*., 2005).

According to this knowledge, in this study, we used the two end-member mixing model with the following assumption, to identify the biological sources of campesterol, stigmasterol, and β-sitosterol in HMW-DOM and sediments in this lake: (1) carbon and hydrogen isotopic compositions of algae-derived sterols (the dark gray zone in Fig. 3) are represented by those of brassicasterol and fucosterol; and (2) the isotopic compositions of terrestrial plant-derived sterols (the light gray zone in broken line in Fig. 3) are available in the isotopic characterization described above. The two end-member model thus can be useful to evaluate biological sources of campesterol, stigmasterol, and β-sitosterol found in HMW-DOM and sediments in the lake, as these sterols are plotted in the mixing zone between the end-member sources, algae and terrestrial plants, with its proportion likely varying among three sampling seasons (Fig. 3).

As a common trend for the three sterols (i.e., campesterol, stigmasterol, and β-sitosterol), the plots for HMW-DOM are gradually moved on the mixing zones between algae- and terrestrial plant-derived sterols, from the former zone in June toward the latter zone in December, through intermediate in October. This trend is particularly remarkable for stigmasterol. A large quantity of stigmasterol in HMW-DOM is found from April to September, but not in October and December (Fig. 2). The isotope signals of stigmasterol are found in the δ^{13}C-δD region of algal sterols in June, but fall in the terrestrial plants region in December, and is intermediate between these two sources in October (Fig. 3). These results suggest that (1) stigmasterol is principally the major sterol indicating terrestrial input of HMW-DOM throughout a year, but (2) an excess stigmasterol from spring to summer HMW-DOM is, however, derived from algal production.

In Lake Kizaki, although stigmasterol is also found in the phytoplankton fraction through seasons as well as in the surface sediments, the dominant sterol is cholesterol and β-sitosterol, respectively, in these samples. These results imply that (1) some minor, specific phytoplankton is as a major source of stigmasterol found in the summer HMW-DOM in the lakes, (2) the stigmasterol from such specific phytoplankton can contribute much into the HMW-DOM but little into the phytoplankton fraction and the surface sediments in this lake, and (3) major phytoplankton species contribute rarely to the stigmasterol in HMW-DOM fraction. Specific species responsible for the abundant stigmasterol observed in the HMW-DOM fraction cannot identified in this study. However, we reveal at least a new finding that autochthonous algae (perhaps some minor phytoplankton) as a major source of the abundant stigmasterol in the HMW-DOM fraction in the lakes.

Sources of HMW-DOM

Source and formation of HMW-DOM and its interaction with POM and LMW-DOM have been poorly understood so far, because molecular and stable isotopic compositions of HMW-DOM are generally far different from those of POM and LMW-DOM (e.g., Benner *et al*., 1997; Ziegler and Fogel, 2003; Yoshiyama *et al*., 2003; McCallister *et al*., 2006a, b; Repeta, 2015). Based on the δ^{13}C and δD values of sterols found in this study, we suggest that the source of HMW-DOM varies in season, as HMW-DOM in summer is mainly derived from autochthonous phytoplankton whereas that in winter is mainly derived from allochthonous terrestrial plants at least in Lake Kizaki. Moreover, as similar to the observation in previous studies (e.g., Benner *et al*., 1997; Repeta, 2015; McCallister *et al*., 2006a, b), the relative proportion of sterols in HMW-DOM is far different from that in POM and phytoplankton as well as sediments, meaning that organic components of HMW-DOM have only minor compatibility or transformability between HMW-DOM and POM and/or between HMW- and LMW-DOM in the lakes, and that HMW-DOM therefore plays a unique carbon cycle apparently independent of the carbon cycle composed of POM, LMW-DOM, and sediments.

Summary

Source and formation of HMW-DOM and its interaction with POM and LMW-DOM have been poorly understood so far, although DOM represents a large reservoir of organic carbon in the hydrosphere and makes up a key component of the global carbon cycle. In this study, we further evaluate a very unique sterol proportion (i.e., a large proportion of stigmasterol) in HMW-DOM (1 kDa << 0.6 μm) during summer in three lake waters, which is far different from the sterol proportion of POM and sediments within the same lakes. Moreover, stable carbon and hydrogen isotope analysis illustrates that this abundant stigmasterol is derived from autochthonous algae (perhaps some minor, specific phytoplankton) in the lake. These results reveal that (1) the source of HMW-DOM varies in season, as HMW-DOM in summer is mainly derived from autochthonous phytoplankton whereas that in winter is mainly derived from allochthonous terrestrial plants; and (2) there is only minor compatibility or transformability between HMW-DOM and POM and between HMW- and LMW-DOM. The uniqueness and apparent difference in the molar and isotopic compositions of sterols in HMW-DOM thus provide that biological sources, formation, and geochemical behavior and function of LMW-DOM are far different from those of POM and LMW-DOM in the hydrosphere, and further suggest that combination analysis of molar and isotopic compositions of other biomarkers (e.g., fatty acids, hopanoids, and etherlipids) in HMW-DOM will clarify the biological sources and formation of HMW-DOM and its interaction with other organic carbon pools in environments.

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Supplementary Materials

URL (http://www.terrapub.co.jp/journals/GJ/archives/data/52/MS528.pdf)
Figures S1 and S2