Holtvoeth, et al. "Improved end-member characterization of modern organic matter pools in the Ohrid Basin (Albania, Macedonia) and evaluation of new palaeoenvironmental proxies"

Supplement 2

A: Compound-specific isotope analyses (CSIA) – Results, potential and methods

B: Additional information on specific biomarkers observed

C: References

A: Compound-specific isotope analyses (CSIA)

Results

- *Carbon ($\delta^{13}C$)*

Carbon isotope composition ($\delta^{13}C$) was determined for even-numbered *n*-alkanoic acids ranging from C_{16}-FA to C_{30}-FA in leaf litter from sites SN, TP and GN and the topsoils from TP and GN. Furthermore, we measured $\delta^{13}C$ for C_{16} FA in *Cladophora* sp., *Potamogeton perfoliatus* and *Chara tomentosa*. The $\delta^{13}C$ values for C_{16} FA range from about -29 ‰ to -22 ‰. The C_{16} FA in *Cladophora* sp. appears the lightest (-28.7 ± 0.3 ‰) while it is slightly heavier in *Chara tomentosa* (-24.6 ± 0.2 ‰) and the heaviest in *Potamogeton perfoliatus* (-22.7 ± 0.4 ‰; Fig. 5). In all leaf litter samples C_{16} FA is the lightest compound, with values ranging from -36 ‰ at SN to -32 ‰ at GN. Leaf litter FAs at GN tend to be heavier while FAs at site SN are the lightest. This is particularly true for C_{16}, C_{18} and C_{22} FA. By contrast, C_{16} FA and, in particular, C_{18} FA are the heaviest compounds in the soil samples at TP and GN. Compared to the corresponding leaf litter samples, C_{16} FA and C_{18} FA are heavier by > 4 ‰ and > 3 ‰, respectively. This significant difference in the $\delta^{13}C$ values of C_{16} FA and C_{18} FA between litter and soil suggests enhanced contributions from an OM source that mainly provides isotopically heavy short-chain compounds, and heavy C_{18} FA in particular, as they are likely biosynthesized by soil OM degraders. Unlike the fractionation occurring during autotrophic CO_2 fixation, fractionation during heterotrophic OM biosynthesis is minor, leading to the view that heterotrophs such as bacterial and fungal soil OM degraders reflect the isotopic composition of their substrate (see, e.g., Boschker and Middleburg, 2002, and references therein). However, during the early stages of soil OM degradation, bacterial and fungal saprotrophs selectively consume OM compounds that are easy to break down, which may lead to significant differences in isotopic composition of lipids biosynthesized *in situ* to those in the substrate. For example, cellulose of C_3 plants is enriched in ^{13}C relative to their lipids (Hobbie and Werner, 2004). Thus, the observed shift in short-chain FA's isotopic composition between leaf litter and topsoil could be explained by selective substrate utilization by soil microbes and enhanced contributions from isotopically heavy microbial biomass. Investigating the relationship between the carbon isotopic composition of certain substrates and of short-chain FAs of 11 strains of microbes, Abraham et al. (1998) found that fungi preferentially utilized glucose from a mixed substrate to form C_{16} FA. They also found that C_{18} FA was more enriched in ^{13}C relative to C_{16} FA. Hence, it is possible that the heavy isotopic signature of both C_{16} and C_{18} FA in the topsoils and of C_{18} FA in the leaf litter results from fungal breakdown of cellulose

(polysaccharide) while C_{16} FA in leaf litter still overwhelmingly derives from plant lipids. FAs of chain lengths ≥ 20 become increasingly depleted in ^{13}C with increasing chain length. Significant amounts of mid-chain C_{22} and C_{24} FAs likely derive from suberin, which may modify their isotopic composition towards slightly heavier values as small differences in the isotopic composition between root and leaf tissues have been observed (Lichtfouse et al., 1995). However, long-chain C_{26}, C_{28} and C_{30} FA probably derive solely from leaf waxes and their $\delta^{13}C$ values still show a robust decreasing trend in all investigated soil samples (average R^2 = 0.93), with the C_{30} FA being 0.9 - 2.5 ‰ lighter than C_{26} FA. Such a trend has been described previously by Lockheart et al. (1997) for long-chain *n*-alkanes (C_{27}, C_{29}, C_{31}) in leaves of *Betula ermanii*, *Quercus castaneifolia*, *Fagus japonica* and *Fagus sylvatica*, notably when the leaves were sampled in autumn. With a decrease in $\delta^{13}C$ of 2 to 4 ‰, the trend was more pronounced for *Quercus castaneifolia* and *Betula ermanii* than in *Fagus japonica* (2 ‰ change for C_{31} *n*-alkane, only). The C_{27} *n*-alkane of *Fagus sylvatica*, the species present in the high-altitude forests of the Ohrid Basin, showed a decrease of about 1 ‰ between May and October. The seasonal trend results from replenishment of leaf waxes over the course of the growing season during which both the isotopic composition of the substrate used for leaf wax biosynthesis and of the leaf wax itself are changing (Lockheart et al., 1997 and references therein). Finally, odd-numbered FAs are depleted in ^{13}C relative to the even-numbered FAs in the soil samples (Fig. 6), with $\delta^{13}C$ values for C_{25}, C_{27} and C_{29} FA that are lower by 0.5 ‰, on average and up to 1.1 ‰ for C_{25} FA in the high-altitude soil, for example. This difference results from the elongation of FAs following the acetyl-coenzyme A (acetyl-CoA) pathway and the fact that the methyl and carboxyl carbon atoms in acetyl-CoA have a distinctly different isotopic composition (Melzer and Schmidt, 1987; van der Meer et al., 2001). Although these patterns should also be reflected in the isotopic composition of the leaf litter lipids, we do not have enough data for the long-chain FAs from litter samples to confirm this.

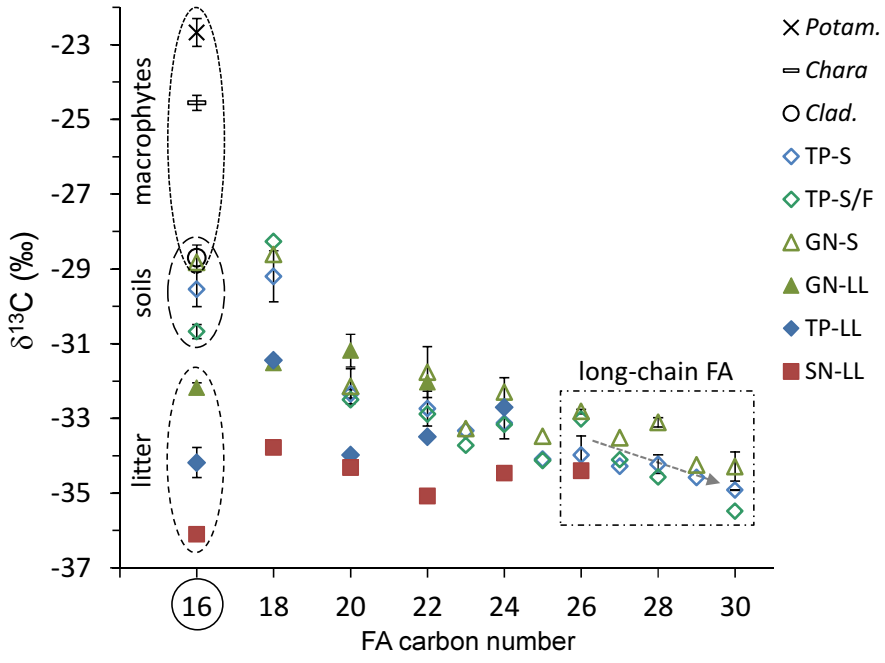

Figure S1. Carbon-isotope composition ($\delta^{13}C$) of *n*-alkanoic acids from macrophytes, leaf litter and topsoils (LL = leaf litter, S = soil, S/F = soil visibly affected by fungi). While the $\delta^{13}C$ values for C_{16} FA are heaviest in macrophytes and lightest in leaf litter, as expected for aquatic and terrestrial biomass, there is a significant shift toward heavier values for C_{16} FA in soils that even overlap with the value for *Characeae* spp.. Also note the trend of decreasing $\delta^{13}C$ values of long-chain FAs (dashed arrow) and the lower values of odd-numbered long-chain FAs.

- *Hydrogen (δ^2H)*

Hydrogen isotope composition was determined for even-numbered FAs ranging from C_{16} to C_{32} FA in topsoils from SN, TP and GN, in leaf litter from sites SN, GN and in samples of *Potamogeton perfoliatus*, *Chara tomentosa* and *Phragmites* roots and leaves. The δ^2H values range from -204 ‰ for C_{16} FA in *Phragmites* roots to -119 ‰ for C_{18} FA in leaf litter from site SN (Fig. 6). On average, lipids from leaf litter and soils from all terrestrial sites are heavier (-161 ± 17 ‰) than lipids from macrophytes (-193 ± 17 ‰) by about 30 ‰, with the exception of lipids from *Phragmites* leaves that show a 'terrestrial' value of -160 ± 12 ‰. The large overall range within the terrestrial lipids is mainly due to the samples from site SN, with the leaf litter being particularly heavy (-135 ± 13 ‰). This likely results from the fact that some plants contributing leaves to the litter layer utilize water vapour from year-round evaporation and condensation over the ponds of the St Naum springs, as opposed to seasonal uptake of precipitation or soil water at the other sites. The spring water is enriched in ^2H relative to the local meteoric water (Matzinger et al., 2006), with very little variation year-round (Jordanoska et al., 2010), and the SN sampling site is placed in a morphologically sheltered area just a few meters above the spring-fed ponds. As the springs are active year-round delivering up to 7500 L s^{-1} with a constant temperature of 10 - 12°C they create a microclimate that is cooler and more humid in the summer compared to the surroundings, with mist frequently forming over the ponds. This microclimate is also responsible for the specific flora occurring around the St Naum springs (Matevski et al., 2011). Even though soil evapotranspiration rates at sites TP and GN are certainly higher than in the surroundings of the St Naum spring, it appears that the vegetation at these sites utilizes water depleted in ^2H compared to the main water source for the vegetation at SN. While higher evaporation rates should increase the amount of the heavy isotope in the soil water, other factors such as seasonal variability of δ^2H in precipitation presumably outweigh this fractionation process leading to the overall lighter isotope composition of plant lipids at sites TP and GN. Without monitoring of precipitation and soil water isotopic composition, we cannot fully explain these observations.

Lipid δ^2H values in soils and litter from sites TP and GN are lower and show a smaller range (-168 ± 10 ‰) compared to site SN (-142 ± 17 ‰). The heaviest compound in the TP soil samples is C_{18} FA (-150 ± 2 ‰) while C_{16} FA is the lightest (-183 ± 9 ‰). Short-chain compounds are likely to derive from mixed terrestrial sources, i.e. leaf waxes as well as fungal and/or bacterial biomass. This may explain both the lighter value of C_{16} FA and the heavy C_{18} FA in the TP samples depending on whether an organism takes in water directly from precipitation or water that is enriched in ^2H after soil evapotranspiration. In the soil from site SN, $C_{18:1}$ FA and C_{16} FA also show significantly lighter values of -177 ± 0.4 ‰ and -170 ± 2

‰, respectively, compared to the other compounds ($C_{18:2}$ FA, C_{18} – C_{28} FA: -143 ± 13 ‰, on average) again suggesting their biosynthesis involves water derived from different sources. At site GN, on the other hand, long-chain C_{26} and C_{30} FA are the lightest compounds (-184 ± 8 ‰ and -184 ± 1 ‰, respectively), which may arise through uptake of deuterium-depleted precipitation at higher altitude.

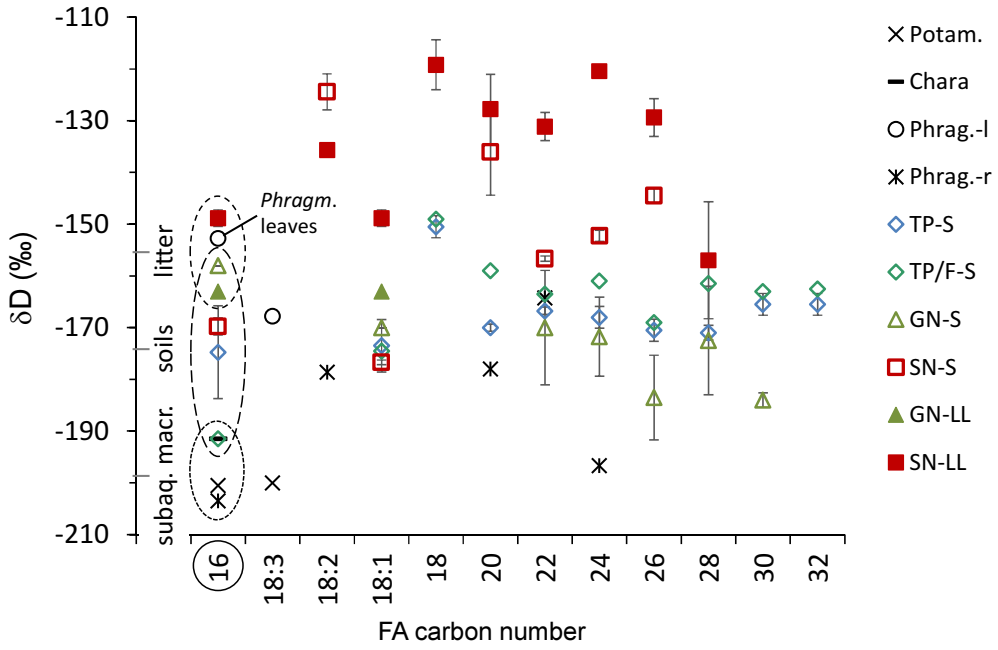

Figure S2. Hydrogen-isotope composition (δ^2H) of *n*-alkanoic acids from macrophytes, leaf litter and topsoils (LL = leaf litter, S = soil, S/F = soil visibly affected by fungi, l = leaves, r = roots). Submerged macrophytes (*Potamogeton* spp., *Chara* spp.) show the lightest values, as expected. Leaves of *Phragmites* spp. plot within the range covered by litter from sites SN and GN. δ^2H values of C_{16} FA from soil and litter overlap, while the sample affected by white rot plots in the range of submerged macrophytes. Note the significantly higher δ^2H values of FAs from site SN that probably result from the intake of water vapor from the St Naum karst springs, which is enriched in deuterium compared to the local meteoric water line (Matzinger et al., 2006).

Potential of carbon and hydrogen isotopes of C_{16} FA for assessments of aquatic and terrestrial origins of sedimentary OM

Interpreting compound-specific isotopic data ($\delta^{13}C$, δ^2H), for example using the C_{16} FA, which is present in all terrestrial and lacustrine samples is not straightforward. Hence, in soil and leaf litter samples there is a range of > 7 ‰ (-29 to -36 ‰) in $\delta^{13}C$. Since both are likely sources of OM to the lake, then change in sedimentary C_{16} FA $\delta^{13}C$ might simply reflect the strength of the soil *vs*. litter supply, rather than any change in terrestrial *vs*. aquatic source. A variation of > 6 ‰ in macrophyte samples adds to the uncertainties. Using a similar approach with δ^2H may be more fruitful as on average the difference between terrestrial and aquatic sources is *ca.* 30 ± 8 ‰ for the C_{16} FA. However, even within the limited number of soil and leaf litter samples

analysed, there is > 40 ‰ variation in δ^2H. Factors such as the amount of microbial biomass contributing to the terrestrial OM, variable input of emergent macrophytes (*Phragmites* leaves), or a local moisture sources (St Naum springs) all enhance overall variability and complexity. Developing a mixing model for aquatic *vs*. terrestrial OM in the sediments using $\delta^{13}C$ and δ^2H thus appears challenging.

Methods

For compound-specific carbon and hydrogen isotopes analyses, *n*-alkanoic acids (FAs) were isolated from the TLEs. TLEs were first taken up on a small amount of alumina, which was dried and transferred onto a silica column (2.5 g of silica in 25 cm Pasteur pipettes). Fractions were successively eluted using solvent mixtures of increasing polarity (3.5 mL each of *n*-hexane (fraction 1), *n*-hexane/toluene 3:1 (fraction 2), *n*-hexane/toluene 1:1 (fraction 3), *n*-hexane/ethyl acetate 9.5:0.5 (fraction 4), 9:1 (fraction 5), 8.5:1.5 (fraction 6) and 8:2 (fraction 7)). Long-chain FAs (> C_{20}) elute in fraction 3 while short-chain FAs (< C_{20}) elute in fraction 4. As C_{16} was the dominant FA in many samples, this chain-length separation was useful as comparable concentrations for isotope analyses were achieved for short and long-chain compounds, thus, avoiding bias related to significant differences in signal/peak size.

Stable carbon isotopic compositions of individual lipids were determined using a Thermo Trace Ultra GC linked to a Delta V Advantage isotope ratio monitoring MS (irmMS) by a ConFlo IV interface (Thermo Fisher, Bremen, Germany). Samples dissolved in hexane were injected in splitless mode onto a DB-5 fused silica capillary column (30 m, 0.25 mm ID, 0.25 µm film thickness, J&W Scientific). The injector temperature was 280ºC. The GC temperature was held at 45ºC (1 min), then programmed to 295ºC at 6ºC min^{-1} and held for 15 minutes. The carrier gas was ultra-high purity grade helium at a constant flow rate of 1.4 mL min^{-1}. The GC column fed directly into a combustion reactor (1000ºC). The combusted compounds then passed through a water separator consisting of a Nafion tube, prior to entering the isotope ratio mass spectrometer. CO_2 reference gas (externally calibrated relative to Vienna Pee Dee Belemnite (VPDB) on a dual inlet mass spectrometer) was automatically introduced into the irmMS in a series of pulses and its $^{13}C/^{12}C$ ratio measured. The accelerating voltage was 3 KV and the trap current and box voltage were set at 0.84mA and 0.66V respectively; the electron energy was 124 eV. The irmMS automatically measured the ion intensities of m/z = 44, 45, 46 corresponding to $^{12}C^{16}O_2$, $^{13}C^{16}O_2$, and $^{12}C^{16}O^{18}O$, respectively. Thermo Isodat 3 software automatically computed the $^{13}C/^{12}C$ and $^{18}O/^{16}O$ ratios of each sample peak, referenced to the standard CO_2 gas and its known $^{13}C/^{12}C$ and $^{18}O/^{16}O$ content. Carbon isotopic compositions represent averaged values of duplicate or triplicate analyses. The results are reported in per mil (‰) relative to VPDB international standard. Standards containing FA methyl esters (FAMEs; Schimmelmann, Indiana University, Bloomington, IN, USA) were used to determine instrument precision (<0.3 ‰) and accuracy (<0.5 ‰).

Stable hydrogen isotopes of individual fatty acid methyl esters (FAMEs) were measured in duplicate or triplicate using a Thermo Trace Ultra GC linked to a Thermo Delta V+ irmMS (HT voltage 3-5kV, Trap current 0.75mA, Box current 0.7mA) via a Combustion III Interface (Thermo Fisher, Bremen, Germany). Samples dissolved in hexane were injected in splitless

mode by a CTC auto sampler onto a DB-5MS UI fused silica capillary column (30 m, 0.25 mm ID, 0.25 μm film thickness, J&W Scientific). The injector temperature was 280°C. The GC temperature was programmed from 50 to 180°C at 10°C min^{-1} and to 300°C at 3°C min^{-1} and held for 3 minutes. Helium flow was 1 mL min^{-1}. The solvent peak was diverted to the FID and H_2 reference gas was initially pulsed into the mass spectrometer. After 7 minutes the back flush valve directed the split sample *via* the combustion furnace (1400°C) into the mass spectrometer and the isotope ratio was measured. Data acquisition was controlled by Thermo Isodat software and raw data was processed using the Isodat dynamic background integration Workspace software. Isotope ratios are given as δ^2H values relative to the reference gas (H_2) calibrated from a reference alkane A4 or FAME F8 mixture with known isotopic values for hydrogen (Schimmelmann, Indiana University, USA). The average standard deviation for duplicate and triplicate analyses of all samples was ± 4 ‰.

B: Biomarkers

Taraxasterol (in leaf litter at SN and TP)

This biomarker occurs in exceptionally high amounts in *Lapsana communis* or nipplewort (Fontanel et al., 1998), which is an element of the "Riverine Forest" vegetation in the area of the St Naum springs (Matevski et al., 2011). Other members of the Asteraceae family such as *Jurinea taygetea* that are present in Macedonia (Ministry of Environment and Physical Planning, 2003) have also been reported to have a high abundance of taraxasterol (Mikolajczak and Smith, 1967; Muley et al., 2009). The absence of taraxasterol in the topsoil samples underlying the leaf litter at sites SN and TP suggests that it is quickly degraded. Taraxasterol was absent in the litter and soil samples at GN.

Steroids

- *in leaf litter and soils*

The main sterol in both leaf litter and topsoils is β-sitosterol accounting for 60 to 80 % of the total sterols. Other steroids include stigmastanol, cholesterol, stigmasterol, campesterol, brassicasterol, ergosterol and β-sitostenone (see Appendix for IUPAC names), all of which are proportionally more abundant relative to β-sitosterol in the topsoils than in the leaf litter samples. The exception is β-sitostenone, which accounts for 12 to 21 % of the total steroids in the leaf litter samples and most likely arises from early plant litter breakdown and has been reported, e.g., in the common wood-decaying fungus, *Xylaria* spp. (Wang et al., 2014).

The leaf litter TLEs from all sites contain *ca.* 3 $\%_{lipids}$ α-tocopherol (vitamin E), which functions as an antioxidant in plants (e.g., Chevolleau et al., 1993) and has also been identified in fungi (e.g., Reis et al., 2011). In the topsoils, the amount of α-tocopherol drops to *ca.* 0.4 $\%_{lipids}$.

- *in macrophytes*

Apart from the main sterols, β-sitosterol, stigmasterol and cholesterol, the investigated macrophytes also contain α-tocopherol and β-sitostenone. In the *Phragmites* sp. samples, the

concentration of α-tocopherol is higher in the leaves (13 µg/g, 0.75 $\%_{lipids}$) and lower in the stem and roots (0.4 µg/g, 0.1 $\%_{lipids}$). By contrast, proportions of β-sitosterol and β-sitostenone are higher in stem and root and reflect the requirement of different cell membrane properties and, perhaps, a demand of moderately antibacterial compounds (Nair et al., 2012; Bumrela and Naik, 2011).

- in water filtrates

Although the polar fraction of the TLEs from the water filtrates generally did not contain non-polar steroids we did find stigmast-3,5-dien-7-one (0.7 % polar lipids) in the polar fraction.

- 5β-stanols in Lz1120 sediments

Coprostanol and epicoprostanol are steroids that are frequently used as biomarkers for human faeces (Sherwin et al., 1993; Bull et al., 2000; Cordeiro et al., 2008). Notably, both compounds are present throughout the sedimentary record of Lake Ohrid, i.e. even in periods when supposedly no substantial human populations were present, such as during the Eemian warm period (Holtvoeth, unpubl. data). Furthermore, their occurrence appears related to that of epicholestanol, with the three stanols showing a similar distribution whenever present (Fig. S3). This distribution appears to represent the background distribution and is likely the result of the presence and activity of anoxic bacteria *in situ* (Mermoud et al., 1985, Holtvoeth et al., 2010). The only exception is the surface sample of sediment core Lz1120 where the amounts of coprostanol and epicoprostanol are significantly increased due to modern sewage supply (Fig. S3).

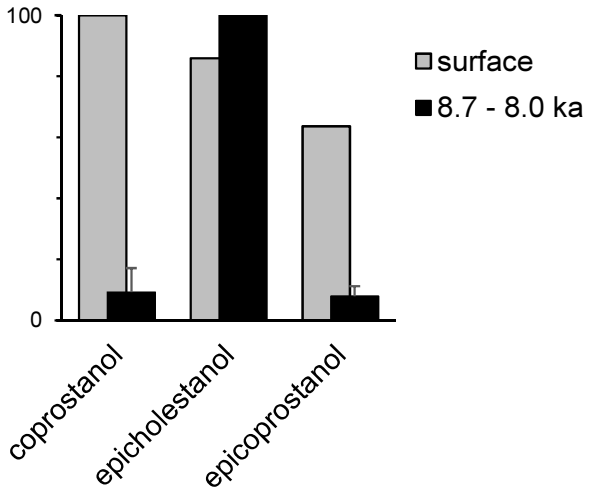

Figure S3. Average distribution of 5β-stanols in surface and Holocene sediments from Lz1120 (in % normalized to main compound).

Neurosporaxanthine

The methyl ester of the pigment β-apo-4'-carotenoic acid (neurosporaxanthin) was detected in the polar lipid fraction of the water filtrates (0.8 $\%_{polar\ lipids}$).

Miscellaneous biomarkers observed in the sediments ("others")

Miscellaneous compounds, summarized as "others" in Table 2 of the main text, account for 17 % of the total lipids in both high- and low-carbonate samples. These include 17β(H),21β(H)-bishomohopanoic acid (9.3 and 6.1 $\%_{lipids}$ in high- and low-carbonate samples), 1,15-triacontadiol (C_{30} 1,15-diol) and 1,15-dotriacontadiol (C_{32} 1,15-diol) (together 2.8 and 3.4 $\%_{lipids}$), 1,15(ω16)C_{30}keto-ol (1.0 and 1.3 $\%_{lipids}$), tetrahymanol (0.7 and 1.5 $\%_{lipids}$), 17β(H),21β(H)-bishomohopanol (0.6 and 1.6 $\%_{lipids}$), β-amyrin (0.7 and 1.2 $\%_{lipids}$) and small amounts of *iso*- and *anteiso*-branched C_{15} OH (< 0.5 $\%_{lipids}$). α-Tocopherol was also present in the sediments in low amounts of 0.7 and 0.4 $\%_{lipids}$ in both high- and low-carbonate samples. A number of compounds appear in the sediments that are absent in the terrestrial materials and the macrophytes. Apart from the methyl ketones, these include steroids (coprostanol, epicholestanol, epicoprostanol, cholestanol, 24-methyl-5α(H)-cholestan-3β-ol, 24-ethyl-5α(H)-cholest-22(E)-en-3β-ol, dinosterol, dinostanol, lanosterol, *iso*- and *anteiso*-branched C_{15} OH, C_{30} 1,15-diol and C_{32} 1,15-diol, 1,15-(ω16)-C_{30}-keto-ol, tetrahymanol, 17β(H),21β(H)-bis-homohopanol, 17β(H),21β(H)-homohopanoic acid and 17β(H),21β(H)-bis-homohopanoic acid. While most of these compounds are minor components (<2 $\%_{lipids}$), the long-chain diols, lanosterol and 17β(H),21β(H)-bis-homohopanoic acid, on average, account for 3, 4 and 8 $\%_{lipids}$, respectively. The absence of these compounds in the modern terrestrial samples implies that they are biosynthesized in the water column or *in situ*. The fact that they were not observed in the in most of the water filtrates, only the 40 m sample from site Co1202 contains a small amount of lanosterol (0.2 $\%_{lipids}$), is probably due to the small sample size. A bacterial origin in the surface sediment for 17β(H),21β(H)-bis-homohopanoic acid, 17β(H),21β(H)-homohopanoic acid and 17β(H),21β(H)-bis-homohopanol as well as for the branched C_{15} OH seems most likely. Epicholestanol concentrations correlate well with those of 17β(H),21β(H)-bis-homohopanoic acid in the high-carbonate samples (R^2 = 0.89), suggesting that it also originates from a bacterial process for example through reduction of cholesterol (in low-carbonate samples 17β(H),21β(H)-bis-homohopanoic acid appears less well preserved). Although long-chain diols are frequently found in sediments, their sources are not fully known. However, they have been found in marine as well as freshwater eustigmatophytes (Volkman et al., 1992, 1999), with the C_{32} 1,15-diol dominating in the marine environment and the C_{30} 1,15-diol dominating in the freshwater environment. We therefore assume that the diols found in Lake Ohrid sediments derive from this microalgal source.

Although not observed in the water filtrates, dinosterol and dinostanol are most likely to derive from organisms living in the water column, in particular dinoflagellates (Robinson et al., 1987). While C_{28} sterols such as 24-methyl-5α(H)-cholestan-3β-ol are often ascribed to diatoms, C_{29} sterols such as 24-ethyl-5α(H)-cholest-22(E)-en-3β-ol are assumed largely to derive from a terrestrial source (Xu et al., 2006), although we did not find the latter in any of our modern samples. Finally, tetrahymanol likely derives from ciliates living at the sediment-water interface. (Schwalb et al., 2013).

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