ORGANIC GEOCHEMISTRY OF STRATIFIED EUTROPHIC ALPINE LAKES (LAKE BLED)

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Doctoral Dissertation
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ORGANSKA GEOKEMIJA STRATIFICIRANIH EVTROFNIH ALPSKIH JEZER (BLEJSKO JEZERO)

Doktorska disertacija

Supervisor: Assoc. Prof. Dr. Nives Ogrinc

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Mojim fantom

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Abstract

The identification and quantification of organic compounds present in the environment is a major area of application in modern analytical chemistry. However, it is still scarcely recognized that, in addition to the chemical identity and concentration of organic compounds, there is more information to be found about their source and fate in the environment from their isotope composition. Isotope ratio mass spectrometry (IRMS) following on-line combustion (C) of compounds separated by gas chromatography (GC-C-IRMS) has been commercially available only since 1990. Our understanding of the isotope composition of organic compounds in different studies is therefore still somewhat limited.

In this doctoral thesis, organic geochemical biomarkers, combined with compound specific isotope composition, has been used to determine the sources and transformation pathways of organic matter (OM) in the anoxic, eutrophic alpine Lake Bled. The stable isotope approach was further used to identify the sources of polycyclic aromatic hydrocarbons (PAHs) in sediments. The determination of sources and transformation of OM has been based on the composition of lipids. Importantly, among the available molecular markers, lipids are used mainly because of their source specificity and higher resistance to bacterial degradation than other classes of organic compounds. Examination of lipid biomarkers in particulate organic matter (POM) and suspended trap material in the water column indicates whether fatty acids (FA) of algal origin were abundant in the epilimnion while, in the anoxic zone, POM and trap material were enriched in bacterial FA. The lowest isotopic composition of carbon ($\delta^{13}C$) value of –51.7‰ was observed in *cis*-11-octadecenoic acid (18:1*n*-7) in trap material and was the only FA linked to methanotrophic bacteria. In addition, zooplankton left a marked imprint on particulate lipids and trap material at 12 m based on the predominance of octadecanoic acid (*n*-$C_{18:0}$) over hexadecanoic acid (*n*-$C_{16:0}$), short–chain, even-carbon *n*-alkenols, the high proportion of cholest-5-en-3β-ol (44.8% of total sterol concentration (TST)), a cholesterol/phytosterol ratio of 0.49 and isotopic composition of particulate nitrogen ($\delta^{15}N_{PN}$) values of 6.8 and 11.7‰. The sterol distribution reflects a primarily plankton source, while 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol) and 24-methylcholest-5-en-3β-ol (campesterol) are both of allochthonous origin, coming from terrestrial plants.

Lipids in sediments from two different locations in western basin and Zaka Bay were also mainly of autochthonous origin, however their distribution and isotope composition differ significantly from those in POM and sedimentary trap material collected 2 m above the sediment in western basin. These results indicate that some biomarkers have different source organisms that produce the same molecule. Thus, isotope composition should be used as a complementary tool for source identification. It is interesting to note that source identification based only on lipid distribution could lead to incorrect conclusions. Low $\delta^{13}C$ values for long-chain *n*-alkanes, *n*-alcohols and FAs and, especially, those determined in C_{29} sterols, indicate that these compounds were derived mainly from microalgae and bacteria and not from higher plants or other terrigenous OM, as previously reported. The $\delta^{13}C$ values observed in long-chain FAs and, especially, sterols, were lower in the western basin sediments comparing to that in Zaka Bay, indicating the

pronounced contribution of an anaerobic, bacterial source. On the other hand, the $\delta^{13}C$ values obtained for short-chain FAs in both environments and for short-chain *n*-alkanes in Zaka Bay indicate a larger contribution of more refractory terrestrial OM. $\delta^{13}C$ values of other lipids were similar in the two sediments showing that identification of their sources did not results from different depositional regimes.

It is believed that the presence of more labile autochthonous OM could also influence the methanogenesis pathway in sediments. Acetate fermentation appears to be associated with more labile OM, whereas reduction of carbon dioxide (CO_2) to methane utilizes more refractory OM. However, the biogeochemical processes and structure of the archaeal community determined in Lake Bled sediments do not support this assumption. It was found that hydrogenotrophic methanogenesis is the dominant pathway in the sediment of alpine Lake Bled, despite the low temperature and prevalence of "fresh" autochthonous derived OM.

Finally, molecular and stable isotope analyses were combined to trace and identify the sources of PAH in sediments. The concentrations of PAH in surface sediments at the two locations (western basin and Zaka Bay) were comparable and higher than those found in previous studies, reaching 4230 and 4380 ng g^{-1}. It was found that retene (Re) and perylene (Per) are both of mainly natural origin in Zaka Bay while, at station D in western basin, the value of $\delta^{13}C$ determined at a depth of 12–14 cm in the 1950s indicates that Re is of pyrolytic origin. The distribution of $\delta^{13}C$ values of other individual PAHs showed that the PAH input to lake sediments was of pyrolytic origin, probably dominated by coal and later, in the 1950s, also by wood burning. The influence of PAH originating from vehicle emissions could be seen at the depth of 12–14 cm in western basin and Zaka Bay. This depth corresponds to the period 1953–1961.

The doctoral thesis clearly indicates that the origin of OM and pollutants in aquatic environments should be verified by stable isotope geochemistry.

Povzetek

Identifikacija in kvantifikacija organskih spojin prisotnih v okolju je glavna aplikacija na področju sodobne analizne kemije. S pomočjo izotopske sestave lahko poleg kemijske identifikacije in koncentracije organske snovi dobimo dodatne informacije o izvoru in usodi organskih komponent v okolju. Masni spektrometer za stabilne izotope sklopljen s plinskim kromatografom je komercialno dostopen od leta 1990, zato je naše razumevanje o izotopskih sestavi organskih komponent v različnih okoljih še vedno omejeno.

Doktorsko delo vključuje geokemijske raziskave z namenom določitve sestave, izvora in kroženja organske snovi v stratificiranem alpskem jezeru – Blejskem jezeru. Pri raziskavah smo se osredotočili na lipidne organske sledilce (biomarkerje) in na osnovi izotopske sestave na molekularnem nivoju določili njihov izvor v vodnem stolpcu in sedimentih Blejskega jezera. Mnogi med njimi omogočajo edinstven vpogled v izvore, diagenetske spremembe, depozicijo in ohranjanje organske snovi v sedimentu. Čeprav so bile prve raziskave biomarkerjev opravljene že v osemdesetih letih, so te študije še vedno aktualne, predvsem zaradi vedno novih odkritij lipidov iz alg, bakterij, zooplanktona in ostalih organizmov, ki jih uporabljamo za zelo specifično identifikacijo izvora organske snovi. Uporaba stabilnih izotopov nam omogoča tudi določiti izvore polutantov v okolju. Prav na podlagi izotopske sestave smo določili izvore policikličnih aromatskih ogljikovodikov (PAH) v sedimentih Blejskega jezera. Ocena lipidnih biomarkerjev v partikulatni organski snovi (POM) in v pasteh v vodnem stolpcu, kažejo na prisotnost alg v epilimniju medtem ko je POM in posedeni material v pasteh v anoksičnem okolju obogaten z lipidi bakterijskega izvora. Najnižja izotopska sestava ($\delta^{13}C$) –51,7 ‰ je bila določena v *cis*-11-oktadecenojski kislini (18:1*n*-7) v pasti in je edina FA, katere izvor so metanotrofne bakterije. Prisotnost zooplanktona na globini 12 m potrjujejo višje vsebnosti oktadekanojske FA (n-$C_{18:0}$) v primerjavi s heksadekanojsko FA (n-$C_{16:0}$), kratkoverižni alkoholi s sodim številom C atomov, visok delež holest-5-en-3β-ol sterola, ki predstavlja 44,8 % celokupne koncentracije sterolov (TST), holesterol/fitosterol razmerje 0,49 in izotopska sestava suspendiranega dušika ($\delta^{15}N_{PN}$) z vrednostmi med 6,8 in 11,7 ‰. Porazdelitev sterolov kaže na izvor v planktonu, medtem ko sta 24-etilholestan-5,22E-dien-3β-ol (stigmasterol) in 24-metilholest-5-en-3β-ol (kampesterol) oba alohtonega izvora, in izvirata iz kopenskih rastlin.

Lipidi v sedimentih so večinoma avtohtonega izvora. Njihovo porazdelitev in izotopsko sestavo pa lahko primerjamo s partikulatno organsko snovjo v sedimentih in pasteh, ki smo jih zbirali 2 m nad sedimentom. Rezultati kažejo, da imajo nekateri biomarkerji različen izvor čeprav izhajajo iz istih molekul. Ti rezultati kažejo na nujno uporabo stabilnih izotopov kot orodje za identifikacijo izvora OM, ker bi identifikacija izvora na osnovi lipidne porazdelitve lahko vodila do napačnih zaključkov. Nizke $\delta^{13}C$ vrednosti za dolgoverižne alkane, alkohole, FA in C_{29} kažejo, da le ti izvirajo iz mikroalg in bakterij in ne iz višjih rastlin in druge kopenske organske snovi, kot je bilo ugotovljeno v prejšnjih študijah. $\delta^{13}C$ vrednosti smo opazovali v dolgoverižnih FA in sterolih in ugotovili, da so nižje v zahodni kotanji kot v zalivu Zaka, kar nakazuje na izrazit prispevek anaerobnega, bakterijskega izvora. Po drugi strani pa kratkoverižne FA v obeh okoljih in kratkoverižni alkani v zalivu Zaka kažejo na večji prispevek kopenske organske

snovi. Izotopske vrednosti ostalih lipidov so podobne v obeh okoljih in kažejo, da identifikacija njihovega izvora ni odvisna od oksidacijskih pogojev.

Domnevamo, da prisotnost bolj labilne, avtohtone organske snovi vpliva tudi na nastanek metanogeneze v sedimentih. Fermentacija acetata je povezana s prisotnostjo in razgradnjo bolj labilne organske snovi, medtem ko je nastanek metana z redukcijo povezan z vsebnostjo težje razgradljive, bolj obstojne organske snovi. Biogeokemijski procesi in struktura združbe arhej določene v sedimentih Blejskega jezera teh ugotovitev ni potrdila. Ugotovili smo, da je hidrogenotrofna metanogeneza prevladujoča pot v sedimentih alpskega jezera kljub nizki temperaturi in prisotnosti svežega, avtohtonega organskega materiala.

Nazadnje smo s kombinacijo molekularne in izotopske analize ugotovili izvor PAH v sedimentih Blejskega jezera. Koncentracije PAH v površinskih sedimentih na dveh lokacijah (v zahodni kotanji in Zaki), so bile primerljive in višje kot v prejšnjih študijah in so dosegale 4230 in 4380 ng g^{-1}. Ugotovili smo, da sta reten (Re) in perilen (Per) iz zaliva Zaka večinoma naravnega izvora, medtem ko na postaji D v zahodni kotanji vrednost $\delta^{13}C$ določena na globini 12–14 cm (iz leta 1950), kaže da je bil Re pirolitskega izvora. Porazdelitev $\delta^{13}C$ vrednosti posameznih PAHov, kažejo da je vnos le-teh v jezerskih sedimentih pirolitskega izvora in se tvorijo pri visokotemperaturnem izgorevanju fosilnih goriv. Vpliv PAH iz avtomobilskih izpuhov je viden na globini 12–14 cm na postaji D in v zalivu Zaka in ustreza obdobju 1953–1961.

Rezultati v doktorski nalogi nazorno kažejo, da je potrebno izvor organske snovi in onesnaževal v vodnem okolju preveriti tudi z uporabo stabilnih izotopov.

Abbreviations

18:1*n*-7	=	*cis*-11-octadecenoic acid
3-PGA	=	3-phosphoglycerate
A	=	anthracene
amu	=	atomic mass unit
BaA	=	benz(a)anthracene
BaP	=	benzo(a)pyrene
BeP	=	benzo(e)pyrene
BbF	=	benzo(b)fluoranthene
BkF	=	benzo(k)fluoranthene
BghiP	=	benzo(g,h,i)perylene
BSTFA	=	N,O-bis(trimethylsilyl)-trifluoroacetamide
BTEX	=	benzene, toluene, ethylbenzene and xylenes
C	=	carbon
C_3	=	C_3 plants, Calvin cycle
C_4	=	C_4 plants, Hatch-Slack cycle
CAM	=	crassulacean acid metabolism
CH_4	=	methane
Chry	=	chrysene
CO	=	carbon monoxide
CO_2	=	carbon dioxide
CO_3^{2-}	=	carbonate
COH	=	aliphatic alcohols
CSIA	=	compound specific isotopic analysis
δ	=	delta, relative isotopic composition, value given in parts per thousand or “per mil” (‰)
$\delta^{13}C$	=	isotopic composition of carbon
$\delta^{15}N_{PN}$	=	isotopic composition of particulate nitrogen
DBahA	=	dibenz(a,h)anthracene
EA-IRMS	=	elemental analyser- isotope ratio mass spectrometer
EPA	=	Environmental Protection Agency
FA	=	fatty acids
FAME	=	fatty acids methyl esters
Fe	=	iron
FID	=	flame ionization detector
Fl	=	fluorene
Fla	=	fluoranthene
GC	=	gas cromatography
GC-C-IRMS	=	gas cromatography combustion isotope ratio mass spectrometry
GC-FID	=	gas cromatograpy with flame ionization detector
GC-MS	=	gas cromatography coupled to mass spectrometry
GDGT	=	glycerol dialkyl glycerol tetraether
H_2	=	hydrogen
H_2O	=	water

HC	=	aliphatic hydrocarbon
HCO_3^-	=	hydrogen carbonate
IcdP	=	indeno(1,2,3–c,d)pyrene
IPCC	=	Intergovernmental Panel on Climate Change
IRMS	=	isotope ratio mass spectrometry
KIE	=	kinetic isotope effect
LULCC	=	land use and land cover change
MS	=	mass spectrometry
n-$C_{16:0}$	=	hexadecanoic acid
n-$C_{18:0}$	=	octadecanoic acid
N	=	nitrogen
NIST	=	National Institute of Standards and Technology
NW	=	north western
OC/TN	=	organic carbon to total nitrogen ratio
OM	=	organic matter
P	=	phosphorus
PAH	=	polycyclic aromatic hydrocarbon
PDB	=	Pee Dee Belemnite
Per	=	perylene
Phen	=	phenanthrene
POP	=	persistent organic pollutants
Py	=	pyrene
Re	=	retene
S	=	sulphur
ST	=	sterol
TLE	=	total lipid extract
TOC	=	total organic carbon
T-RFLP	=	terminal restriction fragment length polymorphism
TST	=	total sterol
VPDB	=	Vienna Pee Dee Belemnite

1 Introduction

1.1 Carbon cycle

During the last two decades, the global biogeochemical cycles of elements have been investigated in considerable detailed (Wollast et al., 1993). Much effort has been invested in the carbon cycle. The interest in this cycle and others, like those of sulphur (S), nitrogen (N), phosphorus (P) and trace elements, has been heightened by problems associated with global, regional and local environmental problems. These problems result in part from increased fluxes of C, N, P, and S compounds into the natural biogeochemical cycles of these elements because of anthropogenic activity. To evaluate various future global environmental changes due to natural or anthropogenic causes, it is necessary to know the past and present behaviour of the Earth-surface system. The data needed for this are generally incorporated into quantitative descriptions of the global biogeochemical cycle of the elements. Therefore, detailed knowladge of the biogeochemical cycling of C, H, N, S, P, Fe, etc. is crucial to understand the bioavailability of toxic elements or toxic compounds, and their natural geochemical variability in different environmental system.

The global carbon cycle is usually divided into the following major reservoirs of carbon interconnected by pathways of exchange:

- The atmosphere
- The terrestrial biosphere
- The oceans, including dissolved inorganic carbon and living and non-living marine biota
- The sediments, including fossil fuels, fresh water systems and non-living organic material, such as soil carbon
- The Earth's interior, carbon from the Earth's mantle and crust. These carbon stores interact with the other components through geological processes.

The construction of the biogeochemical cycle of carbon requires information on relevant reservoir masses, on processes that control the storage and the transfer between the various external reservoirs and on rates of transport (fluxes). These data are difficult to obtain and estimates vary widely. The organic carbon carbon cycle on Earth is divided into two parts (Figure 1, Tissot and Welte, 1984). The biological cycle starts with photosynthesis of organic matter (OM) from atmospheric carbon dioxide in the surface water of oceans or lakes. It continues through the different trophic levels of the biosphere and ends with the metabolic or chemical oxidation of decayed biomass to carbon dioxide. The half-life is usually days to tens of years depending on the age of the organisms. The geological organic carbon cycle has a carbon reservoir several orders of magnitude larger than that of the biological organic carbon cycle (6.4×10^{15} t C compared with 3×10^{12} t C in the biological cycle) and a turn-over time of millions of years. It begins with the incorporation of biogenic OM into sediments or soils. It then leads to the formation of natural gas, petroleum and coal or metamorphic forms of carbon (e.g. graphite), which may be reoxidized to carbon dioxide after erosion of sedimentary rocks or by combustion of fossil fuels (Rullkötter, 2006).

The tiny leak from the biological to the geological organic carbon cycle, particularly if

seen from the point of view of a petroleum geochemist in the context of the formation of petroleum source rocks (Littke et al., 1997), is represented by the deposition and burial of OM into sediments. If looked at in detail, the transition from the biosphere to the geosphere is less well defined. The transformation of biogenic OM to fossil material starts immediately after the decay of living organisms. It may involve processes during transport, e.g. sinking through a water column, and alteration at the sediment surface or in the upper sediments layers where epi- and endobenthic organisms thrive (Rullkötter, 2006).

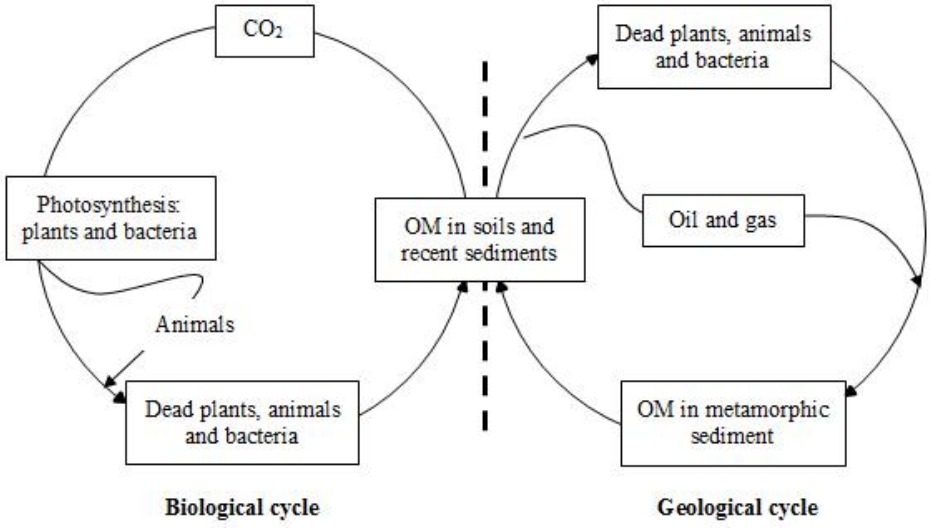

Figure 1: *The two major parts of the organic carbon cycle on Earth* (OM = organic matter) (Tissot and Welte, 1984).

1.1.1 Human influence

Human activity since the industrial era has changed the balance in the natural carbon cycle. Since the industrial revolution, human activity has modified the carbon cycle by changing its component's functions and directly adding carbon to the atmosphere (IPCC, 2007).

The largest and most direct human influence on the carbon cycle is through direct emissions from burning fossil fuels, which transfers carbon from the geosphere into the atmosphere. Humans also influence the carbon cycle indirectly by changing the terrestrial and oceanic biosphere. Over the past several centuries, human-caused land use and land cover change (LULCC) has led to the loss of biodiversity, which lowers ecosystems' resilience to environmental stresses and decreases their ability to remove carbon from the atmosphere. More directly, it often leads to the release of carbon from terrestrial ecosystems into the atmosphere. Deforestation for agricultural purposes removes forests, which hold large amounts of carbon, and replaces them, generally with agricultural or urban areas. Both of these replacement land cover types store comparatively small amounts of carbon, so that the net product of the process is that more carbon stays in the atmosphere (IPCC, 2007).

Other human-caused changes to the environment change ecosystems' productivity and thus their ability to remove carbon from the atmosphere. Air pollution, for example, damages plants and soils, while many agricultural and land use practices lead to higher erosion rates, washing carbon out of soils and decreasing plant productivity. Higher temperatures and CO_2 levels in the atmosphere increase decomposition rates in soil, thus

returning CO_2 stored in plant material more quickly to the atmosphere. However, increased levels of CO_2 in the atmosphere can also lead to higher gross primary production. It increases photosynthesis rates by allowing plants to more efficiently use water, because they no longer need to leave their stomata open for such long periods of time in order to absorb the same amount of carbon dioxide. This type of carbon dioxide fertilization affects mainly C_3 plants, because C_4 plants can already concentrate CO_2 effectively.

Humans also affect the oceanic carbon cycle. The carbonate system (pCO_2, pH, alkalinity, and calcium carbonate saturation state) of the world oceans is changing rapidly due to an influx of anthropogenic CO_2 (Skirrow & Whitfield, 1975; Broecker & Takahashi, 1977; Feely et al., 2004). Ocean acidification may be defined as the change in ocean chemistry driven by the oceanic uptake of chemical inputs to the atmosphere, including carbon, nitrogen, and sulfur compounds. Today, the overwhelming cause of ocean acidification is anthropogenic atmospheric CO_2, although in some coastal regions, nitrogen and sulfur are also important (Doney et al., 2007). Current trends in climate change lead to higher ocean temperatures, thus modifying ecosystems. Also, acid rain and polluted runoff from agriculture and industry change the ocean's chemical composition. Such changes can have dramatic effects on highly sensitive ecosystems such as coral reefs, thus limiting the ocean's ability to absorb carbon from the atmosphere on a regional scale and reducing oceanic biodiversity globally (IPCC, 2007). Also lakes undergo major changes in response to augmented carbon influxes resulting from anthropogenic changes in the catchment area. Carbon and nutrient loading promotes increased productivity and progressive eutrophication of lakes. In global biogeochemical cycles, lakes are now seen as one of the major regulators in the carbon cycle throughout various processes: sedimentation of detrital organic matter, production of autochthonous organic matter, precipitation of carbonates and precipitation of evaporates (Meybeck, 1995).

1.2 Lake

1.2.1 Lakes and water mixing studies

The following classification of lakes is based on their mixing patterns and temperature (Hutchinson & Löffler, 1956; Killops and Killops, 2005).

- **Amictic lakes:** Never mix, are usually ice covered year round and so are found in polar regions or high mountains where the temperature is mostly below freezing.
- **Meromictic lakes:** Mix incompletely, once or more annually (e.g. they can be dimictic). Even when temperature is uniform throughout, mixing occurs only in the upper layer, because the large density contrast between it and the deep layer resists mixing. Over time the deep water layer reaches very high density by accumulating dissolved material and the thickness of this layer also increases.
- **Holomictic lakes:** Mix completely at least once per year and there are four types:
  - Oligomictic: Relatively rare and mostly in the tropics. The poor degree of mixing is irregular or sporadic and usually of short duration. These lakes are generally warm throughout, but the surface waters are even warmer, so stratification can develop. The opportunity for mixing is only provided by infrequent cooling of surface waters.
  - Polymictic: Many mixing periods, with some lakes mixing nearly continuously all year. They are often small and shallow, and located in the tropics or at high altitude. Diurnal variations in surface temperature are often more important than

seasonal changes. Circulation in some lakes occurs mostly at night by the action of convection currents rather than wind.

- Monomictic: One regular period of mixing annually in cold (cold monomictic) or warm (warm monomictic) climates. Cold monomictic lakes are generally at high latitudes; they freeze over during the long winter but are ice-free in summer. Because the surface water does not warm much above 4 °C in summer, frequent mixing may occur during that relatively short season. These lakes would be defined as amictic in years of continuous ice cover. Warm monomictic lakes are typically subtropical and have the opposite cycle to cold monomictic lakes: they have a long summer and short winter, and stratification exists most of the year but for a short period in winter it breaks down and mixing occur.
- Dimictic lakes: Represent the average temperature lake. They have two mixing periods, spring and autumn, when the temperature (and hence density) of the water column becomes uniform throughout, enabling strong winds to effect an overturn.

In general, two types of lakes have to be distinguished: hydrologically open and hydrologically closed lake systems. Important water sources are in both cases precipitation and inflow from the catchment area, the area that is drained by a river and all of its influxes both, on and below the surface into the lake. These sources are balanced by evaporation and outflow. The important feature of closed lake systems is that they do not have a permanent outflow and thus store organic matter more efficiently than those having that outflow. The water body is usually separated into three distinct layers (Figure 2), which are characterized by different water temperatures and temperature gradients (Fischer, 2004).

In a lake, which is stratified of differences in density established by temperature and/or salt concentration changes, the upper, less dense region is called the epilimnion, which is strongly influenced by atmospheric temperatures. It is well mixed and thus oxygenated. The lowermost layer, called hypolimnion, is not affected by atmospheric influences and therefore cooler and less oxygenated to anoxic. Its water temperature is generally lower than that of the epilimnion. Between them, a third layer, called metalimnion, marks the border between the two major water layers. It is characterized by a high temperature gradient.

The layer of maximum density gradient with depth is the pycnocline, which is called the thermocline when it corresponds also to the maximum vertical temperature decrease with depth responsible for the parallel increase of water density. A thermocline is established in most lakes during summer, which constitutes an efficient barrier against vertical mixing. The overturn is the annual mixing process, which lakes normally undergo in autumn, when the epilimnion slowly cools down to a temperature equal to that hypolimnion.

Water has the thermal anomaly of being densest at 4 °C. Once the whole lake cools to this temperature water below 4 °C does not sink but stays at the surface and freezes. This effectively insulates the lake from further cooling and prevents deeper bodies of water from freezing to their bottom. Weak winter stratification with 0-4 °C epilimnion over a 4 °C hypolimnion may develop. This stratification breaks down (Figure 3) in the spring (spring turnover), followed by development of the summer stratification. Thermal stratification has a strong effect on the seasonal availability of and demand for mineral nutrients.

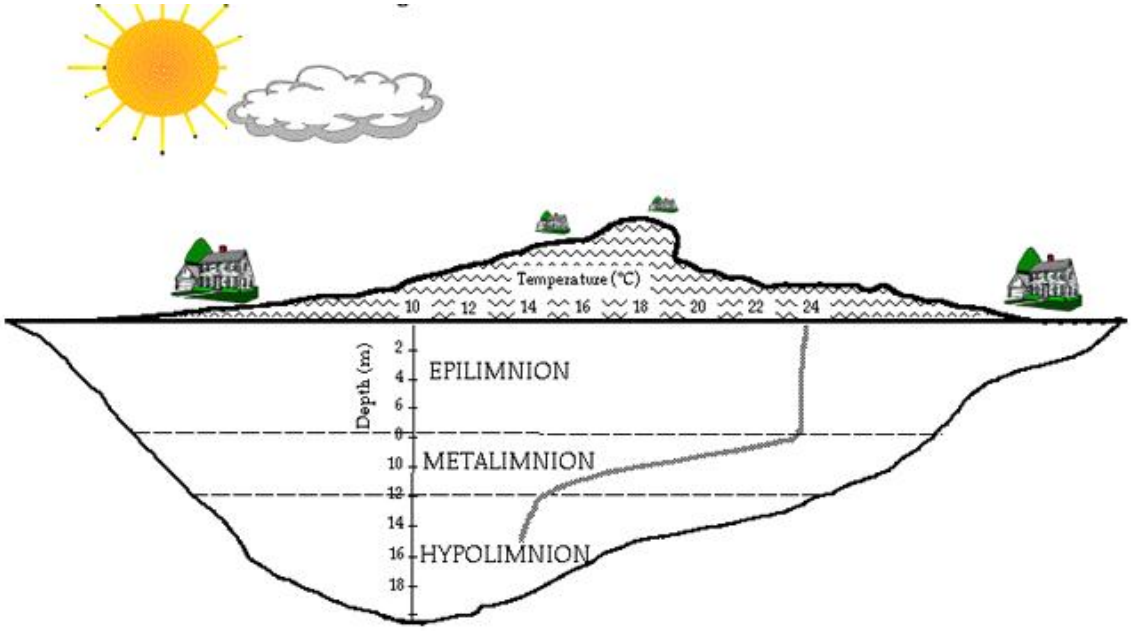

Figure 2: *Generalized temperature stratification* (From: http://capecodcommission.org/water/stratification.gif)

Depending on the degree of the seasonal temperature change, this circulation may affect the whole water body (holomixis), which leads to oxygenated bottom waters. A second circulation may occur during the spring warming; these lakes are called dimictic. Lake Bled is typical dimictic lake. Epilimnion depth is changing throughout the year. The epilimniom layer depends on the season: between 0.5–4 m in spring; 0.5– 6 m in summer; 0.5–10 m in autumn. Metalimnion starts at 5 m depth in spring and later moves down to 10 m.

The isotopic composition of water – like chemical composition and the temperature – is often not uniform throughout lakes, and therefore it is conceivable to use the isotopic differences naturally established and their variations with time to investigate the mixing processes and rates.

Shallow lakes and swamps are in general vertically well mixed, but poorly mixed horizontally. In these lakes, the isotopic composition of the inflowing water (streams, groundwater) is gradually modified by evaporation and by exchange with the atmospheric moisture as the distance from the inflow points increases. The extent of the isotopic variation depends on many parameters, including temperature and atmospheric relative humidity (which governs the evaporation rate), the depth and the mean residence time of water in the lake, the degree of horizontal mixing, etc. In extreme cases, horizontal mixing can be treated as a stream, which evaporates during it flows.

In deep lakes, the horizontal mixing is generally rather fast, and it is mainly due to turbulent mass transport under the wind stirring action. Therefore, deep lakes are often horizontally well mixed, with the exception of restricted areas around the inflow points. The mixing rate, however, decreases with increasing depth; in stratified lakes, the horizontal mixing rate is much greater in the epilimnion, which is the lake region exposed to the wind action, than in the hypolimnion.

Vertical mixing in deep lakes is comparatively rather slow, with the obvious exception of the overturn period. In addition, the thermocline established in summer or permanently occurring as in meromictic lakes, is an efficient barrier hampering mass transport between epilimnion and hypolimnion.

An ecologically useful classification of trophic status as oligotrophic or eutrophic is based on nutrient concentrations and productivity mainly (phosphorous) (Allen, 1971). Oligotrophic lakes have low concentrations of nutrients. Typically, they are deep, have a larger hypolimnion than epilimnion, and have a relatively low primary productivity. In contrast, eutrophic lakes have high nutrient concentrations, are usually shallower and warmer than oligotrophic lakes, and have higher rates of primary production. Oxygen concentrations undergo strong diurnal fluctuations in eutrophic lakes because of the extensive aerobic decomposition of organic nutrients during night when there is no sunlight. Oligotrophic lakes do not experience such low-oxygen periods.

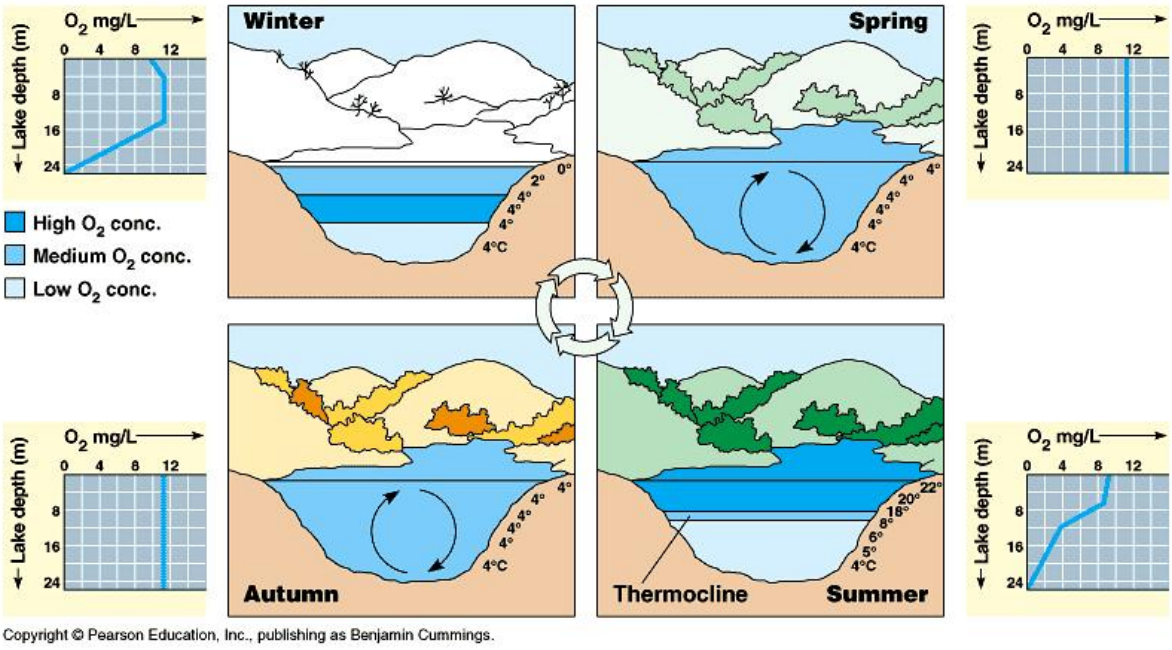

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Figure 3: *Lake stratification and water mixing* (From: http://kentsimmons.uwinnipeg.ca/16cm05/1116/50-15-LakeStratification-L4.gif)

The pH is another important factor influencing which microorganisms inhabit a particular lake. Some lakes are alkaline, some neutral, and still others acidic. Other conditionals being equal, a higher pH favors primary production through higher availability of CO_2 in the forms of HCO_3^- and CO_3^{2-}. Salt concentrations also influence the characteristic autochthonous microorganisms of some lakes. Some landlocked lakes have high salt concentrations (saline lakes).

1.2.2 Composition and activity of microorganisms in lakes

Microorganisms play a key role in both lake productivity and transformation of organic compounds within a lake. The principal ecological functions of microorganisms in freshwater environments include decomposition of dead organic matter, liberating mineral nutrients for primary production; assimilation and reintroduction into the food web of dissolved organic matter; mineral cycling activities; primary productivity; and supplying biomass as a food source for grazers (Killops and Killops, 2005).

Plankton are organisms living primarily in the upper part of the water column and although often capable of some motion, particularly vertical migration in zooplankton, are unable to maintain their overall lateral position and drift with the oceanic currents. These organisms include drifting animals, protists, archaea, algae, or bacteria that inhabit the pelagic zone of oceans, seas, or bodies of fresh water. Classification of the plankton is often made on the basis of size. The ultrananoplankton (or **picoplankton**) is composed

almost entirely of bacteria, the nanoplankton of algae (**phytoplankton**) and macro- and megaplankton of animals (**zooplankton**), mainly invertebrates (Killops and Killops, 2005).

Algae floating or suspended freely in the water are called phytoplankton, those attached to the bottom or sides are called benthic algae. Because these phototrophic organisms use energy from light in the initial production of organic matter, they are called primary producers. In the final analysis, the microbiological activity of an aquatic ecosystem depends on the rate of primary producers by the phototrophic organisms (Madigan & Martinko, 2006). All other organisms gain their energy supplies and organic substrates by feeding, directly or indirectly, upon autotrophes and are termed heterotrophes. In **oxic** areas cyanobacteria and algae prevail and in **anoxic** areas anoxygenic phototrophic bacteria dominate. Number of prokaryotes decrease with depth. In freshwater lakes, once the oxygen is consumed, the deep layers become anoxic. Beside oxygen other factors especially temperature strongly govern microbial activity as well. These bottom layers contain anaerobic prokaryotes and a few kinds of anaerobic eukaryotes, in particular, certain protozoa. In addition there is a conversion from respiratory to fermentative and methanogenic metabolisms, with important consequences for the carbon cycle and other nutrient cycles.

Our knowledge of the microbial world, although expanding dramatically at present, is still very limited. Phylogenetic tree is divided in three kingdoms (or domains): the *Archaea* (or archabacteria), the *Bacteria* (or eubacteria) and the *Eukarya* (or eukaryotes). This classification demonstrates the importance of bacteria, which by this taxonomic system, are divided into the archabacteria (methanogens, halophiles and thermoacidophiles; Woese & Wolfe, 1985) and true bacteria, the eubacteria. The eukaryotes occupy a much less significant position than in older classifications.

The *Bacteria*, one of the two prokaryotic domains, is divided into 30 currently recognized phyla. For example, the cyanobacteria, the proteobacteria (also known as the purple bacteria) and the green sulfur bacteria area all recognized as phyla. Some of these phyla, however, contain relatively few species, and taxonomic designations within the bacterial domain are at present rather imprecise.

The bacterial domain is genotypically (based on genetic information) quite distinct from the *Archaea*, the other prokaryotic domain, but there are many phenotypic characteristics (observable properties) than also distinguish the *Bacteria* from the *Archaea*. First, great metabolic diversity is housed in the *Bacteria*, including many physiologically unique groups of organisms. For example, among the prokaryotes, oxygenic photosynthesis (the cyanobacteria) is unique to the *Bacteria*. The *Bacteria* is also the principal domain for anoxygenic photosynthetic organisms. Other metabolism such as methanotrophy (methane oxidation), methylotrophy (oxidation of one-carbon compounds, not including methane) and nitrification are also, as far as we currently know, restricted to the *Bacteria*. All known forming prokaryotes are found within the bacterial domain (gram-positive bacteria). Organisms from the bacterial domain also contain unique cell wall features.

Other distinguishing features of the bacterial domain include the deep branching of high temperature-adapted organisms. These include the chemolithiautotrophic *Aquifex-Hydrogenobacter* group, sulfate reducers of the genera *Thermodesulfobacterium* and *Thermosulfatator* (Moussard et al., 2003) and the dominantly fermentative organisms of the *Thermotogales* group. All of these organisms have temperature optima in the 80–90°C range (Stetter, 1996). Also deep branching are anoxygenic photosynthetic organisms of the family *Chloroflexiaceae*. It would appear that great metabolic diversity in hot environments accompanied early bacterial evolution (Stetter, 1996).

The *Archaea* is second of the two prokaryotic domains and constitute a domain of

single-celled microorganisms. These microbes have no cell nucleus or any other membrane-bound organelles within their cells. All archaea is chemotrophic and formally subdivided into the two major phyla *Euryarchaeota* (encompassing the methanogens and their phenotypically diverse relatives) and *Crenarchaeota* (comprising the relatively tight clustering of extremely thermophilic archaebacteria, whose general phenotype appears to resemble most the ancestral phenotype of the *Archaea*. *Archaea* have been isolated from environments considered to be the most extreme habitats for life on Earth. These environments include those with high temperature (hyperthermophiles), high salinity (halophiles), and extremes of pH. Recently, a much greater environmental range for *Archaea* has been established by identification of *Archaea* as an important constituent of marine picoplankton (Delong, 1992; Karner et al., 2001) and the discovery of symbiotic associations between *Archaea* and some marine animals (Preston et al., 1996). Subsequent studies explored the presence of *Archaea* in lake systems. Most studies of *Archaea* in lake ecosystem have focused on either the water column or sediment but did not incorporate both into one comprehensive study. Schepler et al. (1997) compared the sediments from both eutrophic and oligotrophic lakes. Their results indicated that archaeal lineages from these lakes were not significantly different from each other but were part of the temperate *Crenarchaeota* identified in marine and soil systems. However, study performed in Lake Superior indicated that the composition of archaeal sequences usually shifted from *Crenarchaeota* found in water column to *Euryarchaeota* (mostly methanogens) in the surface sediment (Kish, 2010).

The organic rich remains of bacteria and archea may make significant contributions to most, if not all, sedimentary organic matter. There are four major sources of sedimentary organic matter, in general order of importance: phytoplankton, higher plants, bacteria and zooplankton. Fungi, perhaps surprisingly, do not appear to make significant contributions to sedimentary organic matter.

1.2.3 Organic matter

Research on biogeochemistry of organic matter (OM) in stratified lacustrine basins is important in predicting the sources, deposition, transformation and preservation of OM, which reflect and influence the trophic status of the lake (eutrophication). Stratified water columns of lakes support multiple layers of biological activity fueled by oxygenic photosynthesis, anoxygenic photosynthesis, chemolithotrophy and heterotrophy. These diverse biological zones in turn generate geochemical stratification in which reduced (sulfide, ammonium, reduced metals, organic matter) and oxidized (oxygen, nitrate, oxidized forms of redox sensitive metals) chemical species coexist in opposing concentration gradients. The chemical composition of OM produced in these biogeochemical zones reflects the planktonic and microbial sources and recycling processes.

Within this complex environment, mixtures of lipid biomarkers are formed. Biomarkers, or molecular markers, are compounds with structures that can be related to specific biological sources due to their own biosynthesis. Many of these biomarkers provide a unique insight into the diagenetic alteration, deposition, preservation and sources of organic matter and, in particular, the microbial community structure present in lacustrine environments. Biosynthetic pathways utilized by living organisms are diverse and, as a result, distributions of biomarkers vary between different types of organisms. For example, the domain archaea is characterized by membrane lipids based on isoprenoid ether (glycerol dialkyl glycerol tetraether; GDGT) structures, whereas acyl-based (fatty acid, acylglycerol) structures are found in the domains bacteria and eukarya. Hopanoids are considered as biomarkers for bacteria (including cyanobacteria), while all

eukaryotes make sterols (Killops and Killops, 2005). In general, chain lengths of hydrocarbons, fatty acids and alcohols characterize the source of lipid matter. For example, the presence of C_{27}, C_{29} and C_{31} *n*-alkanes indicates that terrigenous plants have been important source of lipids (Eglinton and Hamilton, 1967; Cranwell, 1973; Rieley et al., 1991). On the other hand algal contribution is indicated by the presence of *n*-C_{17} and *n*-alkanes between C_{15}-C_{21} (Clark and Blumer, 1967; Blumer et al., 1971) and also by the short-chain length range between C_{14}-C_{18} of *n*-alkanoic acids with a typical even over odd predominance (Cranwell et al., 1987). Increases in OM contributions from aquatic algae and photosynthetic bacteria that accompany eutrophication can be identified by proportional increases of the C_{17} *n*-alkane, the C_{16} and C_{18} *n*-alkanoic acids, the C_{16} to C_{22} *n*-alkanols, and the C_{27} and C_{28} sterols (Cranwell et al., 1987; Neunlist et al., 2002). Long-chain *n*-alkanoic acids, such as C_{24}, C_{26} and C_{28}, are the major components of flowers, waxy coatings of terrestrial plant leaves and pollen (Rieley et al., 1991). Despite this general findings, some deviations from individual species are know, for example higher plants contain more abundant C_{16} and C_{18} *n*-alkanoic acids than long-chain *n*-acids except in the wax coating. Furthermore the higher susceptibility of lipids derived from algal material to degradation in comparison with land-derived OM, which has been microbially reworked before transported into the lake is of general character (Meyers and Ishiwatari, 1993; Meyers, 1997). Some caution is necessary in using the *n*-alkanolic acid biomarkers because the *n*-C_{16} and *n*-C_{18} acids are ubiquitous components of biota and because they are more susceptible to degradation and alteration than other types of lipid biomarkers (Meyers & Eadie, 1993; Meyers, 2006). Studies of settling particles indicated that fatty acids decomposition rates are 10 times higher for *n*-C_{16} comparing to *n*-C_{30} (Meyers and Eadie, 1993)

Sediment biogeochemistry plays an active role regulating carbon cycling in aquatic environments – the final sink for all descending particulate matter that escapes degradation within the water column (Jones and Bowser, 1978; Meyers and Ishiwatari, 1993; Bechtel and Schubert, 2009). Organic matter constitutes a minor but important fraction of lacustrine sediments. It is comprised of a complex mixture of lipids, carbohydrates, proteins and other biochemicals contained in the tissues of living organisms. The major source of primary OM to the sediments is from the detritus of single-celled phytoplankton that have lived in their photic zones. Detritus from land plants can be an important additional contribution to the OM in sediments of many lakes. The relative contributions from these two general sources of OM to sediments are influenced strongly by algal productivity, land-plant productivity and transport processes (Meyers, 1997).

Lake basins, being smaller by far than deep ocean basins, receive proportionally more land-derived clastic sediment particles. The result of this is that sedimentation rates in lakes exceed significantly those of the oceans, and organic matter is buried more rapidly. Land-derived nutrients accompany the sediments washed into lakes, enhancing lake fertility and aquatic production of OM. Lacustrine benthos is not as diverse as those of marine areas, and their depth of bioturbation is less than that of marine fauna. This lesser amount of bioturbation diminishes the exposure of OM to oxidation in lake sediments. Working against the generally greater preservation of OM in lake sediments is the large amount of turbulent mixing of bottom sediments found in the waters of shallower lakes. Turbulent resuspension can be very significant, even in lakes as deep as 100 m (Eadie et al., 1984) and it re-exposes OM to oxidative processes.

Sedimentation rates in deep-sea basins are on the order of a few centimeters per 1000 years. In contrast, sedimentation rates in lakes are about a meter per 1000 years. Lacustrine sediments are thus better suited to high-temporal-resolution studies of short-

term paleoenvironmental changes than for longer-term processes. Furthermore, sedimentary records of lakes are usually more responsive to local and regional environmental conditions than to global factors.

1.2.3.1Degradation of organic matter

Much of the OM that reaches the sediment is remineralized, a process, which may alter the organic matrix in which the pollutants reside. A number of organisms including bacteria, fungi and micro- and macrofauna are responsible for the aerobic degradation of organic carbon (Fenchel et al., 1998). The actual rates of decay depend primarily on OM quality (i.e. the content of protein, cellulose, lignin etc.), age (decomposition stage) and temperature (season) (Fenchel et al., 1998). Almost all of these have the enzymatic capacity to perform a total mineralization of organic substrates. OM is, therefore, completely metabolized by a single organism to H_2O, CO_2 and inorganic nutrients using oxygen as electron acceptor according to the following stoichiometry:

$$(CH_2O)x(NH_3)y\,(H_3PO_4)z + xO_2 \rightarrow xCO_2 + yNH_3 + zH_3PO_4 + xH_2O \qquad (1)$$

A unique feature of aerobic decomposition is the formation and consumption of reactive oxygen-containing radicals such as superoxide anion ($^{*}O_2^{-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{*}OH$). These are capable of breaking bonds and depolymerize relatively refractory organic compounds like lignin (Canfield, 1994). As the oxic (oxygen containing) zone in sediments usually is limited to a thin uppermost layer, a large fraction of the OM is buried in a more or less decomposed form into anoxic layers. Here, anaerobic decomposition is accomplished by mutualistic consortia of bacteria because no single type of anaerobic bacterium seems capable of complete mineralization (Fenchel et al., 1998). Anaerobic decomposition occurs stepwise, involving several different functional types of bacteria (Figure 4). First, the large and normally complex polymeric organic molecules stepwise are split into water-soluble monomers (amino acids, monosaccharides and fatty acids) by hydrolysis and fermentation under the production of energy and release of inorganic nutrients (Kristensen & Hansen, 1995), e.g. mixed propionate and acetate formation:

$$8(CH_2O)x(NH_3)y(H_3PO_4)z \rightarrow$$
$$xCH_3CH_2COOH + xCH_3COOH + 3xCO_2 + 3xH_2 + yNH_3 + zH_3PO_4 \qquad (2)$$

The small organic acids are then oxidized completely to H_2O and CO_2 by a number of respiring microorganisms using a variety of inorganic compounds as electron acceptors. The individual anaerobic respiration processes generally occur in a sequence with depth in the sediment according to the availability of electron acceptors: $Mn^{4+} \leftrightarrow NO_3^{-}$, Fe^{3+}, SO_4^{2-} and CO_2 respiration (Figure 5). The actual sequence is determined by the ability of each electron acceptor to receive electrons, and thus the energy output per degraded organic carbon atom (Fenchel et al., 1998), e.g. nitrate respiration (denitrification, Equation 3) is favored energetically compared to sulfate reduction. The suboxic zone contains the most potent anaerobic electron acceptors, NO_3^{-}, Mn^{4+} and Fe^{3+}. Examples of anaerobic degradation stoichiometries, denitrification, manganese oxide reduction (Equation 4), iron oxide reduction (Equation 5) and sulfate reduction (Equation 6), will be presented here:

Denitrification:

$$CH_3COOH + 1.6NO_3^{-} + 1.6H^{+} \rightarrow 2CO_2 + 0.7N_2 + 2.8H_2O \qquad (3)$$

Manganese oxide reduction:

$$CH_3COOH + 2MnO_2 + 4H^+ \rightarrow 2Mn^{2+} + 2CO_2 + 4H_2O \quad (4)$$

Iron oxide reduction:

$$CH_3COOH + Fe_2O_3 + 4H^+ \rightarrow 2Fe^{2+} + 2CO_2 + 4H_2O \quad (5)$$

Sulfate reduction:

$$CH_3COOH + SO_4^{2-} \rightarrow 2CO_2 + S^{2-} + 2H_2O \quad (6)$$

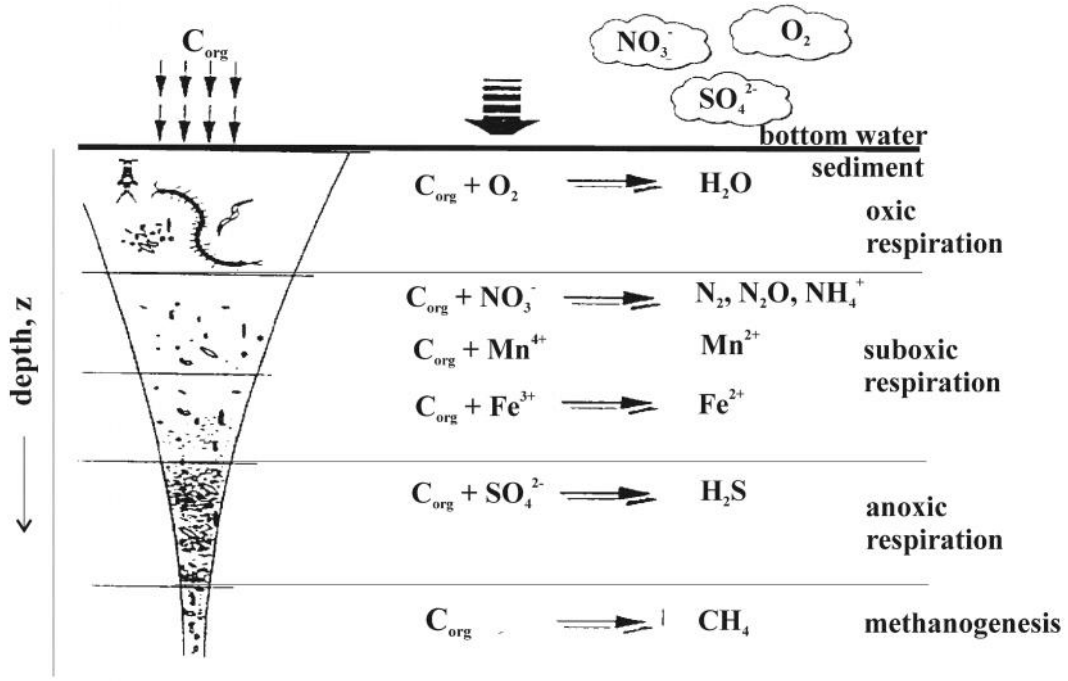

Figure 4: *Schematic diagram of vertical distribution areas in breaking down organic matter in the sediment.* (Froelich, 1979; Ogrinc, 1997).

In the absence of such secondary electron acceptors, further energy may be extracted from some of the fermentation products by methanogenesis (Zeikus et al., 1980), which reduce CO_2 using H_2 produced. The single carbon unit of methane is an unusual substrate, available only to a specialized group of microorganisms, the methylotrophs (Haber et al., 1983). Many of these are obligate methylotrophs, meaning that they are restricted to the utilization of methane, methanol, formate, carbon monoxide, and a few additional reduced single-carbon compounds.

Molecular carbon isotope analysis has recently become particularly important in the investigation of the methane cycle. Biogenic methane produced by archaea is isotopically particularly light ($\delta^{13}C$ = –60 to –100 ‰). Methanotrophic organisms using this biogenic methane as their carbon source biosynthesize, among others, membrane lipids which may isotopically be as light as –100 ‰) (Rullköter, 2006). The combination of molecular and isotope analysis has, thus, helped a great deal in unraveling the complex processes particularly involved in anaerobic methane oxidation (e.g. Orphan et al., 2001, 2002; Elvert et al., 2003; Wakeham et al., 2003). Methanotrophs involved in the anaerobic oxidation of methane are presently thought to be a consortium composed of sulphatoreducers and methanogens, the latter operating in a reverse direction of acetotrophic methanogenesis, producing ^{13}C depleted lipids from ^{13}C–depleted methane (Canfield et al., 2005).

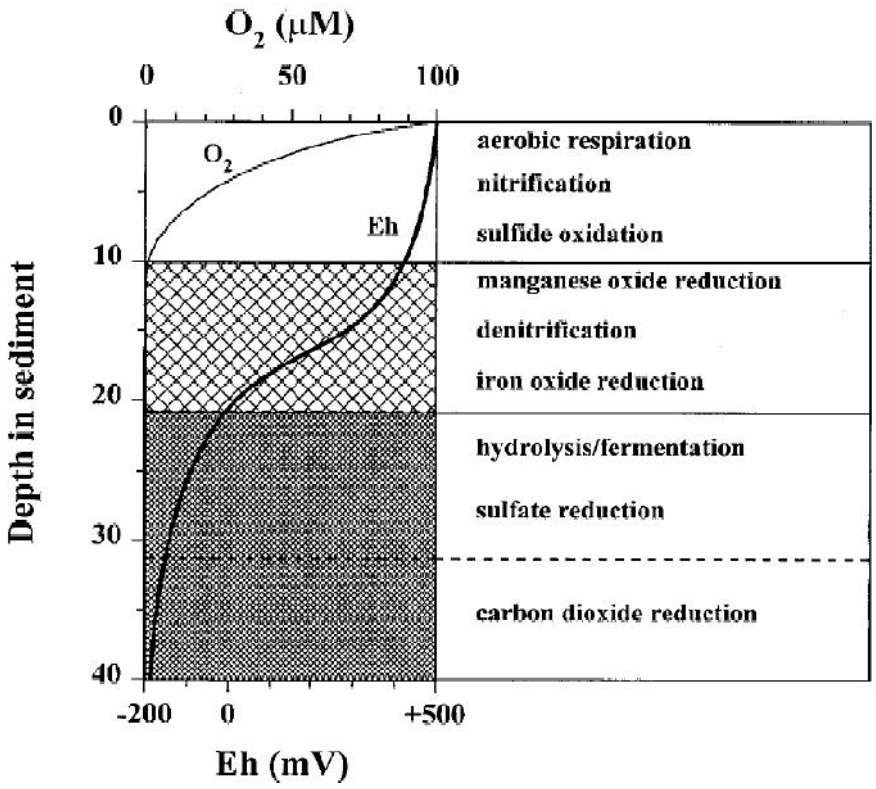

Figure 5: *The vertical distribution of diagenetic processes in sediments.* The oxic zone is illustrated by an oxygen profile (white zone), the suboxic zone is shown as the layer, where the redox discontinuity is evident (light cross-hatched), the reduced zone is shown as the layer where Eh is below zero (dark cross-hatched) (Kristensen, 2000).

1.2.3.1.1 Methane

Methane (CH_4), a highly reduced form of carbon, plays an important role in many geochemical processes in the Earth's crust. It is the third most abundant greenhouse gas and it is approximately 25 times more effective than CO_2 (Denman et al., 2007). Sum of CH_4 is comprised of approximately equal contributions from (Crutzen, 1991):

- natural wetlands;
- rice paddies;
- ruminants and termites;
- coal and gas mining;
- oceans, freshwaters and biomass burning.

Microbial CH_4 emissions from deep subsurface igneous and sedimentary rocks were not included in the estimates. CH_4 is a common constituent in the deep subsurface. Stratified lakes constitute a smaller source of atmospheric CO_2 than estuaries are important as a source of CH_4, with activity localized in the anoxic hypolimnion and sediment. Recently, some existing estimates of global CH_4 emissions have not taken small lakes into account, resulting in underestimates of total lake area by a factor of more than 2 (Walter et al., 2007), and according to Bastviken et al. (2011), CH_4 emissions from lakes and freshwater environments can substantially affect the global greenhouse gas balance. CH_4 is produced by strictly anaerobic Euryarchaeota (Liu and Whitman, 2008), and biogenic CH_4 production is the terminal step in the decomposition of OM in lacustrine systems. Methanogenesis involves primarily two pathways —H_2 oxidation coupled with CO_2 reduction (hydrogenotrophic methanogenesis) and acetate fermentation (acetoclastic methanogenesis). About two-thirds of the naturally produced CH_4 in anaerobic sediments appears to originate from acetoclastic methanogenesis (Oremland,

1988), which is believed to dominate in lacustrine sediments, while hydrogenotrophic methanogenesis is considered to be typical of marine environments (Canfield et al., 2005). However, exceptions are not uncommon, and both processes may occur in both environments (Conrad, 2005). It was recently suggested that the quality of organic substrates and temperature may control the prevalence of the two methanogenic pathways (Glissman et al., 2004; Canfield et al., 2005; Nozhevnikova et al., 2007), but only a few studies have addressed environmental drivers that influence these pathways in anoxic lacustrine sediments. Acetate is considered to be the most important carbon intermediate in anaerobic systems and is produced by fermentation of OM and acetogenesis (Wu et al., 1997). In cold, deep stratified lakes, acetogens have been found to outcompete methanogens for H_2, and H_2 : CO_2 was found to be converted to CH_4 by a two-step process, with initial formation of acetate by reduction of CO_2, followed by acetoclastic methanogenesis (Wand et al., 2006). Acetate fermentation also appears to be associated with the more labile autochthonous OM, whereas CO_2 reduction to CH_4 utilizes the more refractory allochthonous OM (Whiticar et al., 1986; Sugimoto and Wada, 1993).

Isotopic analysis of methane and analyses of methanogenic community in tropical freshwater sediments suggested that CH_4 originates mostly from the hydrogenotrophic pathway and that acetate may also be consumed by acetotrophic fermenting bacteria (Conrad et al., 2010). Only hydrogenotrophic methanogens (Methanomicrobiaceae and Methanobacteriaceae) were found in sediments of Lake Kinneret (Israel), and the syntrophic association with acetate-oxidizers performing hydrogenotrophic methanogenesis was also proposed for these sediments (Nüsslein et al., 2001). Previous studies of CH_4 in anoxic sediments of stratified eutrophic lakes in the Julian Alps (W Slovenia), Lake Planina (Ogrinc et al., 2008), and Lake Bled (Ogrinc et al., 2002) suggested the prevalence of hydrogenotrophic methanogenesis and indicated low pore-water acetate concentrations (Lojen et al., 1999).

1.2.4 Organic pollutants in sediments

It is genarally recognized that hydrophobic organic pollutants are associated with particulate OM in aquatic systems. The exact nature of this association is still being studied, but it is likely that there are many types of associations possible and that the ultimate fate of pollutants will depend on the type of association. Thus sediments represent also the final sink of organic pollutans such as polycyclic aromatic hydrocarbons (PAH). PAH are ubiquitous contaminants in the environment found in measurable concentrations even in remote locations such as Arctic ice (Kawamura and Suzuki, 1994) and snow (Masclet et al., 2000), high altitude lake sediments (Fernandez et al., 1999) and deep-sea sediments (Ohkouchi et al., 1999).

PAH constitute a large group of Persistent Organic Pollutants (POP) containing from two to six fused benzene rings (Figure 6). Certain PAH are among the most carcinogenic substances known and can be acutely toxic or genotoxic, depending on the number and configuration of the benzene rings and the presence and position of their substituents (Hervey, 1997). PAH exhibit different molecular distribution according to their origin formed during incomplete OM combustion (pyrolytic origin) or natural and anthropogenic fossil fuel combustibles (petrogenic origin) (Lima, 2004).

The United States Environmental Protection Agency (EPA) has designated 32 PAH compounds as priority pollutants. The 16 EPA priority PAHs is often targeted for measurement in environmental samples and present in Figure 6. They are naphthalene, acenaphthene, acenaphthylene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benzo(b)fluoranthene (BbF), benzo(k)flouranthene (BkF), benzo(a)pyrene (BaP), dibenz(ah)anthracene (DBahA), benzo(ghi)perylene (BghiP), indeno(1,2,3-

cd)pyrene (IcdP) and benzo(a)anthracene (BaA).

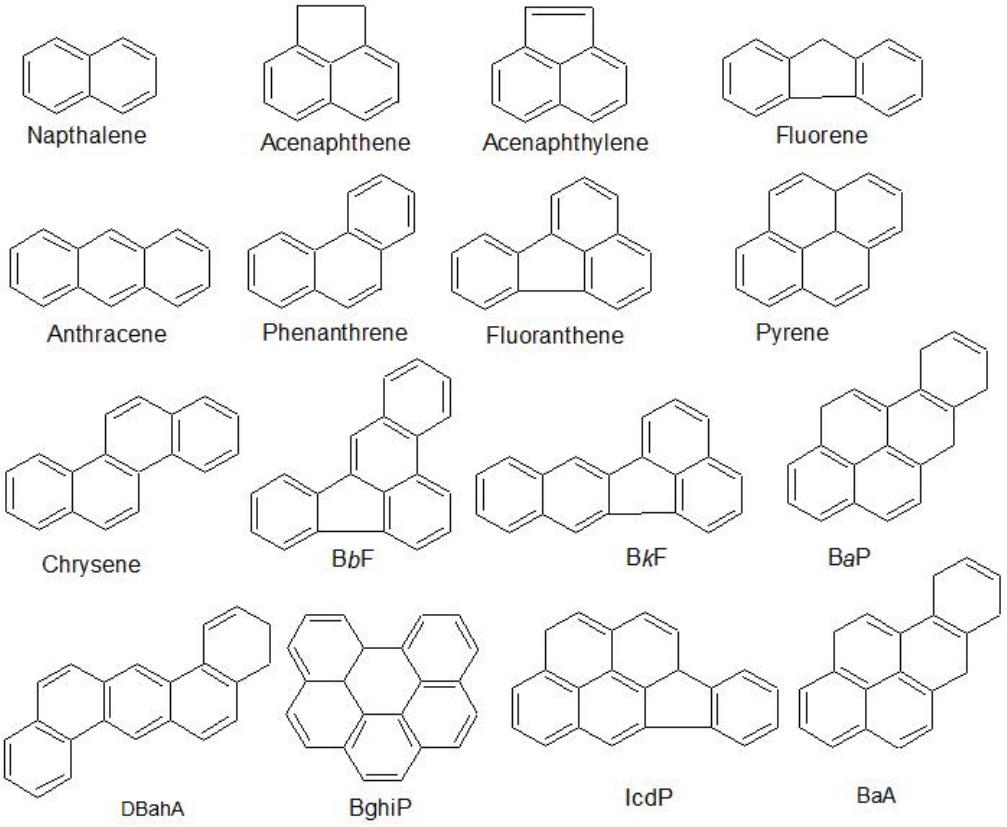

Figure 6: *The chemical structure of the 16 EPA priority PAHs*. benzo(b)fluoranthene (BbF), benzo(k)fluoranthene (BkF), benzo(a)pyrene (BaP), dibenz(a,h)anthracene (DBahA), benzo(g,h,i)perylene (BghiP). indeno(1,2,3-c,d)pyrene (IcdP), benz(a)anthracene (BaA).

PAH reach the environment by several different pathways. These compounds are present in unburned petroleum (petrogenic PAH) and can be released directly to the environment both by human activities (oil spill) and natural processes (oil seepage). Even though oil spills attract a lot of attention from the media and public in general, due to the visible and acute effects that they produce, they usually do not significantly contribute to the PAH concentration inventory. Diagenetic processes are also suspected to generate certain PAH (e.g. perylene) from biogenic precursors (Laflamme and Hites, 1978; Tan and Heit, 1981), although conclusive evidence for this mechanism is still lacking. In general, biosynthesis is considered a localized source, with little impact on global concentrations. The most prominent and ubiquitous source of PAH to the environment is the incomplete combustion of modem (wood) and fossil (petroleum and coal) biomass (Lima, 2004).

1.2.4.1Formation of polycyclic aromatic hydrocarbons

Much effort has been placed in understanding the experimental conditions that favor efficient combustion, so as to minimize the formation of products of environmental and health concern (Frenklach et al., 1984; Macadam, 1997; Palotas et al., 1998; Ritchter and Howard, 2000). During combustion, the organic compounds present in the fuel are fragmented into smaller unstable molecules (free radicals) that can react, through a number of different chemical pathways, to produce the first aromatic ring (Ritchter and Howard, 2000). Further reaction of this aromatic ring with small molecules (2-3 carbons; e.g. C_2H_2 - acetylene) leads to growth of the aromatic system and formation of larger and more stable multi-ring structures. It is well established that mechanisms of formation of

PAHs and of soot are closely intertwined (Macadam, 1997; Vander Wal et al., 1997; Ritchter and Howard, 2000) with high molecular weight PAHs (~ 500-1000 amu) functioning as molecular precursors of soot particles (Ritchter and Howard, 2000). In general, an inverse correlation is seen between the amount of PAHs and soot in flames, where a decrease in PAHs concentration is linked to the start of soot formation (Prado and Lahaye, 1982). A limit to the amount of PAHs produced and emitted during combustion is imposed by either the incorporation of high molecular weight PAHs into the solid phase (soot) and/or their destruction by direct burnout (Prado and Lahaye, 1982; Macadam, 1997). The later process corresponds to the pyrolytic oxidation of PAHs to CO and CO_2. Under fuel-rich conditions OH^* radicals are usually the main oxidant responsible for this convertion, while under fuel-lean conditions O_2 dominates (Ritchter and Howard, 2000; Lima, 2004).

1.2.4.2Biodegradation

It is well documented that low molecular weight PAHs (such as naphthalene) are more likely to undergo microbial degradation than higher molecular weight compounds (Cerniglia and Heitkamp, 1989; Budzinski et al., 1998). Typically, susceptibility to biodegradation decreases as the number of fused rings in the PAH increases. Microbial degradation experiments have also demonstrated that alkyl substituted PAH degrades more slowly than parent compounds. For example, Heitkamp and collaborators (Heitkamp and Cerniglia, 1987) reported faster degradation rates for Phen than for 2-methylnaphthalene in sediments from a pristine and an oil-exposed ecosystem. Similarly, we observed in our analysis that was not possible to determine napthalene, 1-methylnapthalene and 2-methylnapthalene, because they are too volatile and fast degredable.

Experiments using crude oils have yielded similar results (Garrett et al., 1998). Because sediments are usually the final destination of PAHs in the environment, extensive research has been conducted on the aerobic degradation of sedimentary PAHs (Bauer and Capone, 1988; Cerniglia and Heitkamp, 1989; Yuan et al., 2001) and potential pathways for bacterial oxidation of several compounds have been reported (Cerniglia and Heitkamp, 1989). Interestingly, prior exposure to PAHs seems to enhance the capacity of a microbial population to degrade these compounds (Bauer and Capone, 1988). Apparently, microbial communities can adapt to metabolize a compound after prolonged exposure to it (Cerniglia and Heitkamp, 1989). The faster degradation rates reported for certain PAHs in previously exposed sediments therefore result from the selection and proliferation of microbial communities capable of degrading these compounds (Bauer and Capone, 1988). Until the late 1980s it was assumed that PAHs deposited in anoxic sediments were not affected by biodegradation (Cerniglia and Heitkamp, 1989; Rothermich et al., 2002). However, microbial mediated transformations of PAHs in anaerobic environments are now known to occur under denitrifying and sulfate reducing conditions. Marine surface sediments incubated under denitrifying conditions have resulted in degradation of PAH from 3- to 5-rings. As in aerobic degradation, the more soluble, lower molecular weight PAHs (acenaphthene and Phen) degraded faster than less soluble, higher molecular weight compounds (BaA and BaP) (MacRae and Hall, 1998). Moreover, when the biodegradation rate of compounds of the same size is compared, it becomes clear that the microbial community preferentially degrades the most soluble isomer (e.g, Phen was shown to degrade faster than the less soluble A). The main reason for the preferential biodegradation of more soluble compounds is presumed to be the preference of microorganisms to assimilate substrates from the water phase (MacRae and Hall, 1998). That implies that particle-bound pyrogenic PAHs, which are less available to dissolution than PAHs derived from petroleum spills (Farrington et al., 1983; McGroddy

et al., 1996; Gustafsson and Gschwend, 1997) are also less susceptible to degradation by microorganisms. In fact, treatment of PAH contaminated sediments dredged from Milwaukee Harbor showed that PAH sorbed onto coal-derived particles underwent minimal biodegradation, while those sorbed on clay/silt particles were readily biodegraded (Talley et al., 2002).

1.3 Stable carbon isotopes

The identification and quantification of organic compounds present in the environment is a major application area of modern analytical chemistry. However, it is still hardly recognized that in addition to the chemical identity and the concentration of organic compounds there is more information available about their sources and the fate in the environment by including isotopic composition. The following subchapters represent the basis including definitions and calculations, while in the second part the use of stable C isotopes in source apportionment will be outlined. In this part we provide an overview of recent application of stable isotopes in environmental studies emphasis on biomarker studies to identify the source and transformation pathways of OM in lake systems and identification of contaminant sources such as PAHs.

1.3.1 Definitions and calculations

Stable isotope data are usually expressed in delta (δ) values in per mil (‰) according to:

$$\delta(‰) = [(R_{sample} - R_{standard})/R_{standard}] \times 1000 \quad (5)$$

where R_{sample} and $R_{standard}$ represent the isotope ratio, such as $^{13}C/^{12}C$, in sample and an international standard, respectively. For carbon Vienna Pee Dee Belemnite with $R_{standard}$ = 0.0112372 is used (Table 1). Isotopic reference standards can be obtained from two organizations: National Institute of Standards and Technology, Standard Reference Materials Program in USA and International Atomic Energy Agency, Section of Isotope Hydrology, Vienna in Austria. The δ value for carbon, for example, is a convenient means to describe small variations in the relative abundance of the ^{13}C in OM. A negative δ value implies that the sample is depleted in the heavy isotope relative to the standard. For carbon, we avoid the terms "light" and "heavy" and use instead "^{13}C-depleted" and "^{13}C-enriched", to describe relative isotope composition.

Table 1: Isotopic reference standards and guidelines for reporting carbon ($^{13}C/^{12}C$) isotopic composition (Coplen, 1996).

Element	Standard abbreviation	Guidelines for reporting
Carbon in OM (old method)	PDB[a]	$\delta^{13}C$ on scale where PDB = 0‰ (PDB primary standard is now depleted)
Carbon in carbonate	VPDB[b]	$\delta^{13}C$ on scale where NBS19 calcite (secondary standard) = +1.95‰
Other carbon		Same as above, but also report $\delta^{13}C$ of NBS22 oil or other appropriate reference standard

[a]**PDB** - This reference standard for ^{13}C has been Belemnite of the Pee Dee Formation in South Carolina, USA (PDB). Because PDB is no longer available, a new reference standard, Vienna-PDB ([b]**VPDB,** with the same $^{13}C/^{12}C$ ratio as PDB), has been defined by its relationship to NBS19 (National Bureau of Standards)

The difference in mass between isotopes of the same elements results in measurable isotopic fractionation during physical and chemical processes. These fractionations are more pronounced for light elements because their isotopes (e.g. hydrogen, ^{1}H *vs.* deuterium, ^{2}H) show proportionally larger differences in mass than isotopes of heavier elements (e.g. ^{12}C *vs.* ^{13}C).

The main mechanisms for fractionations include:

- equilibrium (thermodynamic or exchange) fractionation and
- kinetic isotope effects associated with irreversible physical or chemical reactions.

Equilibrium fractionation reactions are those in which the distribution of isotopes differs between chemical substances (reactant vs. product) or phases (e.g., vapor *vs.* liquid) when a reaction is in equilibrium. In these reactions the reactants and products remain in close contact in a closed, well-mixed system such that back reactions can occur and chemical equilibrium can be attained. Kinetic isotope effects rise in irreversible or unidirectional reactions because for some reason the reverse reaction is inhibited or not occurring. In kinetic reactions, both bond strength and isotope velocity are important. Kinetic fractionation reactions are normally associated with processes such as evaporation, diffusion, dissociation reactions, and enzymatic effects. Kinetic fractionations are often quite large, usually much larger than equilibrium fractionations, and result in the lighter isotope accumulating in the product (“*lighter goes faster”*) (Sulzman, 2007).

The isotope fractionation between two compounds (Reactants ⇔ Products) can be expressed either with the fractionation factor α or the enrichment factor ε according to Equations. 6 and 7:

$$\alpha_{p-r} = \frac{R_{product}}{R_{reactant}} = \frac{10^{-3}\delta_p + 1}{10^{-3}\delta_r + 1} \quad (6)$$

and

$$\varepsilon_{p-r} = \left(\frac{R_{product}}{R_{reactant}} - 1\right) \times 1000 = (\alpha - 1) \times 1000 [\text{per mil}] \quad (7)$$

where subscript r and p refer to Reactant and Product respectively, and $R_{reactant}$ and $R_{product}$ are the ratios of $^{13}C/^{12}C$ in the substrate and the degradation product respectively, that appear in a infinitely short period of time.

If studies at the low natural abundance level of heavy isotopes are carried out or the fractionation is very small (i.e. degradation processes), the classical Rayleigh-type of relation can be used to describe fractional distillation of mixed liquids:

$$\frac{R_t}{R_0} = f^{(\alpha - 1)} \quad (8)$$

where R_t and R_0 are the ratios of the heavy isotope to the light isotope in the reactant *r* at time $t = 0$ and *t*, respectively and *f* is the remaining fraction of the reactant at time *t*.

1.3.2 Ecological significance of stable carbon isotopes and source apportionment

1.3.2.1Natural processes

The ratio of two stable isotopes of C (^{12}C and ^{13}C) in natural materials varies as a result of isotopic fractionation during physical, chemical and biological processes. All plants assimilate ^{12}C in preference to ^{13}C and their isotopic composition is the results of enzymatic processes and different sizes of the metabolic carbon pools (Hayes, 1993).

To give an example, in most plants CO_2 fixation, results in the formation of a C_3 body, 3-phosphoglycerate (3-PGA). This pathway of CO_2 fixation is known as the Calvin cycle.

Plants using the 3-PGA pathway for CO_2 fixation are commonly called C_3 plants. Most photosynthetic plants incorporate carbon into OM using the C_3, Calvin pathway, which biochemically discriminates against ^{13}C to produce a $\delta^{13}C$ shift about −20‰ from the isotope ratio of the inorganic carbon source. However, some plants mainly in tropic environments make use of a different pathway. Here, CO_2 fixation yields a C_4-dicarboxylic acid, oxalo acetate, hence the term C_4 plants (the C_4-dicarboxylic acid pathway is also known as the Hatch-Slack cycle) and use the C_4 Hatch-Slack pathway, which creates a diffusional isotope shift of approximately −7 ‰. Other plants, mostly succulents, utilize the crassulacean acid metabolism (CAM) pathway, which discriminates variably against ^{13}C depending on growth dynamics. Differences in $\delta^{13}C$ values were reported for total leaf tissue, total surface lipids extracts and individual n-alkanes isolated from plants utilizing C_3, C_4 and CAM pathways for carbon fixation. The average $\delta^{13}C$ values obtain from C_4 and CAM plant material. The ranges of $\delta^{13}C$ values for *n*-alkanes were: from −31.4 to −38.6‰ for C_3 plants from −18.5 to −24.5‰ for C_4 plants; and from −25.2 to −26.8‰ for CAM plants (Lockheart, 1997).

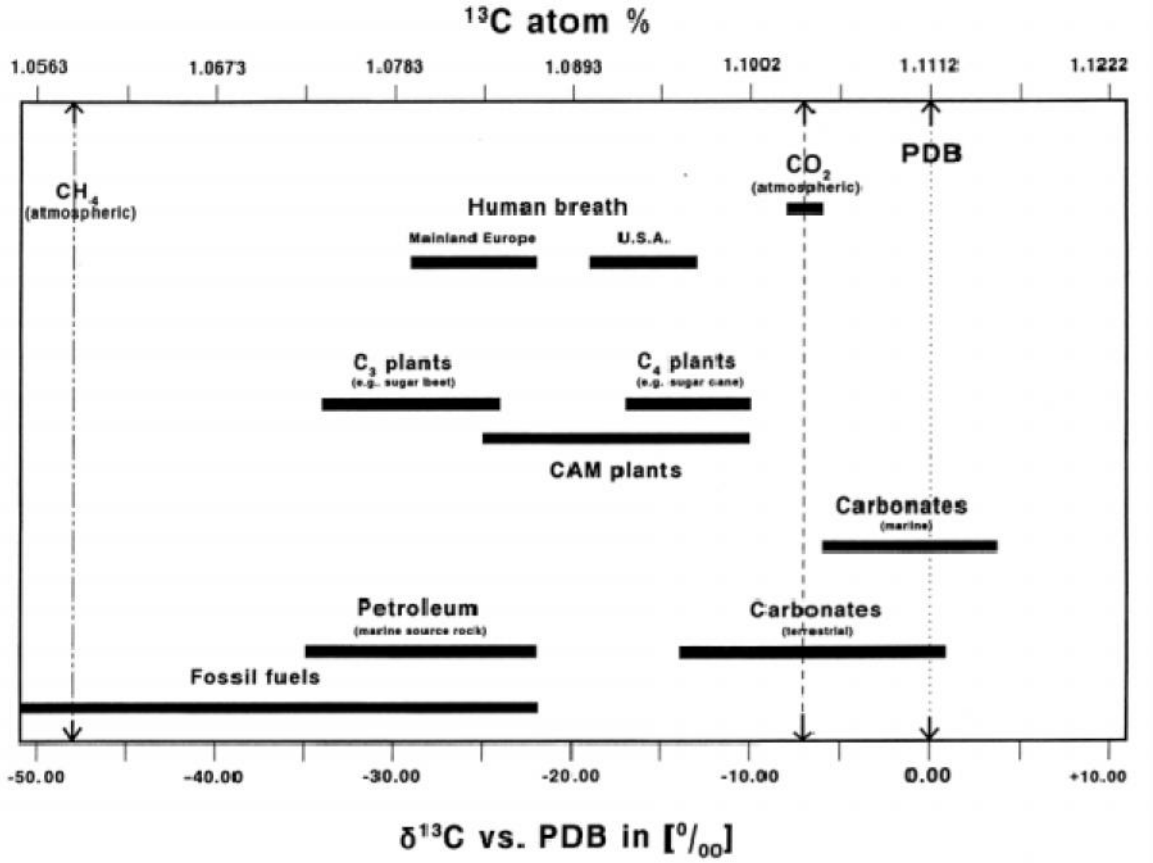

Figure 7: *Some typical examples of natural $\delta^{13}C$ values grouped according to origin along the scale of ^{13}C natural abundance* (Meier-Augenstein, 1999).

OM produced from atmospheric CO_2 ($\delta^{13}C \approx -7$‰) by land plants using the C_3 pathway has an average $\delta^{13}C$ (PDB) value of approximately −27‰ and by those using the C_4 pathway approximately −14‰ (O'Leary, 1988). Freshwater algae utilize dissolved CO_2, which is usually in isotopic equilibrium with atmospheric CO_2.

The carbon isotopic ratios are useful to distinguish between marine and continental plant sources of sedimentary OM and to identify OM from different types of land plants. The carbon isotopic compositions of OM reflect principally the dynamics of carbon assimilation during photosynthesis and the isotopic compositions of the carbon source (Hayes, 1993). The source of inorganic carbon from marine algae is dissolved

bicarbonate, which has a $\delta^{13}C$ value of approximately 0 ‰. Marine OM consequently typically has $\delta^{13}C$ values between −20 and −22‰ (Meyers, 1994). The ≈7‰ difference between OM produced by C_3 land plants and marine algae has successfully been used to trace the sources and distribution of OM in coastal ocean sediments (e.g. Meyers, 1997; Tolosa et al., 1999; Boschker et al., 2002). Figure 7 shows some typical examples of natural $\delta^{13}C$ values grouped according to origin along the scale of ^{13}C natural abundance.

1.3.2.2Lipid biomarkers and stable carbon isotopes

Biomarkers, or molecular markers, are compounds with structures that can be related to specific biological sources due to their own biosynthesis. An important fact in considering their fate is that the transformation of dissolved, suspended and deposited lipids in any environment (oxidizing or reducing) tends towards a selective preservation of low-polar compounds, including hydrocarbons, fatty acids and sterols. Hence, organic biomarker compounds are often used as tracers of sedimentary OM sources. Long-chain n-alkanes, n-alkanols and n-fatty acids are used as biomarkers for terrigenous input; unsaturated alkenones and dinosterol as tracers for marine production and branched-chain fatty acids of the iso- and anteiso series as bacterial markers.

Lipid biomarkers provide quantitative information about the structure of extant microbial communities without the need for culturing and isolation (White, 1988). Lipids are also one of the most useful biochemical measures of in situ interactions between microbial species and their environments because lipid compositions can indicate temperature-, redox-, stress-, or nutritional conditions (Ray et al., 1971, Fork et al., 1979, Jahnke, 1992). Furthermore, bacteria commonly have ester-linked lipids and some bacterial species contain additional non-phytanyl ether lipids (De Rosa et al., 1988). Thus, analysis of lipid structures permits identification of these two microbial domains in unknown environmental samples. In Table 2 most probable source organisms of lipid biomarkers are presented.

Table 2: Most probable source organisms of lipid biomarkers (Neunlist et al., 2002).

Lipid biomarkers (structural group)	Probable source organisms according to literature
Long-chain linear compounds	Plant waxes[a], microalgae[b]
C_{27} sterols	Algae[c], zooplankton, animals
C_{28} sterols	Microalgae[b]
C_{29} sterols	Higher plants, algae[b,d]
Hopanoids	Bacteria and cyanobacteria[e]

Sources of lipids attributions:

[a] Eglinton and Hamilton, 1967; Rieley et al., 1991, [b] Volkman et al., 1998, [c] Weete, 1976, [d] Huang and Meinschein, 1979; Wette, 1976, [e] Ourisson and Rohmer, 1992.

Different microorganisms have different biosynthetic pathways. In particular, autotrophic microorganisms can use several pathways for CO_2 fixation (Zhang et al., 2004). The use of lipid biomarkers has permitted useful perspectives in organic biogeochemical studies, but some care about the assumptions used in the different relationships is required. Over recent years, it has been apparent that some biomarkers are more widely distributed in the environment than previously thought, and thus, their specificity has been reduced. Sterols are a good example of this; some of them have been found only in a few classes, while others are known to be quite widely distributed. Certain general markers are still considered unambiguous, but the usefulness of those derived from a variety of sources needs to be explored. The advent of compound specific isotopic analyses (CSIA) through

the development of gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) allows the determination of the $\delta^{13}C$ composition of individual compounds. Consequently, $\delta^{13}C$ of specific biomarkers should be useful to improve the veracity of source identification, thereby allowing the origin of ubiquitous compounds with multiple potential sources to be determined (Tolosa et al., 2001, Simoneit et al., 1998). Lipids are generally depleted in ^{13}C relative to bulk biomass as a consequence of the biosynthetic pathways for fatty acid (FA). However, it is first necessary to determine how the isotopic composition of the lipids relates to the biomass as a whole and to growth substrates, as well as to determine whether there are species-specific variations that might affect potential depletion during synthesis of FA (Londry, 2004).

Results of this study show that lipid biomarkers and their isotopic compositions are only slightly affected by abundance and isotopic compositions of dissolved inorganic carbon in different facies. Instead, lipid biomarkers mostly reflect the changing microbial communities in different environments with temperature being the dominating factor; the isotopic compositions of biomass and fatty acid biomarkers, on the other hand, provide a valuable insight into the mechanisms of CO_2 fixation by the extant microbial communities on the changing environments.

Proxy parameter evaluations using distributions and isotopic composition of lipid biomarkers have often focussed on certain compound classes, as they will be introduced here.

n-Alkanes are widespread lipid biomarkers in marine and terrestrial archives. Due to the relatively simple structure of the members of this compound class, *n*-alkane distributions have been widely investigated in various types of possible source organisms as well as in sedimentary settings. *n*-Alkanes distribution in the lake sediments changes with depth. Aliphatic hydrocarbons (HC) of terrestrial origin are resistant on microbial degradation of OM and thus dominate at the bottom of lake sediments. In lake sediments we found two different source of HC: (1) algae, zooplankton, vascular plants that live within a lake and (2) anthropogenic contamination. At the surface of sediments C_{17} *n*-alkane dominates (e.g., Blumer et al., 1971; Giger et al., 1980; Cranwell et al., 1987), which indicate a presence of many aquatic algae and photosynthetic bacteria. At the bottom of lake sediments increase in the long-chain CH indicates a presence of vascular land plants and epicuticular waxy coatings (e.g., Eglinton and Hamilton, 1963, 1967; Cranwell, 1973; Cranwell et al., 1987; Rieley et al., 1991).

Pristane and phytane are isoprenoid hydrocarbons and usually found in young lake sediments. Pristane is produced primarily in the digestive tracts of copepods, from phytol derived from the side chain of chlorophyll a (Shi et al., 2001) or with erosion of sedimentary rocks (Meyers, 1994). Pristane is occasionally not found in the sediments of a lake (e.g., Ho and Meyers, 1994), which indicates that the types of animals that produce this hydrocarbon are absent from the food chain of the lake and that the watershed has not contributed ancient hydrocarbons to the sediments. Methanogenic bacteria, which exist beneath the zone of bioturbation of most lake sediments, are an important source of phytane (Risatti et al., 1984). Phytane therefore records methanogenesis in the lake bottom. Cyanobacteria contribute 7- and 8-methyl heptadecane to lake sediments (Filley et al., 2001), which indicates OM production under low-oxygen conditions in the photic zone. However, it is important to remember that hydrocarbons normally constitute a very small fraction of the total OM in both biota and in sediments. With $\delta^{13}C$ of individual *n*-alkanes from lake sediments enabled to distinguish between fresh-water algae and terrestrial plants. Ficken et al. (1998, 2000, 2002) extensively use abundances and isotopic compositions of normal chain lipids (*n*-alkanes, *n*-fatty acids, *n*-alcohols) for OM source assignment in lacustrine archives. Relatively high proportions of mid-chain

homologues in the chain length range from C_{23} to C_{25} were detected for the submerged macrophytes. By comparing the $\delta^{13}C$ of individual *n*-alkanes and *n*-alkanols from leaves of lake side trees with those from the lake's sediments, it was possible to discriminate between the fresh-water algae and terrestrial plants. Other classes of biomarkers, such as the hopanes, are also not always derived from a common precursor.

Alternatively, the $\delta^{13}C$ obtained for biomarkers commonly associated with terrestrial sources (e.g., long-chain *n*-alkanes, *n*-alkanols and $C_{29}\Delta^5$ sterol) have elucidated sources other than higher plants. Other possible algal origin of long chain odd *n*-alkanes in immature sediments was revealed by distributions and carbon isotopic composition (Simoneit, 1998).

n- Fatty Acids (FAs) in lake sediments are constituents of the widespread triglycerides and typically originate from multiple sources. They are among the most abundant biomarkers. Gillan et al. (1986) and Wakeham et al. (1989) reported that FA are one of the few indicators of bacterial contribution and may also distinguish marine from terrigenous and algal from zooplanktonic inputs. The unsaturated *n*-C_{16} and *n*-C_{18} acids are major constituents of the lipids of freshwater algae, yet they are rapidly degraded by microbes during and after sedimentation (Cranwell, 1976). Longer even-chain C_{24}-C_{30} FAs may point to an important autochthonous contribution to the sedimentary OM. The n-C_{15} and anteiso-C_{15} FAs have been used as indicators of microbial biomass in lake sediments (Cranwell et al., 1987; Goossens et al., 1989), and they represent *in situ* production of secondary lipids at the expense of primary OM. Kawamura et al. (1980) reported, that some FA are more susceptible to diagenesis than other. For example, ratios of $C_{16:1}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$ to their corresponding alkanoic acids decrease by a factor of 10 in the upper 8 cm of sediment in Lake Haruna, Japan.

Simoneit (1998) reported, that the isotopic compositions of long-chain fatty FAs ($C_{20:0}$–$C_{26:0}$) ranged from −31.0 to −30.7‰ and reflect their sources from higher-land plants. Isotopic composition of $C_{16:0}$ and $C_{18:0}$ acids ranged from −28.7 to −27.7‰ and presented marine plankton. $C_{14:0}$ and $C_{15:0}$ acids with isotopic composition from −38.7 to −37.2‰ originated from bacteria (Simoneit, 1998).

Sedimentary **alcoholic compounds** are usually subdivided into two general subgroups: *n*-alcohols and sterols. **n-Alcohols** are presented in epicuticular waxes of land plants and contain an even number of carbon atoms from C_{22} to C_{30}. Their distributions are usually dominated by the C_{26}, C_{28}, and C_{30} (Eglinton and Hamilton, 1967; Rieley et al., 1991), but some sediment horizons contain large proportions of the C_{24} component, which may indicate enhanced importance of submerged macrophytes or cyanobacteria at the times corresponding to these horizons. In contrast, aquatic algae and bacteria contribute shorter chain length by C_{16} to C_{22} components (Robinson et al., 1984; Volkman et al., 1999). The common mono-unsaturated isoprenoid alkenol, phytol, is derived from chlorophyll and can be diagenetically reduced to dihydrophytol over time.

Simoneit (1998) reported, that $\delta^{13}C$ of *n*-alkanols from saline sediment enabled to distinguish between the terrestrial long-chain *n*-alkanols (*n*-C_{24}, *n*-C_{26}) ranged from −30 to −32‰ and the marine short-chain n-alkanols (*n*-C_{16} to *n*-C_{22}) ranged from −18 to −23 ‰.

Sterols and their derivatives are important paleoenvironmental biomarker compounds. Sterols build up a highly complex group of biomarkers. They are in general synthesised from squalene epoxide by cyclisation in all eucaryotic organisms, but biosynthetic pathways differ between land plants and algae on the one hand and animals and fungi on the other hand (Figure 8). The presence or absence of double bonds and methyl groups at

various positions on the carbon framework, the length of the branched side chain at the C_{27} position, and the stereochemistry of the substituent bonds create a variety of compounds (e.g., Volkman, 1986). The structural diversity of sterols and the stanols and steranes derived from them provides information about the origins and diagenetic alterations of OM in lake sediments.

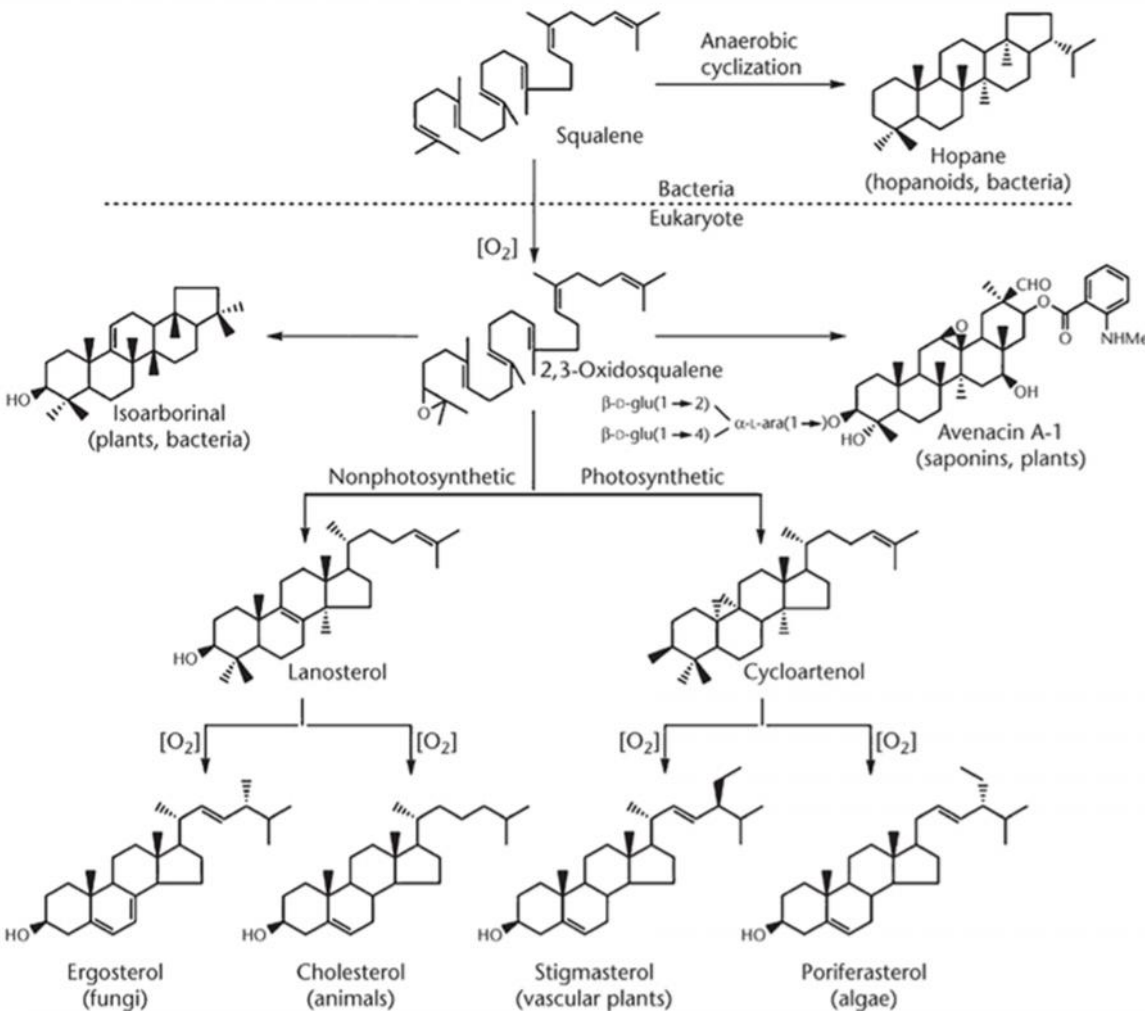

Figure 8: *Biosynthetic pathway of sterol synthesis* (Devarenne, 2009).

The relative amounts of C_{27} and C_{29} sterols help to identify the contributions of algal and land-plant OM in marine sediments. Huang and Meinschein (1976, 1979) have proposed that this way of distinguishing also use the lake. Volkman (1986) reported that sterols can trace the contribution from different algae, higher animals, vascular plants and sewage contamination. Nishimura and Koyana (1977) are found that is cholesterol the dominant C_{27} sterol in algae and 24-ethylcholesterol (β-sitosterol) is the major C_{29} sterol in vascular plants in and around from Lake Suwa (Japan). The abundance of 5α-cholestan-3α-ol (epicholestanol), 24-ethylcholesta-5,22(E)-dien-3β-ol (stigmasterol) and 24-methylcholest-5-en-3β-ol (campesterol) indicated that these compounds could be the product of anaerobic lacustrine microbes. Further Matsumoto et al. (1982) for example detected 24-ethylcholesterol, a C_{29} sterol, to be a major constituent in lake sediments of Victoria Land, Antarctica despite the lack of higher land plants in the catchment area. Thus, blue-green algae were identified as appropriate sterol sources. More recently, Matsumoto et al. (2001) investigated the $\delta^{13}C$ isotopic composition of this compound in Japan Sea sediments over the last 30,000 years. The results yield a shift in the isotopic composition in correlation to climatic change in that region. Accordingly, the data suggest

a marine source of 24-ethylcholesterol after the Last Glacial period whereas a terrestrial origin was taken into account for glacial sediments.

1.3.2.3Source apportionment of polycyclic aromatic hydrocarbons

The molecular isotopic signature of environmental contaminants can often be used to trace their sources on local to global scales. On a local scale it is often necessary to allocate a contamination to a specific source in order to allow appropriate means of risk reduction and/or to identify responsible parties in litigation. These techniques also exploit the unique molecular and isotopic compositions of PAHs (Wilkes, 2009).

Several different methods are reported in the literature for apportionment of the sources of PAHs encountered in the environment. Some of these methods include the use of historical records (Heit et al., 1988; Latimer and Quinn, 1996; Gevao et al., 1998; Van Metre et al., 2000; Schneider et al., 2001; Lima et al., 2003), source diagnostic ratios (Colombo et al., 1989; Yunker et al., 1996), principal component analysis (Yunker et al., 1999; Dickhut et al., 2000), multiple linear regression (Simcik et al., 1999), chemical mass balance (Gordon, 1988; Christensen et al., 1999; Zhang et al., 2002; Li et al., 2003), stable carbon isotopic composition (OMalley et al., 1994; McRae et al., 1999; Okuda et al., 2002) and more recently, the radiocarbon content of specific PAHs (Currie et al., 1997; Reddy et al., 2002; Reddy et al., 2003). We will focus mainly on the use of isotopic composition of carbon, since was also used in our study.

O'Malley and collaborators (1994) were the first to measure the carbon isotopic composition of individual PAHs from environmental samples. Compound-specific isotope analysis (CSIA) allows the determination of isotopic signatures of individual compounds and was initially developed to help reconstruct biogeochemical processes (Hayes et al., 1989). This technique has been widely employed for the discrimination of the sources of hydrocarbons encountered in modem and ancient sediments (Freeman et al., 1990; Rieley et al., 1991). O'Malley and collaborators (1994) have suggested that the isotopic composition of PAHs present in environmental samples is not altered by weathering processes. Evaluation of the effects of evaporation, photodecomposition and microbial degradation of PAH standards under controlled laboratory conditions revealed no significant alteration of the isotopic composition of individual compounds (O'Malley et al., 1994). Similar results were obtained for the aerobic biodegradation of an Arabian crude oil sample (Mazeas et al., 2002).

The use of compound-specific carbon isotope characterization of PAHs as a source apportioning technique relies on the premise that combustion-derived compounds retain the isotopic signature of their original precursors. It is thought that the range in $\delta^{13}C$ values of PAHs generated during pyrolysis is correlated to the isotopic signature of the source. Carbon isotopic measurements of individual PAHs showed different $\delta^{13}C$ values for an automobile exhaust and a wood soot sample (O'Malley et al., 1994). In general, the automobile soot exhibited more ^{13}C -depleted (i.e., "lighter" or more negative $\delta^{13}C$ values) for 3- and 5-ring compounds (Phen, A, benzofluoranthenes and BaP) *vs.* 4-ring PAHs (Fl, Py, BaA and Chry). However, BaA present in the wood soot sample was ^{13}C -enriched relative to Fl and Py (Figure 9). The initial samples analyzed by O'Malley and collaborators (O'Malley et al., 1994) demonstrated that the isotopic composition of PAHs generated by wood burning varied with ring size, with 3- and 5-ring PAHs being more C-depleted than 4-ring compounds. The $\delta^{13}C$ values of individual PAHs were observed to correspond to more ^{13}C-depletion with increased temperature of formation. PAHs released by low temperature combustion processes exhibited $\delta^{13}C$ values similar to those of the parent coal (−24‰ to −25‰). However, combustion of different fuels can yield similar mixtures of pyrogenic PAHs, and the ranges of $\delta^{13}C$ values of the main energy

sources (coal, petroleum and wood) greatly overlap in the range of −30 to −20‰. Hence, the difficulty in relying solely on $\delta^{13}C$ values to separate the contributions of PAHs derived from two combustion processes.

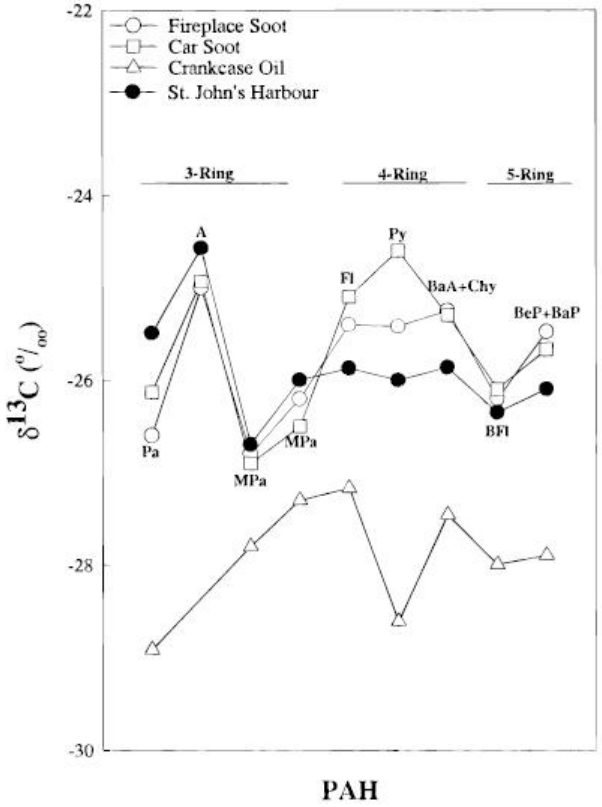

Figure 9: *Isotopic composition of individual PAHs in three potential contamination sources and in sediments from the St. John's harbour, Newfoundland*; anthracene (A); phenanthrene (Pa); methyl-phenanthrene (MPa); fluorene (Fl); pyrene (Py); benzoanthracene (BaA); chrysene (Chry); benzofluoranthenes (BFl); benzo(e)pyrene (BeP) and benzo(a)pyrene (BaP) (O'Malley et al., 1996).

The sedimentary records of PAH concentrations have frequently been studied in order to develop historical records of combustion, (Fernandez et al., 2000, Schneider et al., 2001, Rose et al., 2002, Lima et al., 2003, Guo et al., 2011) but studies of stable carbon isotope composition of PAH in sediment cores are limited (Smirnov et al., 1998, McRae et al., 1996). Studies in aquatic systems, focused mainly on surface sediments (McRae, 1999). McRae et al. (1996) have shown that PAH from other anthropogenic sources in urban lakes are more enriched in ^{13}C than combustion derived PAH. Various sources of PAH have also been distinguished in sediments along the St. Lawrence River (Stark et al., 2003). Localized ^{13}C-enrichment in the stable isotope signature of PAH was shown to be due to petroleum-related and aluminium smelter contributions against the regional backdrop of combustion-dominated PAH sources.

1.4 Determination of the isotopic composition of organic compounds

Isotope ratio mass spectrometry (IRMS) following on-line combustion (C) of compounds separated by gas chromatography (GC) is used to determine the isotopic composition of organic compounds. GC-C-IRMS instruments are commercially available only since 1990; therefore our understanding of the isotopic composition of organic compounds in

different studies is still somewhat limited. This chapter presents the basics of GC-C-IRMS instrument, sample preparation scheme for sediments samples, data treatment and error analysis.

1.4.1 Sample preparation scheme

Sample preparation schemes usually comprise the following steps:

- extraction,
- separation, and
- derivatization (in the case of functionalized compounds).

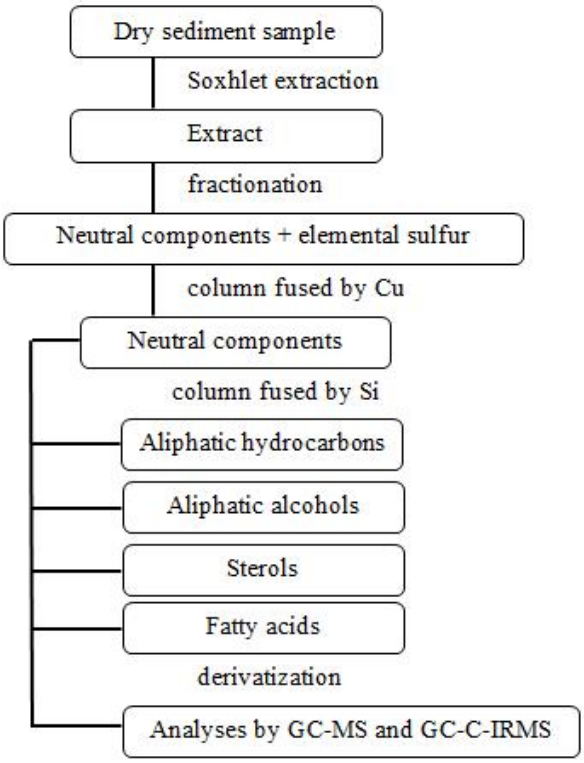

Figure 10: *A schematic overview of an isolation procedure.*

Figure 10 shows a typical protocol for the isolation of the most common compounds targeted for compound-specific stable isotope analysis. Before extraction, samples are dried, crushed and homogenized to increase the effectiveness of solvent penetrating the sample matrix.

Methods available for lipid extraction include: Soxhlet (large samples; 10-100 of grams; heat stable), ultrasonication (small samples, <10g) and liquid/liquid extraction. The resulting total lipid extract (TLE) is then further separated into different compound classes using various chromatographic methods. First it is important to verify whether or not aspects of the analytical protocol will introduce an isotopic fractionation effect by analysis of reference compounds of known stable isotopic composition. In addition many important components of ecological materials cannot be analyzed directly by GC. For example, complex lipids require chemical cleavage to yield GC amenable components, i.e. commonly occurring phospholipids are saponified and methylated to generate fatty acid methyl esters (FAMEs; Crossman et al., 2004).

GC-C-IRMS instrumentation enables the compound-specific isotope analysis of individual organic compounds, for example, n-alkanes, fatty acids, sterols extracted and

purified from bulk organic materials. Thus compounds containing polar functional groups should be chemicaly modified, or derivatised, to enhance their volatility prior to introduction to the GC-C-IRMS. Derivatization of FA, alcohols and sterols, to FAMEs, alcohol and sterol trimethylsilyl ethers, respectively, is relatively straightforward since the resulting sample-to-derivative carbon molar ratio is high, resulting in minimal analytical error. Additionally, derivatization reactions, such as esterification and silylation are typically rapid and quantitative thereby precluding kinetic isotope effects (Rieley, 1994).

The single most important requirement in performing a valid and robust determination of the $\delta^{13}C$ value of an individual compound by GC-C-IRMS is good chromatographic separation of the target compound(s), achieved by optimization of the GC operating conditions and judicious column selection. Peaks that are not fully resolved (up to 25% co-elution) can still be integrated separately as long as a minimum estimate of analytical error is gained by running the sample at different concentrations (Ricci et al., 1994). For any larger overlap, co-eluting components must be integrated as one peak using the integration software, although the errors associated with such determinations may be substantial.

In addition to the reference gas calibration it is important that the performance of the GC-C-IRMS instrument is constantly monitored using a suite of compounds of known relative stable isotopic composition. Such references should ideally belong to the same compound class as the target compounds since the performance of the instrument for one particular class of compounds will not necessarily replicate that of a different class of compounds (Meier-Augenstein et al., 1996). In addition, a range of homologues should be utilized as a standard mixture thereby assessing the performance of the instrument across the entire temperature range utilized by the GC. By doing this factors such as leaks and/or blockages at varying temperatures can be quickly identified and resolved. However it should be emphasised that there are very limited international standards available for GC-C-IRMS measurements. Usually the isotopic composition of standards containing a single compound is determined with an elemental analyser and isotope ratio mass spectrometer (EA-IRMS). The same compound is then analysed with GC-C-IRMS. The isotopic composition of the EA-IRMS measurements is taken as a ‘true values’ of the standard and thus the accuracy of the GC-C-IRMS evaluating by comparing the $\delta^{13}C$ values determined with both methods.

1.4.2 GC-C-IRMS spectrometer

Gas chromatography- isotope ratio mass spectrometry (GC-C-IRMS) has become easy and powerful approach that may be used to trace the flow of organic compounds at the molecular level. In contrast to organic mass spectrometer (MS) that yield the structural information by scanning a mass range for characteristic fragment ions, IRMS instruments achieve highly precise measurements of isotopic abundance at the expense of the flexibility of scanning MS. For isotope measurements, the analyte must be converted into a simple gas before entering the ion source of an IRMS. Therefore, it is obvious that GC cannot be directly coupled to an IRMS. The combustion interface where the GC effluent is fed into a combustion reactor is used to convert separated organic compounds into a simple gas. This reactor, either a quartz glass or ceramic tube, is filled with CuO/Pt or CuO/NiO/Pt and maintained at a temperature of approximately 820 or 940 °C respectively (Rautenschlein et al., 1990; Merritt et al., 1995). Generalized schematics of a GC-C-IRMS configured for $\delta^{13}C$ analysis is presented in Figure 11.

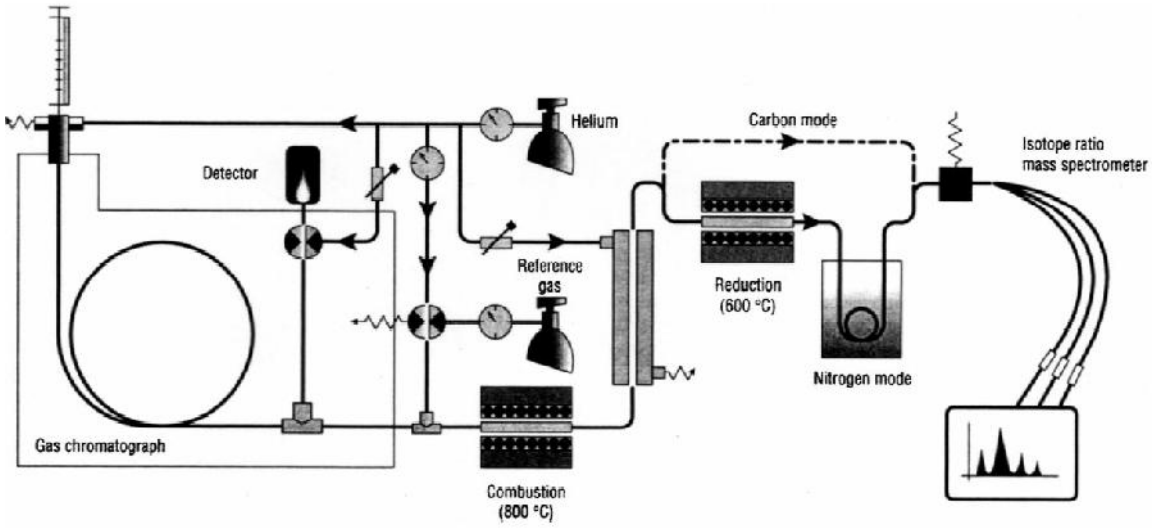

Figure 11: *A generalized schematics of a GC-C-IRMS configured for $\delta^{13}C$ analysis.* (Meier-Augenstein, 1997).

The seamless interfacing of the GC module to the IsoPrime mass spectrometer that is available in our laboratory (Figure 12) provides selective, sensitive and accurate on-line $\delta^{13}C$ measurements of individual components in complex sample matrices. In Table 3 are compared external precision, accuracy, sample size and linearity for GC-C-IRMS for $\delta^{13}C$ analysis.

The first step in this analytical technique is gas chromatography and this is followed by complete combustion of the carbon-bearing substances in the GC effluent, producing CO_2 and water. The water is removed from the effluent stream, and the mass spectrum of the CO_2 is measured. The amount of CO_2 containing C-13 (total molecular weight of 45) can be compared to the amount of CO_2 containing the more common C-12 (total molecular weight of 44). Gas chromatography (GC) is used to separate a complex mixture of volatile substances into its respective components.

Table 3: Precision, accuracy, sample size and linearity for GC combustion.

	13C
External precision	≤ 0.2 ‰
Accuray	≤ 0.3 ‰
Sample size	> 1.0 nmol CO_2 (0.1 nmol C_{10} injected onto column)
Linearity	≤ 0.3 ‰
Hopanoids	≤ 0.2 ‰

Compared to conventional dual inlet techniques, continuous flow GC-IRMS offers significant advantages in the analysis of complex extracts, eliminating the need for tedious purification procedures and offering a high throughput, selective and sensitive alternative. GC-IRMS can be used routinely for the analysis of nanomole quantities of chromatographically resolved components, reducing sample preparation time and expense. The ability to analyze enriched samples open up new investigative opportunities, with the quantity of tracer required reduced by a factor 100 compared with conventional MS methods.

The GC combustion system includes:

- Hewlett Packard GC6890 EPC gas chromatograph with split/splitless capillary injector;
- flame ionization detector (FID);
- oxidation furnace, interface oven and controller;
- heated GC-furnace interfaced to a micro-bore oxidation furnace;
- on-line capillary water trap immersed in liquid nitrogen;
- pressure regulators for control of gas flow throughout the interface.

Features of the GC-combustion system

- automated sample introduction;
- sample components chromatographically resolved by the capillary GC;
- eluent switched between the FID and the mass spectrometer;
- components of interest combusted in a micro-furnace;
- water removed from sample eluent by a cryogenic trap;
- automatic introduction of pulses of pure reference gas to the IsoPrime MS for best possible precision and accuracy.

Chromatographic separation: The GC system uses capillary chromatography to efficiently separate the most complex matrices prior to on-line measurement of stable isotopes using the IsoPrime high performance mass spectrometer.

'Heart Cutting' Technology: Individual components eluting from the GC column can be simply switched, using the 'Heart Cutting' valve, between the flame ionization detector (FID) and the IsoPrime mass spectrometer. This facility allows only those components of interest to the analyst to be directed to the combustion furnace and finally the mass spectrometer, venting unwanted eluent, eliminating flooding/overloading the source and providing a full gas chromatogram if required.

Water traps: Removal of the water of combustion is achieved using the standard cryogenic water trap or a Nafion water trap. The standard water trap comprises an on-line capillary immersed in liquid nitrogen. The trap temperature is fully programmable, with a temperature range of –100 °C to +100 °C. The optional Nafion trap is a hydrophilic membrane that absorbs water from the gas stream and transfers it to a drying gas flow passing over the outside of the membrane.

Microfurnace: Chromatographically separated effluent is fully combusted to CO_2 in a long-life, low maintenance microfurnace.

Reference gas injector: Running in parallel with the sample line is Micromass unique reference gas injector system, which allows the introduction of pulses of reference gas to the MS as required. This arrangement provides the analyst with the most accurate and precise isotope ratio measurements.

Figure 12: *Isoprime GC-C-IRMS instrument available at Department of Environmental Science at Jožef Stefan Institute.*

1.4.3 Data treatment and error analysis

1.4.3.1 Correcting for derivative groups

In case of derivatization account must be taken of exogenous atoms added to the analyte molecule during analysis. The contribution of the derivative atom to the measured δ value of the derivatized compound for FAMEs, alcohols and sterols can be calculated using a simple mass balance equation 9, where n is number of moles of the isotope of interest, F is the fractional abundance of the isotope of interest, c refers to compound of interest, d refers to the derivative group, and cd refers to the derivatized compound (Rieley, 1994). For compounds at natural abundance, F can be replaced with the corresponding δ value (Equation 10):

$$n_{cd}F_{cd} = n_cF_c + n_dF_d \quad (9)$$

$$n_{cd}\delta_{cd} = n_c\delta_c + n_d\delta_d \quad (10)$$

The application of these equations requires the isotope value of the derivatizing molecule to be established. If all the atoms of interest in the derivatizing reagent are transferred to the analyte, the contribution can be measured directly offline. However, if this is not the case or if the reagent is purchased in numerous small batches thereby precluding off-line analysis, the contribution of the derivatizing group can be measured indirectly by derivatizing a compound of known isotope value. However, this will have implications for the errors associated with the measurement, as discussed below.

1.4.3.2 Estimating kinetic isotope effects (KIEs)

If the observed δ values for derivatized analytes do not equal those predicted by equation 6, then a kinetic isotope effect is present. Correction for the KIE can be made through the use of correction factors, as long as the KIE is proved to be reproducible across a range of analyte concentrations. These correction factors can be defined as the “effective” stable isotope composition of the derivative carbon introduced during derivatization taking into account the isotopic fractionation associated with the reaction. Correction factors are determined indirectly by measuring the δ value of an underivatized standard of the molecule of interest (by EA-IRMS), the value of the standard after derivatization (by GC-C-IRMS), and using a rearranged Equation 10 to determine δ_d. The δ_d term can then be replaced with δ_{corr} to represent the correction factor for the analyte of interest (Silfer et al.,

1991; Macko et al., 1998).

1.4.3.3Isotopic calibration

For reasons mentioned before, it is not possible in GC–C–IRMS to calibrate target compounds against a standard of known isotopic composition, intro compared ducing the standard in exactly the same way as the analyte. There are only three feasible means of introducing a standard (Meier-Augenstein, 1999):

- addition of reference compounds to the sample,
- introduction of refer tracer ence gas pulses to the carrier gas stream, or
- introduction of reference gas pulses directly into the ion source.

Caimi et al. (1996) comprehensively listed all the desirable properties internal reference compounds should possess:

- high chemical stability;
- conveniently available in high purity;
- readily soluble in high-purity solvents;
- low vapour pressure at room temperature and atmospheric pressure;
- environmentally rare;
- ideally useful for GC and liquid chromatography (LC) techniques; and
- sufficiently different chromatographic characteristics to avoid partial or complete co-elution with sample analytes.

The results of an extensive study into methods of isotopic calibration by Merritt et al. (1994) emphasised these demands. Comparing the use of internal reference compounds with the introduction of reference gas pulses directly in the ion source of the IRMS, Merritt et al. (1994) found an offset of >2‰ between the two methods in the case of incomplete combustion and other systematic errors affecting only the analytes. These systematic errors affected both the analytes and the co-injected reference compounds but were not reflected by the external reference gas pulses. Similar observations were made by other groups interested in isotopic calibration. In the absence of such systematic errors, Merritt et al. (1994) found that both methods of isotopic calibration gave consistent results as long as multiple reference peaks were used to permit drift correction. Only one reference peak for isotopic calibration, albeit from an internal reference compound, is not enough to compensate for the influence of GC parameters, such as analyte / stationary phase interaction, column temperature on measured isotope ratios (Meier-Augenstein, 1999).

Within the GC–C–IRMS system, seven potential sources for mass discrimination and, hence, systematic errors can be identified (Meier-Augenstein, 1997):

- isotopic fractionation during sample injection (which can be overcome by on-column or time programmed split- less injection);
- chromatographic isotope effect;
- chromatographic peak distortion (leading and trailing peak tail);
- combustion process;
- peak disortion of N_2/CO_2 gas peak during passage of the combustion interface;
- changing flow conditions at the open split prior to the IRMS; and
- the IRMS itself.

Obviously, the external reference gas pulses only compensate for item, whereas internal reference compounds reflect all of the aforementioned. Recently, a method for isotopic calibration was reported that, provided a combustible gas was used, could reflect the

systematic errors caused by items. This method combines the convenience and practicability of external reference gas calibration with the advantage of reflecting the majority of physical influences to which analytes are subjected in a GC–C IRMS system (Meier-Augenstein, 1999).

2 Hypothesis and aims

The main purpose of the thesis project is to identify the source and transformation pathways of OM using analysis of organic geochemical markers combined with compound specific isotope composition in the anoxic, eutrophic alpine Lake Bled. This is the first detailed organo-geochemical characterisation of OM using the stable isotope approach currently available.

We assume that, on the basis of distribution of biomarkers and by the isotope composition of individual compounds, we can assess the contributions of land, lake and anthropogenic origins of OM. The research included investigations performed in water columns (particulate organic matter and sediment trap material) and sediments. Results on POM are present in scientific paper »Lipid biomarkers of suspended particulate OM in Lake Bled (NW Slovenia)«, while the results of sediments are present in scientific paper »Methanogenesis pathways in a stratified eutrophic alpine lake (Lake Bled, Slovenia)«. The examination of individual biomarker compounds has been focused mainly on the more long-lived lipid groups such as hydrocarbons, alcohols, sterols, and fatty acids. Previous research (Muri and Wakeham, 2006) has suggested that the origin of OM in sediments, as determined by molecular distributions, was mixed, in sharp disagreement with the origin of bulk OM, probably due to selective degradation of lipids. Our aim was to verify this assumption using the stable isotope approach and these results are present in scientific paper »Stable isotopes and source identification of lipids in oxic and anoxic sediments of Lake Bled (NW Slovenia)«. The latter was further used to determine the origin of polycyclic aromatic hydrocarbons (PAHs) in Lake Bled sediment. Previous studies suggested that these PAHs (excluding Per and Re) are of pyrolytic origin and derived mainly from coal combustion (Muri and Wakeham, 2009). Results of our analyses are present in scientific paper »Source identification of polycyclic aromatic hydrocarbons in Lake Bled (NW Slovenia) sediments using stable carbon isotopes«.

Thus the application of stable isotope analysis performed within this thesis has three major objectives:

- to investigate the sources and compositional changes of lipid biomarkers in particulate organic matter (POM), sedimentary trap material and sediments of Lake Bled (NW Slovenia);
- to identify the sources of lipid biomarkers in two sediment cores deposited, one under oxic (Zaka Bay) and the other, under anoxic (western basin) conditions;
- to trace and identify the sources of PAHs in Lake Bled sediments.

3 Publications

3.1 Scientific paper: »Lipid biomarkers of suspended particulate OM in Lake Bled (NW Slovenia)«

In this chapter, the paper entitled “Lipid biomarkers of suspended particulate OM in Lake Bled (NW Slovenia)” by Marinka Gams Petrišič, and Nives Ogrinc is presented. The paper was published in Geomicrobiology Journal, 2013.

Lipid biomarkers provide a unique insight into the diagenetic alteration, deposition, preservation and sources of OM and, in particular, the microbial community structure present in lacustrine environments. In our study we used molecular and stable carbon isotope ratios of specific lipid biomarkers to evaluate their sources and to explore variations in the biogeochemistry of the POM and sediment trap material positioned at three depths throughout the water column at the deepest part of the subalpine Lake Bled, NW Slovenia. In October 2006, samples of POM were collected. POM samples were taken at two depths, 12 and 28 m, while sediment trap material was collected at three depths in the water column: in the upper layer (5 m), thermocline (12 m) and lower, anoxic layer (28 m). In these samples aliphatic hydrocarbons (CH), aliphatic alcohols (COH), sterols (ST) and fatty acids (FA) were determined after extraction and derivatization by GC-FID analysis and identify by GC-MS. Stable isotope analysis were obtained using GC-C-IRMS. The abundance of lipid biomarkers in trap material was two to four times higher than in POM indicating that OM was extensively reprocessed by microbes during sinking which was more intensive under thermocline. Fatty acids were more abundant in POM, while in trap material the contribution of *n*-alkanes to the particulate organic carbon (POC) was larger than that of FAs. Autochthonous lipid material accounted for the major part of POM and trap material, supported by $\delta^{13}C$ values, the prevalence of shorter chain saturated *n*-alkenes and *n*-alcohols from *n*-C_{14} to *n*-C_{19}, saturated and unsaturated C_{27} sterols and shorter chain saturated and unsaturated FAs from *n*-C_{14} to *n*-C_{19}. Zooplankton left a marked imprint on particulate lipids and trap material at 12 m by predominance of *n*-$C_{18:0}$ over *n*-$C_{16:0}$ FA, short-chain, even-carbon *n*-alkenols, the high proportion of cholest-5-en-3β-ol (44.8% of total sterol concentration (TST)), cholesterol/phytosterol ratio of 0.49 and $\delta^{15}N_{PN}$ (isotopic composition of particulate nitrogen) values of 6.8 and 11.7‰. The distribution and composition of fatty acids were influenced by three processes: (1) algal production in the oxic epilimnion; (2) preferential degradation of labile algal components and presence of zooplankton in metalimnion and (3) production of bacterial fatty acids in the anoxic hypolimnion. The lowest $\delta^{13}C$ value of −51.7‰ was observed in 18:1*n*-7 FA in trap material at 28 m, which was the only FA that could be linked to methanotrophic bacteria contributing 58%. It was shown that lacustrine phytoplankton biosynthesize 24-ethylcholest-5-en-3β-ol, which is often used as a marker of terrigenous organic matter. Phytoplankton represented an important source of cholest-5-en-3β-ol and 24-methylcholest-5,22(E)-dien-3β-ol, while 24-ethylcholesta-5,22E-dien-3β-ol and 24-methylcholest-5-en-3β-ol were of terrestrial origin. Low stanol/stenol ratios determined in the epilimnion are typical for plankton. A marked increase was observed in 24-ethyl-5α-cholestan-3β-ol/24-ethylcholest-5-en3β-ol ratio to 1.8 at the depth of 28 m in trap material. Such high stanol/stenol ratios usually

appear in anoxic zones, which represent an ideal environment for production of Δ-stenols to 5α-stanols. 24-ethyl-5α-cholestan-3β-ol was the most ^{13}C-depleted sterols with $\delta^{13}C$ value of −58.0‰ indicating bacterial origin.

Part of this work was also presented and published at scientific conference; 20th International Symposium on Environmental Biogeochemistry (ISEB); September 27-30 2011, Istanbul, Turkey and 9th International Symposium on Environmental Geochemistry (ISEG); July 15-21 2012, Aveiro, Portugal.

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Lipid Biomarkers of Suspended Particulate Organic Matter in Lake Bled (NW Slovenia)

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The fatty acid, hydrocarbon and sterol composition were used to investigate the organic sources and compositional changes in particulate organic matter (POM) and trap material in the water column in the deepest part of Lake Bled (NW Slovenia) in October 2006. Fatty acids distribution of algal origin were abundant in particles and trap material in epilimnion, while in anoxic zone POM and trap material were enriched in bacterial fatty acids (e.g. 16:1*n-7*, 18:1*n-7*, 18:1*n-9*, *iso*-C_{14}-C_{16} and *anteiso*-C_{15}). The lowest $\delta^{13}C$ value of −51.7‰ was observed in 18:1*n-7* FA in trap material and was the only FA which could be linked to methanotrophic bacteria. In addition Zooplankton left a marked imprint on particulate lipids and trap material at 12 m by predominance of *n*-$C_{18:0}$ over *n*-$C_{16:0}$ FA, short-chain, even-carbon *n*-alkenols, the high proportion of cholest-5-en-3β-ol (44.8% of total sterol concentration (TST)), cholesterol/phytosterol ratio of 0.49 and $\delta^{15}N_{PN}$ values of 6.8 and 11.7‰. It was shown that lacustrine phytoplankton biosynthesize 24-ethylcholest-5-en-3β-ol which is often used as a marker of terrigenous organic matter. Phytoplankton represented an important source of cholest-5-en-3β-ol and 24-methylcholest-5,22(E)-dien-3β-ol, while 24-ethylcholesta-5,22E-dien-3β-ol and 24-methylcholest-5-en-3β-ol were of terrestrial origin. There was an evidence of microbial transformation of Δ^5-stenols to 5α(H)-stanols in POM and trap material in hypolimnion.

Keywords: lake, lipids, POM, stable isotopes

Introduction

Research on biogeochemistry of organic matter in stratified lacustrine basins is important in predicting the sources, deposition, transformation and preservation of organic matter which reflect and influence the trophic status of the lake (eutrophication). Stratified water columns of lakes support multiple layers of biological activity fueled by oxygenic photosynthesis, anoxygenic photosynthesis, chemolithotrophy and heterotrophy. These diverse biological zones in turn generate geochemical stratification in which reduced (sulfide, ammonium, reduced metals, organic matter) and oxidized (oxygen, nitrate, metal oxides) chemical species coexist in opposing concentration gradients. The chemical composition of organic matter produced in these biogeochemical zones reflects the planktonic and microbial sources and recycling processes. Organic matter (OM) cycling in the water column determines the chemical and biological character of underlying sediments, with the sediments recording environmental changes that take place over time (Wakeham 1999).

Within this complex environment, mixtures of lipid biomarkers are formed. Many of these biomarkers provide a unique insight into the diagenetic alteration, deposition, preservation and sources of organic matter and, in particular, the microbial community structure present in lacustrine environments. Biosynthetic pathways utilized by living organisms are diverse and, as a result, distributions of biomarkers vary between different types of organisms. For example, the domain archaea is characterized by membrane lipids based on isoprenoid ether (glycerol dialkyl glycerol tetraether; GDGT) structures, whereas acyl-based (fatty acid, acylglycerol) structures are found in the domains bacteria and eukarya. Hopanoids are considered as biomarkers for bacteria (including cyanobacteria), while all eukaryotes make sterols (Killops and Killops 2005).

In general, chain lengths of hydrocarbons, fatty acids and alcohols characterize the source of lipid matter. For example, the presence of C_{27}, C_{29} and C_{31} *n*-alkanes indicates that terrigenous plants have been important source of lipids (Cranwell

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1973; Eglinton and Hamilton 1967; Rieley et al. 1991). On the other hand algal contribution are indicated by the presence of *n*-C_{17} and *n*-alkanes between C_{15}-C_{21} (Blumer et al. 1971; Clark et al. 1967) and also by the short-chain length range between C_{14}-C_{18} of *n*-alkanoic acids with a typical even over odd predominance (Cranwell et al. 1987).

Long-chain *n*-alkanoic acids, such as C_{24}, C_{26} and C_{28}, are the major components of flowers, waxy coatings of terrestrial plant leaves and pollen (Rieley et al. 1991). Despite this general finding, some deviations from individual species are know, for example higher plants contain more abundant C_{16} and C_{18} *n*-alkanoic acids than long-chain *n*-acids except in the wax coating. Furthermore the higher susceptibility of lipids derived from algal material to degradation in comparison with land-derived OM which has been microbially reworked before transported into the lake is of general character (Meyers 1997; Meyers and Ishiwatari 1993).

Studies of settling particles indicated that fatty acids decomposition rates are 10 times higher for *n*-C_{16} comparing to *n*-C_{30} (Meyers and Eadie 1993). The stable carbon isotope composition of lipids allows a more detailed reconstruction of terrestrial, algal and microbial sources of these compounds. Introduction of compound specific ^{13}C analysis as a tool to include microbial communities has only recently begun (Freeman et al. 1990). Isotope analysis of biomarkers provides the possibility to link directly microbial identity (biomarker), biomass (concentration of the biomarker) and activity (isotope assimilation) (Boschker and Middelburg 2002).

Most research on particulate organic material (POM) relates to marine environments and less is known about lacustrine ecosystems. Several studies in marine systems combine biomarker analysis with a stable isotope approach (Pancost et al. 1997; 1999; Tolosa et al. 2003; 2004; 2008; Wakeham et al. 2003, 2007). A few studies have been undertaken to study variability and transformation processes of molecular organic matter from the surface water to the sediment, using traps or POM in lacustrine environments (Beehtel and Schubert 2009; Meyers and Eadie 1993; Parrish et al. 1992; Russell and Rosell-Melé 2005), while studies of stable carbon isotope composition of specific biomarkers in POM and trap material are limited (Hartgers et al. 2000).

In our study we used molecular and stable carbon isotope ratios of specific lipid biomarkers to evaluate their sources and to explore variations in the biogeochemistry of the POM and sediment trap material positioned at three depths throughout the water column at the deepest part of the subalpine Lake Bled, NW Slovenia.

Materials and Methods

Study Site and Sample Collection

Lake Bled is an urban dimictic subalpine lake situated at an elevation of 475 m above sea level in the north-west part of Slovenia (Figure 1).The lake has a surface area of 1.44 km^2 (volume 26×10^6 m^3, max. depth 30.1 m, mean depth 17.9 m) and can be divided into two basins separated by an island—a deeper western basin (depth of 30 m), and a 24 m deep eastern basin. The surficial inflows are two small streams, Mišca and Solznik, in the western basin, while the water outflow proceeds through Jezernica into the river Sava. The lake is stratified with an anoxic hypolimnion most of the year, with a temperature range between 4 and 6°C. In the shallower parts, oxidizing conditions in the whole water column prevail.

Two amelioration projects were previously undertaken: introduction of a fresh water inflow from the river Radovna, and pumping of anoxic water from the eastern basin into Jezernica. In addition, Bled's sewage system was renovated in 1985, resulting in decreased inflow of wastewater into the lake. All these improvements have contributed to the increase of water quality in the lake and Lake Bled is now classified as a mesotrophic lake according to OECD criteria (OECD 1982). In addition, the residence time in the lake was shortened from

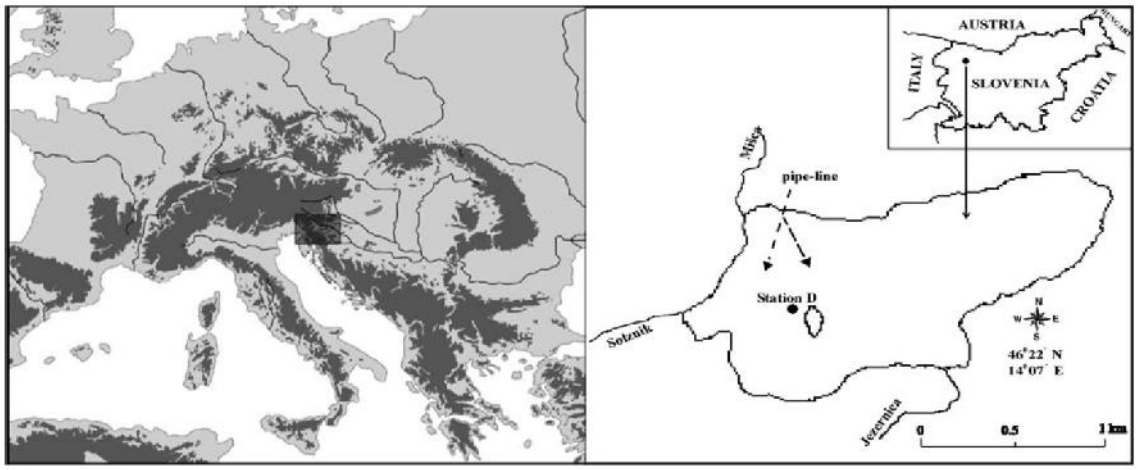

Fig. 1. Map showing the location of Lake Bled, SW Slovenia with sampling site D in the West Basin.

3.6 years to 1.5 years. The predominant phytoplankton community in the water column is cyanobacteria (*Cyanophyta*) for most of the year, except in spring when golden algae (*Chrysophyta*) are the most abundant.

The deepest part (station D; Figure 1) of the lake the depth of the water column is 30.5 m—was selected as a study site. Profiles of temperature, pH, and concentration of dissolved oxygen, conductivity and redox potential were measured with a CTD Hydrolab H2O probe. Settling particulate material was collected in sediment traps deployed in the water column in October 2006.

Material was obtained from three depths: in the upper part (5 m), in the thermocline (12 m), and in the lower, anoxic part of the water column (28 m) for determination of particulate organic matter. The traps consisted of two cylinders, each 50 cm long by 10 cm diameter, with a 500 ml sample collection bottle at the bottom of each trap. Traps were retrieved after 14 days of deployment. At the same time water samples were collected at 12 and 28 m with a Van Dorn sampler, transferred into pre-cleaned plastic bottles and stored at 4°C during transport to the laboratory within the next 3 h. Aliquots for determining alkalinity were stored in acid washed HDPE bottles without pre-treatment, while samples for dissolved organic carbon (DOC) analysis were filtered, acidified, and stored at 4° C. Samples for determining the isotope composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) were also taken. 6 ml water samples were introduced at the sampling site directly into gas-tight vials (VACUTAINER Septum Tubes, Labco Limited, UK) containing 100–200 μL of H_3PO_4. Vials were previously flushed in the laboratory with pure He to remove any air contamination.

Sedimentary material collection bottles and water samples were filtered in the laboratory through pre-combusted (480°C for 4 h) Whatman GF/F glass-fiber filters and dried at 40°C.

Water Analysis

Total alkalinity (TA) was determined within 24 h of sample collection by Gran titration with a precision of ±1%. Concentrations of DOC were measured with a Shimadzu TOC-5000 instrument with an analytical precision of ±2%. $\delta^{13}C_{DIC}$ values were determined directly from the headspace by Isotope Ratio Mass Spectrometry (IRMS; Europa Scientific 20 20) with an ANCA-TG preparation module for trace gas samples, equipped with a Gilson autosampler. In order to determine the optimal extraction procedure for water samples, a standard Na_2CO_3 solution was prepared with a known $\delta^{13}C$ value of 10.8 ± 0.2‰.

Solid Phase Analysis

The concentration and isotope composition of particulate organic carbon (POC) and total particulate nitrogen (PN) were determined on filters. Samples used for determining POC concentration and isotope composition were acidified with 1M HCl to remove carbonate minerals, then dried. Concentrations of POC and PN were measured with a Carlo Erba EA1108 elemental analyzer at a combustion temperature of 1020°C. The precision of measurements was ±3%. The isotope composition of POC ($\delta^{13}C_{POC}$) and PN ($\delta^{15}N_{PN}$) was determined on an IRMS Europa 20 20 with a solid-liquid preparation module ANCA-SL. Analyses were calibrated against reference material - IAEA-CH6 and IAEA-CH7 for carbon and IAEA–N1, IAEA-N2 and IAEA-NO-3 for nitrogen. All stable isotope results are reported using conventional delta (δ) notation in per ml (‰) relative to the VPDB standard ($\delta^{13}C$) or AIR ($\delta^{15}N$). The precision of measurements was usually ±0.2‰ for $\delta^{13}C_{POC}$ and $\delta^{13}C_{DIC}$ and ±0.3‰ for $\delta^{15}N_{PN}$.

Lipid Extraction and Fractionation

All solvents and reagents (JT Baker, Mallinckrodt Baker) were of analytical grade or higher. Dry particulate organic matter on filters was weighed into cellulose extraction thimbles followed by the extraction procedure described by Muri et al. (2004). Thimbles were extracted with dichloromethane for 8 10 h in a Soxhlet extractor. The extracts were concentrated in a rotary evaporator, the solvent exchanged to hexane, then evaporated to 1 mL under a stream of dry nitrogen gas. These extracts were fractionated on a glass column packed with 5% deactivated silica (Sigma-Aldrich, 70–230 mesh) and eluted with various solvents.

Aliphatic hydrocarbons, aliphatic alcohols, sterols and polar fatty acids were eluted successively with 25 mL hexane, 20 mL 15% ethyl acetate in hexane, 20 mL 20% ethyl acetate in hexane and 20 mL MeOH, respectively. Elemental sulfur was removed from the aliphatic hydrocarbon using activated copper. Aliphatic alcohols and sterols were isolated as non-esterified compounds, derivatized using BSTFA [bis(trimethylsilyl)trifluoroacetamide] and pyridine to yield trimethylsilyl (TMS) ethers. Polar lipids were saponified with 0.5 M KOH in MeOH and the fatty acids thus released methylated using BF_3-methanol to yield fatty acid methyl esters (FAME). All fractions were dried in a stream of N_2 and the residue dissolved in *iso*-octane.

Gas Chromatography

Lipids (aliphatic hydrocarbons, aliphatic alcohols, sterols and polar fatty acids) were analyzed using an Agilent model Hewlett-Packard 6890N gas chromatograph fitted with a DB 1ms capillary column (60 m, 0.32 mm i.d., 0.25 μm) and an FID detector. Helium was used as a carrier gas with a flow rate of 1.5 mL/min. The injection volume was 1 μL. The temperature of injector was set at 250°C and temperature of detector was set at 280°C.

Component identifications were performed with a Hewlett-Packard 6890 GC coupled to an MSD mass spectrometer using a DB-5ms (30 m×0.25 mm i.d., 0.25 μm) capillary column. The oven temperature program for hydrocarbon analysis was started at 40°C, followed by heating to 90 140°C at 10°C/min and rapid heating up to 320°C at 4°C/min with a final holding time of 30 min. The oven temperature program for alcohols, sterols and fatty acids (FA) was programmed from 70 to 130°C at 10°C/min and subsequently to 300°C at 4°C/min and a final holding time of 15 min. The MSD operated in scan mode,

covering a range of 50 to 500 Dalton for sterols, FA and alcohols; for hydrocarbon analysis we used SIM mode. Peak identity was confirmed using GC with mass spectrometer detection (GC-MS).

Internal standards were used for quantification, with 5α-cholestane as standard for aliphatic hydrocarbons, aliphatic alcohols and sterols, and C_{19} FAME for FA. The precision of the method was 5–10%. Concentrations of the selected lipid biomarkers were normalized to the POC.

Compound-Specific Isotope Analysis

Compound specific stable isotope ratios were determined using an Isoprime GV GC/C/IRMS system. The gas chromatograph was fitted with a DB 1ms capillary column (60 m, 0.32 mm i.d., 0.25 μm) and used helium as carrier gas. Samples dissolved in isooctane were injected at 120°C, and the oven was programmed to 300°C at 3°C/min, followed by an isothermal hold for 20 min. $\delta^{13}C$ values were determined by duplicate analyses and averaged, with errors of about 0.4‰. Corrections for the isotope change introduced in the derivatization of sterols and FA were determined through derivatization of standards of known isotope composition and applying the equation of Jones et al. (1991).

Results and Discussion

Physical and Chemical Characterization of the Water Column

The vertical distributions of temperature (T), dissolved oxygen (DO), DOC, TA and $\delta^{13}C_{DIC}$ values are presented in Figure 2. During the study the lake was stratified with the thermocline at 12 m. In the hypolimnion the temperature was 6.1°C. Concentrations of DO were fairly constant above the thermocline (10.6 mg/L) and reached a maximum value at a depth of 12 m (11.5 mg/L). They then dropped steadily, until reaching a value of <0.2 mg/L at 28 m, where anaerobic processes occurred. At this depth anoxic conditions are present throughout the year (Remec-Rekar and Bat 2007). Anoxic conditions were further reflected in enhanced alkalinity (4.87 mEq/l) and a low $\delta^{13}C_{DIC}$ value of −9.9‰, probably as a result of greater OM respiration. DOC concentrations varied between 1.3 and 2.2 mg C/l.

Phytoplankton in Lake Bled during the sampling was composed mainly of cyanobacteria (up to 75% of total phytoplankton biomass) followed by dinoflagellates (10%), diatoms (7%), golden algae (crysophyte) (6%), and green algae (2%) present at the lake surface (Remec-Rekar and Bat 2007). Zooplankton represented only 1% of total plankton biomass in the W basin of Lake Bled composed mainly by *Daphnia hyalina in Daphnia galeata* and their hybrids. The highest amount of zooplankton was determined at the depth of 12 m.

Solid Phase Analysis

The C/N ratios in all samples were less than 10.2, indicating the prevalence of autochthonous POM. The $\delta^{13}C_{POC}$ and $\delta^{15}N_{PN}$ of trap material ranged between −34.9‰ and −31.9‰ and between 1.7‰ and 6.8‰, respectively. The $\delta^{13}C_{POC}$ and $\delta^{15}N_{PN}$ values in suspended material were −32.0‰ and 11.7‰ at a depth of 12 m and −32.5‰ and 3.4‰ at 28 m. The

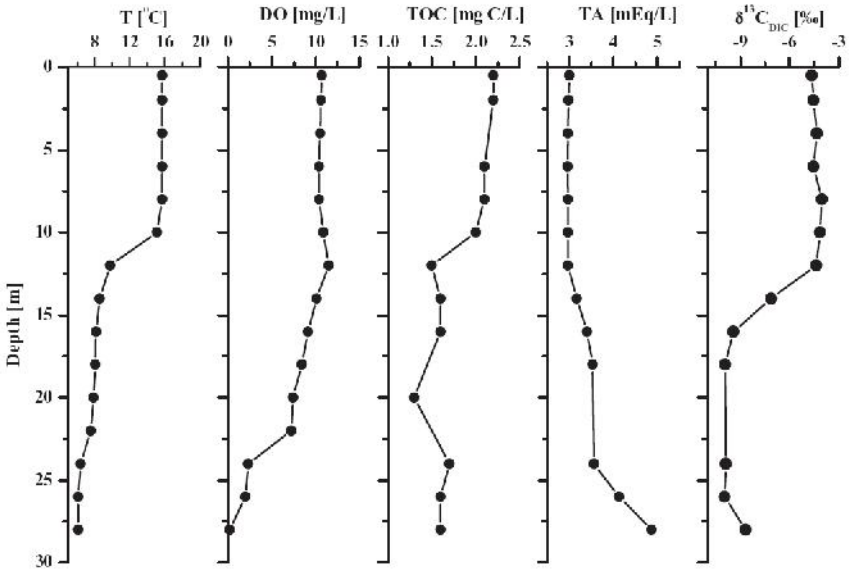

Fig. 2. The vertical distribution of temperature (T), dissolved oxygen (DO), concentrations of dissolved organic carbon (DOC), total alkalinity (TA) and isotope composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) at sampling site D in Lake Bled in October 2006.

lowest values were found at the bottom of the water column. The $\delta^{13}C$ value of −27.7‰ for phytoplankton determined in Lake Bled (Čermelj et al. 1996) was still higher than that for $\delta^{13}C_{POC}$ determined in the epilimnion. The phytoplankton studies from other lakes indicated that a substantial difference was observed in $\delta^{13}C$ values in phytoplankton, depending on phytoplankton taxa, season and lake. They ranged between −34.4‰ and −5.9‰ in mesotrophic and eutrophic southwest Finnish lakes (Vuorio et al. 2006).

Low $\delta^{13}C$ values for diatoms have also been reported from Loch Ness in autumn (−31.2‰, Jones et al. 1998) and from Lake Kinneret in winter (−32.0‰, Zohary et al. 1994). As expected, a significant shift in $\delta^{13}C_{POC}$ values was observed as POM sank, indicating preferential losses of carbohydrates and proteins during remineralization. Low $\delta^{13}C_{POC}$ values determined in trap and suspended materials at the bottom of the water column could also reflect the contribution of methanotrophs. Biomass originating from methanotrophic bacteria has very low $\delta^{13}C$ values, because the CO_2 utilized by bacteria is strongly depleted in ^{13}C following CH_4 oxidation (−70 ± 15‰) (Lehmann et al. 2002).

The bacterial origin of POM in the hypolimnion is additionally supported by low $\delta^{15}N_{PN}$, with a minimum value of 1.7‰ measured in trap material. Our $\delta^{15}N_{PN}$ values, ranging from 1.7 to 11.7‰, were well within the range reported by Vuorio et al. (2006) for diatoms (from −2.1‰ to +12.8‰) but higher than values reported for cyanobacteria (from −2.1‰ to −1.6‰) in southwest Finnish lakes. The highest $\delta^{15}N_{PN}$ value of 11.7‰ was observed in POM at a depth of 12 m. This may be attributed to microzooplankton grazing, release of nitrogen compounds during degradation, or regeneration of nitrogen in the euphotic layer. The composition of the lipid fraction from the sediment trap and from suspended particulate material provided more detailed information about the sources of OM and degree of alteration.

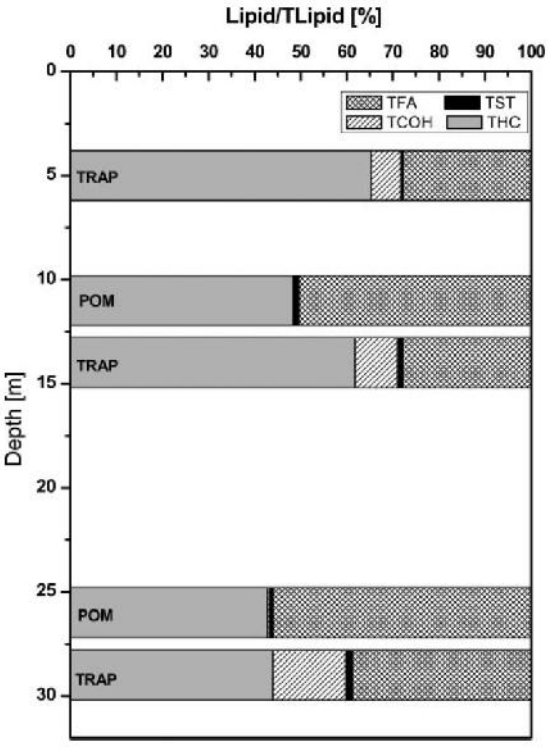

Fig. 3. Relative distribution of selected lipids expressed as the ratio between selected lipids and total lipids (Lipid/TLipid) in POM and in trap material from the water column of Lake Bled. Total sterols (TST), total aliphatic alcohols (TCOH), total aliphatic hydrocarbons (THC) and total fatty acids (TFA).

Lipid Composition

The dominant lipids were carboxylic acids (FA), *n*-alkanes, *n*-alkenols and sterols. The amount of lipid components associated with particles decreased with increasing depth more than their POC content. The contribution of total FA was greatest in POM, while in trap material, the contribution of the *n*-alkanes to the POC is larger than that of FA (Figure 3) probably due to higher sensitivity of FA to degradation processes. Cranwell (1981) and Hoefs et al. (2002) reported that *n*-alkanes are being the most resistant compounds in anoxic lacustrine environments as well as in oxic marine sediments. These compounds lack the oxygen-containing functional groups and the double bonds that are susceptible to microbial attack. A similar distribution of lipid compounds was also observed in Lake Michigan (Meyers and Eadie 1993), yet in Lake Baikal (Russell and Rosell-Melé 2005). FAs were more abundant in trap material. The largest amount of lipid components was observed at the thermocline in trap material at the depth of 12 m.

Carboxylic Acids

The total FA concentrations in POM were 5.4 and 3.6 mg/g TOC at depths of 12 and 28 m, respectively comparable with data obtained in Lake Lugano in autumn (Bechtel and Schubert 2009) (Table 1). Total FA concentrations in trap material ranged from 4.9 to 10.3 mg/g TOC and were in the same range as results obtained for Lake Baikal (Russell and Rosell-Melé 2005), but higher than those in POM and trap material obtained in oligotrophic Lake Michigan (Meyers and Eadie 1993). The distribution of fatty acids at 5 m in trap material gives an indication as to likely sources of the OM (Figure 4). All samples showed an *n*-alkanoic FA distribution from C_{12} to C_{26}, with a strong even/odd preference and maximum at *n*-C_{16} and *n*-C_{18} (Table 2).

This distribution pattern indicated a predominance of authochthonous OM (Cranwell et al. 1987; Stefanova and Disnar 2000). The detailed analysis of the *n*-alkanoic FAs' distribution in trap material and POM are presented in Table 2. *n*-C_{18} saturated and monounsaturated compounds were the most abundant alkanoic acid with the dominance of 18:0, reflecting zooplankton, algae and cyanobacteria

Table 1. Dominant lipid characteristics of POM and trap material from Lake Bled during autumn

Sample	Total FAs [μg/g TOC]	Total *n*-alkanes [μg/g TOC]	Total *n*-alcohols [μg/g TOC]	Total sterols [μg/g TOC]	Odd/even n-alkanes
Trap material					
5 m	4909	11550	1123	90	1.4
12 m	10430	23129	3469	423	1.5
28 m	7533	8550	3063	288	1.7
POM					
12 m	5449	13383		371	0.7
28 m	3602	6007	72	95	7.4

contributions. The highest proportion of 18:0 comparing to 16:0 fatty acid was observed on POM at 12 m suggesting a greater contribution of zooplankton grazing at this depth in autumn. This indication was further supported by the highest $\delta^{15}N_{PN}$ value of 11.7‰ determined in POM. Fatty acid 16:0 has also been found at a higher abundance; however it is difficult to assign its origin. It can derive from algae (Cranwell et al. 1987), but it is also found in higher plants (Rieley et al. 1991), bacteria (Ueki and Suto 1979) and fungi (Weete 1976).

The highest content determined in the trap material at 5 m was consistent with the presence of diatoms in the photic zone. It is known that diatoms are dominated by 14:0, 16:0 and 16:1*n*−9 (Volkman and Hallegraeff 1988) fatty acids. All these alkanoic acids were present in all samples, but mainly at 12 m in trap material. The $\delta^{13}C$ values of 16:0 ranged from −24.9 to −24.9‰ at all three depths in trap material showing that the source of this FA is not changing with depth. The $\delta^{13}C$ values of 18:0 were more variable ranging from −30.6 to −26.4‰ with the lowest value determined at the depth of 28 m (Table 3).

The relative proportions of mono-unsaturated *n*-FAs in POM and trap material increased with depth. Mono-unsaturated FAs (16:1 and 18:1; (*n*-7) and (*n*-9) isomers) contributed up to 19.2% to the total FAs. The fatty acid 18:1

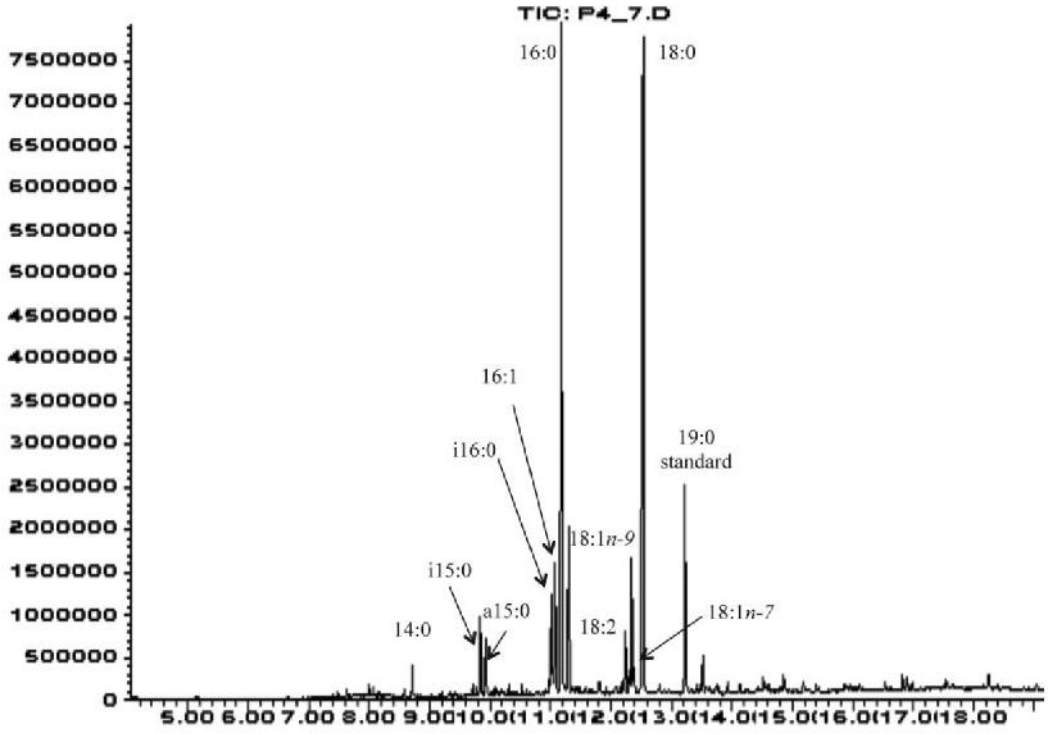

Fig. 4. Partial capillary gas chromatogram of free fatty acids (methyl esters) at 5 m in trap material at the station D in the deepest part of Lake Bled in October 2006. The nomenclature used is number of carbon atoms: number of double bonds.

Table 2. Relative percentage composition of fatty acid distribution in POM and trap material in Lake Bled

	Trap material			POM	
Fatty acid	0 5 m	12 m	28 m	12 m	28 m
Straight-chain saturates					
12:0	1.2	12.3	1.9	1.7	1.3
14:0	2.1	27.4	0.4	1.4	2.1
16:0	36.6	12.5	26.8	28.9	29.3
17:0	TR	0.3	TR	TR	TR
18:0	36.8	18.7	31.9	43.1	39.7
23:0	0.3	0.2	0.5	0.3	0.4
24:0	0.9	0.8	0.4	0.7	1.0
25:0	0.2	0.3	0.3	0.4	0.1
26:0	1.1	0.3	0.4	1.3	1.0
total:	79.1	72.9	62.8	77.9	74.9
Branched-chain saturates					
iso 14:0	3.2	0.9	1.9	5.7	0.8
iso 15:0	0.4	0.6	1.1	0.4	0.5
anteiso 15:0	0.2	0.5	1.0	0.2	0.4
iso 16:0	0.7	2.8	6.8	2.3	7.4
anteiso 17:0	TR	0.6	TR	0.3	0.2
total:	4.4	5.4	10.8	8.9	9.3
Monosaturates					
16:1*n-7*	5.0	2.0	15.0	7.6	7.1
18:1*n-9*	5.6	9.3	2.3	2.6	3.8
18:1*n-7*	1.1	1.7	1.9	0.7	0.7
total:	11.7	13.0	19.2	10.9	11.7
Polysaturates					
18:2	1.0	1.1	1.0	0.5	0.3
20:1	0.6	1.0	1.1	0.8	0.8
22:1	0.3	0.9	0.6	0.2	0.5
26:1	1.1	0.3	0.4	1.3	1.0
Total:	2.9	3.3	3.1	2.9	2.6
18:1*n-7*/18:1*n-9*	0.21	0.19	0.83	0.25	0.19
Branched-chain/ polyunsat. FAs	4.61	4.97	10.4	16.8	26.9

TR: trace (less than 0.2%).

Table 3. Stable isotope compositions of selected biomarkers in trap material.

	$\delta^{13}C$ [‰]		
Compound	5 m	12 m	28 m
Fatty acid			
iso-15:0	−37.3	−27.6	−42.2
15:0	−22.4	−29.3	−28.8
16:0	−24.2	−24.5	−24.9
18:1*n-7*	−30.9 (0.08)	−31.0 (0.08)	−51.7 (0.58)
18:0	−26.4	−28.0	−30.6
pristane	−28.1	−29.5	−24.9
n-alkanes (C_{15} - C_{22})	from −26.2 to −32.8	from −28.1 to −33.3	from −24.1 to −30.4
n-alkanes (C_{22} C_{28})	from −25.2 to −35.8	from −24.1 to −32.6	from −26.5 to −33.3

The contribution of methanotrophic bacteria to selected FAs is presented in brackets.

is generally either (*n-9*), which is common in animals, higher plants and algae or (*n-7*), which is particularly abundant in bacteria (Killops and Killops 2005). 16:1 fatty acid is also widely distributed and has similar differentiation between predominantly bacterially derived (*n-9*) and algally derived (*n-7*) isomers although 16:1*n-9* is a FA of diatoms. Monounsaturated C_{16}-FAs are also abundant in manganese-, iron- and sulfate-reducing bacteria (Wakeham et al. 2007).

Cyanobacteria produced different fatty acid distribution, some dominated by 16:0 and 16:1*n-7* and others containing high proportion of 18:1*n-9* (Killops and Killops 2005). Anaerobic photosynthetic bacteria often contain 16:1*n-7* and 18:1*n-7* as a major fatty acids and high concentrations of these compounds, especially 16:1*n-7*, was found in anoxic lakes (Cranwell et al. 1987; Fredrickson et al. 1986; Volkman et al. 1998) and the Black Sea (Wakeham and Beier 1990) and also in our study in hypolimnion in trap material (Table 2).

It is interesting to note that 18:1*n-9* was the major isomer in all samples and not 18:1*n-7*, which could be expected to predominate in bacteria using anaerobic biosynthetic pathway. Similar observation was also found in Ace Lake in Antarctica (Volkman et al. 1988) indicating that this acid was probably derived from heterotrophic or sulphate reducing bacteria. Higher proportion of 18:1*n-9* found at 12 m in trap material in our study could come from cyanobacteria. The increased ratios of branched in the water depth profiles indicated enhanced bacterial biomass and OM degradation.

The bacterial reworking of phytoplankton material can also be traced by the ratio of 18:1*n-7* to 18:1*n-9*, since the former is the major bacterial FA (Table 2). It was found that Type II methanotrophs are enriched in 18:1*n-7* and 18:1*n-8* (Niggemann and Schubert 2006; Wakeham et al. 1997). The higher contribution of bacterial biomass to the OM was confirmed by the low $\delta^{13}C_{POC}$ and $\delta^{15}N_{PN}$ values. Other FAs of bacterial origin present in all our samples were *iso*-acids in the range from C_{14} to C_{17} and *anteiso*-C_{15}, but at a lower abundance than C_{16} and C_{18} FAs.

The highest concentrations of these FAs whose sources could be related to cyanobacteria and sulfate-reducing bacteria (Wakeham et al. 2003) were observed in trap material at 28 m amounted 10.8% of the total FAs. Stable isotope compositions of FAs might aid in confirming bacterial sources, however $\delta^{13}C$ values of the major FAs were found to range from –51.7‰ to –22.4‰ (Table 3), with a pronounced depletion in the hypolimnion. The lowest $\delta^{13}C$ value of −51.7‰ was observed in 18:1*n-7* FA in trap material at 28 m, which was the only FA whose source could be assigned to methanotrophic bacteria, since the $\delta^{13}C$ of methane determined in the anoxic part of the lake was found to be around −70‰ (Lojen et al. 1999; Mandić-Mulec et al. 2012; Ogrinc et al. 2002). The contribution of methanotrophic bacteria to 18:1*n-7* was estimated using simple mass balance calculation using $\delta^{13}C$ value of −27.7‰ as a phytoplankton source (Čermelj et al. 1996), while $\delta^{13}C$ value of −70‰ was used as a source of methanotrophic bacteria. It was estimated that 58% of 18:1*n-7* is coming from bacteria (Table 3). The presence of methanotropic bacteria was also observed in surface sediments by the hopanoid distribution in the neutral lipid fraction, in which hop-17(21)-ene, with an average $\delta^{13}C$ value of −76.2 ± 5.1‰,

was the most abundant. The stable isotope composition of *iso*-C_{15} FAs ranged from −42.2‰ to −27.6‰, with the highest value observed in trap material at 12 m.

The culture experiments showed that *iso*- and *anteiso*-C_{15} FAs in sulfate-reducing bacteria are lower, by about 15‰, than their substrate (Mather et al. 1997). Thus our stable isotope data suggest that the isotope composition of organic substrates used by these bacteria is greater than −27.2‰, indicating that they were living either on isotopically enriched breakdown products (i.e., derived from sugars) or on terrestrial material or possibly algal cell material. Similar conclusions were drawn in monomictic Lake Cisó in Spain (Hartgers et al. 2000).

Neutral lipids

n-Alkanes. The hydrocarbon distribution in POM and trap material contained large proportions of shorter chain-length components (n-C_{16} – n-C_{20}), indicating their algal origin. The C_{17} n-alkane, which is produced by cyanobacteria and other eukaryotic algae, was present in all samples in POM and trap material, with the highest abundance found at 12 m in trap material, in parallel with TOC maxima (Figure 2). At this depth also the distribution of different n-alkanes was the most abundant. n-C_{18} and n-C_{20} were present in all samples, while the most abundant n-alkane was n-C_{21} in POM at 12 m.

Isoprenoid pristane was determined only in trap material, while phytane was present in all samples. Pristane is produced primarily in the digestive tracts of copepods, from phytol derived from the side chain of chlorophyll *a* (Shi et al. 2001). Phytane is produced by anaerobic microbial reprocessing of phytol (Risatti et al. 1984). These two isoprenoid n-alkanes has multiple sources including petroleum, however the isotopic composition of pristine (Table 3) indicated that pristine is mainly of phytoplankton origin. The contamination with petroleum in Lake Bled was also excluded in the study performed in sediments, since little if any UCM was found (Muri and Wakeham 2006).

The presence of both these derived hydrocarbons was further evidence of early diagenetic alteration of algal debris while sinking to the bottom of Lake Bled. The odd/even ratios for n-alkanes were significantly lower than those of FAs (Table 1). Furthermore, these ratios differ significantly from the leaf-wax values of 8 to 14 reported by Rieley et al. (1991); however, there are still greater than 1 except on POM at 5 m. These differences imply that the FAs have been synthesized more recently than the hydrocarbons. The most probable explanation is microbial production during particle settling. The bimodal distribution and the range of long chain length hydrocarbons observed in trap material at the depth of 12 m reflect the combination of autochthonous and allochthonous inputs (Ficken et al. 2000). The isotope composition of n-alkanes ranged from −35.8‰ to −24.1‰, reflecting the input of both autochthonous and allochthonous OM (Table 3). It was also not possible to identify possible sources based on the chain length of n-alkanes.

n-Alkanols and steroidal alcohols

Low concentrations of n-alkanols were observed in POM, while in trap material their total concentration ranged from 1.1 to 3.5 mg/g TOC. The proportion of n-alkanols to total lipid concentrations increased with depth, being around 15.8% at 26 m in trap material. The short chain n-alkanols (C_{13}-C_{20}) present in our samples are indicative of algal or bacterial inputs (Albro 1976; Weete 1976). The even/odd predominance in the C_{13}-C_{17} range supports the conclusion of *in situ* microbial synthesis (Cranwell 1980). The short chain n-alkanols of even carbon number (n-C_{14}, n-C_{16} and n-C_{18}), that are associated to zooplankton markers (Sargent et al. 1977), were more abundant in trap material at 12 m. Phytol was the only branched alkanol present in trap material at 12 and 28 m. Lower concentrations were found at 28 m depth, indicating its degradation under anoxic conditions.

Sterols originate from a variety of eukaryotic organisms, including phytoplankton, e.g., diatoms, coccolithophorids, dinoflagellates, zooplankton and vascular plants (Volkman 1986). C_{29} was assigned primarily to higher terrestrial plants while C_{27} is derived mainly from plankton. The source of C_{28} sterols is less specific, since they are relatively abundant in both algae and terrestrial higher plants (Volkman 1986). Sterols were present in highly variable concentrations in both the POM (145 and 44 μg/g TOC at depths of 12 and 28 m) and in the trap material (90, 424 and 288 μg/g TOC at depths of 5, 12 and 28 m) with the highest concentrations found in thermocline.

Of the sterols, C_{27} were dominant in POM samples and at 12 m in trap material, constituting up to 78% of the total sterol concentration (TST) (Table 4). Cholesterol (cholest-5-en-3β-ol) was the most abundant sterol in trap material at 12 m comprised 44.8% of TST and in POM (38% and 41.5% of TST at 12 m and 28 m, respectively), but somewhat less at 5 m and 28 m in trap material. 24-ethylcholest-5-en-3β-ol (β-sitosterol) was the most abundant C_{29} sterol representing up to 32.8% at the depth of 5 m in trap material (Table 4).

The highest proportion of cholest-5-en-3β-ol at 12 m could indicate an increased input from zooplankton grazing. This interpretation is consistent with the observed predominance of n-$C_{18:0}$ n-FA, the short chain of n-alkanols and $\delta^{15}N_{PN}$ values of 6.8‰ observed in this sample. If the zooplankton biosynthesize wax esters, then 20:1 and 22:1 FAs are present. These two FA were also found in our study (Table 2). However, a wide diversity of phytoplankton also contains cholest-5-en-3β-ol and in many species is the major sterol present (Volkman 1986; Volkman et al. 1998).

It is present in many diatoms and it appears to be a major sterol in most cyanobacteria although reliable quantitative data are lacking. Recent studies have shown that the absolute abundance of sterols in cyanobacteria is usually low and probably attributable to contamination (Summons et al. 2002). The cholesterol/phytosterols ratio was estimated to evaluate the ratio of the contribution of zooplankton to that of phytoplankton (Muhleback and Weber 1998; Tolosa et al. 2003) in all samples. Phytosterols were taken to include 24-methylcholesta-5,22(E)-dien-3β-ol, 24-ethylcholesta-5,22(E)-dien-3β-ol and 24-ethylcholesta-5-en-3β-ol (Tolosa et al., 2008). The highest ratio of 0.49 was obtained in trap material at 12 m indicating higher contribution coming from zooplankton.

Similar ratios of 0.14 and 0.17 were determined at 28 m in POM and trap material, while the lowest ratio of 0.05 was

Table 4. Relative percentage composition of sterol distribution in POM and trap material in Lake Bled

	Trap material			POM	
Sterols	0–5 m	12 m	28 m	12 m	28 m
27-nor-24-methylcholesta-5,22E-dien-3b-ol	22.5	5.3	6.7	17.1	6.9
5α-cholest-3α-ol	TR	TR	2.7	TR	TR
cholesta-5,22E-dien-3β-ol	18.0	6.2	5.0	12.0	2.3
5α(H)-cholest-22-en-3β-ol	TR	2.2	9.9	3.3	TR
cholest-5-en-3β-ol	5.1	44.8	17.4	38.0	41.5
5α(H)-cholestan-3β-ol	4.0	10.0	TR	TR	18.6
27-nor-24-methyl-5α(II)-cholestan-3β-ol	TR	3.2	TR	TR	11.5
C_{28} Δ^{22} stenol	TR	3.1	TR	TR	0.5
24-methylcholesta-5,22E-dien-3β-ol	8.3	3.5	4.7	10.2	TR
24-methylcholest-5-en-3β-ol	TR	1.2	TR	TR	TR
24-methyl-5α-cholestan-3β-ol	TR	1.4	4.7	TR	TR
24-ethyl-5β-cholestan-3β-ol	TR	1.2	10.6	TR	TR
24-ethylcholesta-5,22E-dien-3β-ol	TR	2.2	11.9	TR	11.5
24-ethylcholest-5-en-3β-ol	32.8	13.1	5.0	11.8	0.5
24-ethyl-5α(II)-cholestan-3β-ol			21.9		
C_{27}/TST	0.59	0.71	0.46	0.78	0.76
C_{28}/TST	0.08	0.11	0.05	0.10	0.12
C_{29}/TST	0.33	0.18	0.49	0.12	0.12
5α(H)stanols/Δ^5stenols	0.07	0.23	0.99	0.05	0.43

TR: trace (less than 0.2%).
TST – total sterol concentration.

observed in trap material at 5 m. On the other hand the diatoms and green algae could represent a potential source of cholest-5-en-3β-ol (5% of TST) and 24-ethylcholest-5-en-3β-ol (32.8% of TST) at 5m in trap material. Unfortunately the isotope composition of sterols was only determined in trap material at 28 m, where $\delta^{13}C$ value of cholest-5-en-3β-ol was found to be −38.3‰, while $\delta^{13}C$ value of 24-ethylcholest-5-en-3β-ol was −30.5‰. These results indicated that these two sterols originated from phytoplankton.

In general 24-ethylcholest-5-en-3β-ol has been reported to be a typical marker of inputs of higher plant and marsh grasses (Canuel et al. 1997), but may be partly derived from algae and cyanobacteria (Rontani and Volkman 2005; Volkman et al. 1999) as was also shown in our study. Other C_{27} sterols showed $\delta^{13}C$ values ranging from −29.8‰ to −26.5‰, reflecting a mixed origin for these hydrocarbons. In trap material at the depth of 28 m 5α(II)-cholestan-3α-ol (epicholestanol) was also present comprising 2.7% of TST. It seems that the anoxic conditions present in hypolimnion enabled anaerobic microbes to produce this uncommon C_{27} sterol. This sterol was also found in surface sediments in the anoxic part of Lake Leman, Switzerland (Mermoud et al. 1985).

The contribution of C_{29} sterols was the highest at the bottom of the water column and comprised 49% of the TST concentration in trap material. The major sterols found in higher plants (Goad and Goodwin 1972) are 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol) and 24-methylcholest-5-en-3β-ol (campesterol). The $\delta^{13}C$ values of these two sterols determined in our study were −25.8‰ and −27.0‰, respectively, indicating that indeed these two compounds are of terrestrial origin coming from higher plants. The presence of 24-ethyl-5β-cholestan-3β-ol (24-ethylcomprostanol) is often taken as evidence for the presence of faecal-derived organic matter from a variety of animals. This sterol was present at the depth of 12 m and 28 m in trap material.

Stanols were also present in our samples with the increase of stanol/stenol ratio with depth (Table 4). Low stanol/stenol ratios determined in the epilimnion are typical for plankton. A marked increase was observed in 24-ethyl-5α-cholestan-3β-ol/24-ethylcholest-5-en3β-ol ratio to 1.8 at the depth of 28 m in trap material. Such high stanol/stenol ratios were also common in the Black Sea (Wakeham 1989) and usually appear in anoxic zones, which represent an ideal environments for production of Δ-stenols to 5α(II)-stanols. 24-ethyl-5α-cholestan-3β-ol was the most ^{13}C-depleted sterols with $\delta^{13}C$ value of −58.0‰ indicating bacterial origin.

C_{28} sterols were the least abundant sterols in samples and were dominated by 24-methylcholest-5,22(E)-dien-3β-ol (either epibrassicasterol or brassicasterol) which is considered as a diatom biomarker (Brasell et al. 1982; Killops and Killops 2005). This sterol was most abundant in POM at 12 m. The isotope composition of 24-methylcholest-5,22(E)-dien-3β-ol was found to be −28.4‰ indicated a relatively constant microalgal source in Lake Bled and was comparable with the data reported by Neunlist et al. (2002).

Conclusions

Lipid biomarkers determined in particulate organic matter (POM) and in trap material have provided new insights into organic carbon cycling in Lake Bled and reflect complex

biogeochemical source and alteration processes. The abundance of lipid biomarkers in trap material was two to four times higher than in POM indicating that OM was extensively reprocessed by microbes during sinking which was more intensive under thermocline. Fatty acids were more abundant in POM, while in trap material the contribution of *n*-alkanes to the particulate organic carbon (POC) was larger than that of FAs.

Autochthonous lipid material accounted for the major part of POM and trap material, supported by $\delta^{13}C$ values, the prevalence of shorter chain saturated *n*-alkenes and *n*-alcohols from *n*-C_{14} to *n*-C_{19}, saturated and unsaturated C_{27} sterols and shorter chain saturated and unsaturated FAs from *n*-C_{14} to *n*-C_{19}. The predominance of $C_{18:0}$ *n*-FA, short chain, even carbon (*n*-C_{14}, *n*-C_{16} and *n*-C_{18}) *n*-alkenols, the high proportion of cholesterol, higher cholesterol/phytosterols ratio and $\delta^{15}N_{PN}$ values indicated zooplankton grazing at 12 m in POM and trap material. In the hypolimnion more active reprocessing induced by bacteria was observed. The distribution and composition of fatty acids were influenced by three processes: (1) algal production in the oxic epilimnion; (2) preferential degradation of labile algal components and presence of zooplankton in metalimnion and (3) production of bacterial fatty acids in the anoxic hypolimnion.

The lowest $\delta^{13}C$ value of −51.7‰ was observed in 18:1*n*-7 FA in trap material at 28 m, which was the only FA that could be linked to methanotrophic bacteria contributing 58%. Sterol distribution, reflect primarily a plankton source. It was suggested that much of 24-ethylcholest-5-en-3β-ol and cholest-5-en-3β-ol in trap material at 28 m might derived from phytoplankton and not higher plants and zooplankton, respectively. 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol) and 24-methylcholest-5-en-3β-ol (campesterol) were both of allochthonous origin coming from terrestrial plants, while 24-methylcholest-5,22(E)-dien-3β-ol the most abundant C_{28} sterol originated from constant microalgal sources.

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3.2 Scientific paper: »Methanogenesis pathways in a stratified eutrophic alpine lake (Lake Bled, Slovenia)«

In this chapter, the paper entitled “Methanogenesis pathways in a stratified eutrophic alpine lake (Lake Bled, Slovenia)” by Ines Mandić-Mulec, Katja Gorenc, Marinka Gams Petrišič, Jadran Faganeli and Nives Ogrinc is presented. The paper was published in Limnology and Oceanography (ASLO Publications).

Stratified lakes constitute a smaller source of atmospheric CO2 than oceans and estuaries, but are important as a source of CH_4, with activity localized in the anoxic hypolimnion and sediment. Recently studies suggested that CH_4 emissions from lakes and freshwater environments could substantially affect the global greenhouse gas balance. CH_4 is produced by strictly anaerobic Euryarchaeota (Liu and Whitman, 2008) and biogenic CH_4 production is the terminal step in the decomposition of organic matter in lacustrine systems. Methanogenesis involves primarily two pathways - H_2 oxidation coupled with CO_2 reduction (hydrogenotrophic methanogenesis) and acetate fermentation (acetoclastic methanogenesis). It is believed that acetoclastic methanogenesis dominate in lacustrine sediments, while hydrogenotrophic methanogenesis is considered to be typical of marine environments. However, exceptions are not uncommon and both processes may occur in both environments (Conrad 2005). It was recently suggested that the quality of organic substrates and temperature might control the prevalence of the two methanogenic pathways (Glissman et al. 2004; Canfield et al. 2005; Nozhevnikova et al. 2007). The aim of the published study was to evaluate the relative contribution of hydrogenotrophic and acetoclastic methanogenesis in the sediment profile of the dimictic eutrophic Lake Bled, using a combination of biogeochemical and molecular approaches. We tested whether the prevalence of hydrogenotrophic methane is reflected in the archaeal community composition determined by T-RFLP profiling and 16S rDNA cloning. In addition the quantity and quality of organic matter available for decomposition was estimated by lipid biomarker analysis. It was found that hydrogenotrophic formation of CH_4 prevailed in the lake sediment in both study periods. At the surface, the contribution of hydrogenotrophic methanogenesis was less pronounced, but it still accounted to more than 50% (51% in 2006 and 56% in 2007). Its contribution increased with depth accounting up to 90% at the depth of 15 cm. The higher contribution of acetotrophy observed at the surface compared to other sediment depths could be associated with the presence of more labile organic matter. The proportion of ‘‘fresh’’ autochthonous lipids in total extractable lipids in sediment decreased from 62% at the surface to 41% at a depth of 20 cm. The contribution of lipids of bacterial origin was more pronounced at the surface, comprising 13%. Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S ribosomal ribonucleic acid (rRNA) sequences of bacterial and archaeal community members that the majority of archaeal sequences belonged to Euryarchaeota. The methanogenic population accounted for 73% and 38%of the archaeal community at depths of 0–2 cm and 10–12 cm, respectively. In the upper 2 cm, hydrogenotrophs, mostly Methanomicrobiaceae, were dominant. In the deeper sediment, archaeal sequences were mostly those of unknown affiliation with Euryarchaeota, Thermoplasmatales, and related linkages, and only 21% of the hydrogenotrophic methanogenic archaea were detected. Somewhat lower percentages (18%) of sequences representing acetotrophic archaea (Methanosaetaceae) were present in the two layers. Biogeochemical and archaeal community analyses support the hypothesis that hydrogenotrophic methanogenesis is the dominant pathway of CH_4 production in sediment of the alpine Lake Bled, despite its low temperature and the prevalence of autochthonous, and therefore more labile, organic carbon. This study also showed that the processes and methanogenic archaeal communities were in many ways

similar to those in other mid-altitude lakes, and even to tropical lakes, indicating that temperature could not be the main factor controlling the methanogenetic pathway.

My contribution to this work was related to sampling, preparation and determination of the concentration of lipid biomarkers in sediments using GC-MS techniques. Further I actively participated in the evaluation of analysis. Part of this work was also presented and published at scientific conference; 11th Symposium on Aquatic Microbial Ecology, August 30 - September 4 2009, Piran, Slovenia, Goldschmidt 2010, June 13-18 2010, Knoxville, Tennessee and 20th International Symposium on Environmental Biogeochemistry (ISEB); September 27-30 2011, Istanbul, Turkey.

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Methanogenesis pathways in a stratified eutrophic alpine lake (Lake Bled, Slovenia)

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Abstract

The production pathway and carbon source of CH_4 in the surface sediment of a eutrophic alpine lake (Lake Bled, northwest Slovenia), in which the hypolimnion is anoxic for most of the year, were determined from molecular and biogeochemical studies. The average $\delta^{13}C_{CH_4}$ value of $-69.5‰ \pm 1.2‰$, associated with low acetate concentrations, suggested that CH_4 should be formed, predominantly, hydrogenotrophically. The proportion of "fresh" autochthonous lipids in total extractable lipids in sediment decreased from 62% at the surface to 41% at a depth of 20 cm. The contribution of lipids of bacterial origin was more pronounced at the surface, comprising 13%. Terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S ribosomal ribonucleic acid (rRNA) sequences of bacterial and archaeal community members suggested that larger sediment-depth dependent changes occurred in the latter. The majority of archaeal sequences belonged to *Euryarchaeota*. The methanogenic population accounted for 73% and 38% of the archaeal community at depths of 0–2 cm and 10–12 cm, respectively. In the upper 2 cm, hydrogenotrophs, mostly *Methanomicrobiaceae*, were dominant. In the deeper sediment, archaeal sequences were mostly those of unknown affiliation with *Euryarchaeota*, *Thermoplasmatales*, and related linkages, and only 21% of the hydrogenotrophic methanogenic archaea were detected. Somewhat lower percentages (< 18%) of sequences representing acetotrophic archaea (*Methanosaetaceae*) were present in the two layers. The biogeochemical processes and structure of the archaeal community support the hypothesis that hydrogenotrophic methanogenesis is the dominant pathway in the sediment of alpine Lake Bled, despite low temperature and the prevalence of "fresh" autochthonous-derived organic matter.

Although methane (CH_4) is the third most abundant greenhouse gas, it is approximately 25 times more effective than CO_2 (Denman et al. 2007). Stratified lakes constitute a smaller source of atmospheric CO_2 than oceans and estuaries, but they are important as a source of CH_4, with activity localized in the anoxic hypolimnion and sediment. Recently, some existing estimates of global CH_4 emissions have not taken small lakes into account, resulting in underestimates of total lake area by a factor of more than 2 (Walter et al. 2007), and according to Bastviken et al. (2011), CH_4 emissions from lakes and freshwater environments can substantially affect the global greenhouse gas balance.

CH_4 is produced by strictly anaerobic Euryarchaeota (Liu and Whitman 2008), and biogenic CH_4 production is the terminal step in the decomposition of organic matter in lacustrine systems. Methanogenesis involves primarily two pathways—H_2 oxidation coupled with CO_2 reduction (hydrogenotrophic methanogenesis) and acetate fermentation (acetoclastic methanogenesis). About two-thirds of the naturally produced CH_4 in anaerobic sediments appears to originate from acetoclastic methanogenesis (Oremland 1988), which is believed to dominate in lacustrine sediments, while hydrogenotrophic methanogenesis is considered to be typical of marine environments (Canfield et al. 2005). However, exceptions are not uncommon, and both processes may occur in both environments (Conrad 2005). It was recently suggested that the quality of organic substrates and temperature may control the prevalence of the two methanogenic pathways (Glissman et al. 2004; Canfield et al. 2005; Nozhevnikova et al. 2007), but only a few studies have addressed environmental drivers that influence these pathways in anoxic lacustrine sediments.

Acetate is considered to be the most important carbon intermediate in anaerobic systems and is produced by fermentation of organic matter and acetogenesis (Wu et al. 1997). In cold, deep stratified lakes, acetogens have been found to outcompete methanogens for H_2, and H_2:CO_2 was found to be converted to CH_4 by a two-step process, with initial formation of acetate by reduction of CO_2, followed by acetoclastic methanogenesis (Wand et al. 2006). Acetate fermentation also appears to be associated with the more labile autochthonous organic matter, whereas CO_2 reduction to CH_4 utilizes the more refractory allochthonous organic matter (Whiticar et al., 1986; Sugimoto and Wada 1993). Although lipids usually constitute only few percent of total organic matter (OM), their diversity and specificity make them useful compounds to distinguish the origin of OM (autochthonous vs. allochthonous). In the complex biogeochemical environment in lakes, mixtures of lipid "biomarkers" are biosynthesized. Biosynthetic pathways utilized by living organisms are diverse, and, as a result, distributions of biomarkers vary among types of organisms (Killops and Killops 2005). Chain lengths of hydrocarbons, fatty acids, and alcohols generally differ between aquatic (short-chains) and terrestrial (long-chain) plants. Furthermore, fatty acids are one of the few indicators of bacterial contribution to the OM and may also help to distinguish aquatic sources such as plankton. Ratios of longer-chain to shorter-chain lipids can be used to assess relative contribution of

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allochthonous vs. autochthonous components (Cranwell 1982; Meyers et al. 1984; Meyers 2003). Research over several decades has clearly demonstrated that biomarker distributions in stratified water columns and sediments reflect the sources of organic matter and, in particular, the microbial community structure in these environments.

Very few studies that address the origin of CH_4 in lake sediments combine process analysis with microbial community composition analysis (Zepp-Falz et al. 1999; Chan et al. 2005; Conrad et al. 2010). Molecular analysis of archaeal 16S ribosomal ribonucleic acid (rRNA) genes in the Swiss Lake Rotsee (Zepp-Falz et al. 1999) and in the German eutrophic Lake Dagow sediments (Glissman et al. 2004) indicated acetoclastic *Methanosaetaceae* and hydrogenotrophic *Methanomicrobiaceae* representatives, which was consistent with chemical process analysis suggesting that CH_4 originates from acetoclastic and hydrogenotrophic methanogenesis in lake sediments. However, in Lake Rotsee, hydrogenotrophic species dominated the upper sediment layer (Zepp-Falz et al. 1999), while in the upper layer of Lake Dagow sediment, the acetoclastic *Methanosaetaceae* dominated (Chan et al. 2005). In tropical freshwater sediments, the methanogenic community consisted of acetotrophic *Methanosaetaceae* and hydrogenotrophic *Methanomicrobiales*, *Methanobacteriales*, and *Methanocellales*. Chemical analysis suggested that CH_4 originates mostly from the hydrogenotrophic pathway and that acetate may also be consumed by acetotrophic fermenting bacteria (Conrad et al. 2010). Only hydrogenotrophic methanogens (*Methanomicrobiaceae and Methanobacteriaceae*) were found in sediments of Lake Kinneret (Israel), and the syntrophic association with acetate-oxidizers performing hydrogenotrophic methanogenesis was also proposed for these sediments (Nüsslein et al. 2001).

Previous studies of CH_4 in anoxic sediments of stratified eutrophic lakes in the Julian Alps (W Slovenia), Lake Planina (Ogrinc et al. 2008), and Lake Bled (Ogrinc et al. 2002) suggested the prevalence of hydrogenotrophic methanogenesis and indicated low pore-water acetate concentrations (Lojen et al. 1999). Alpine Lake Bled (NW Slovenia), with its anoxic hypolimnion and cold temperatures persisting for most of the year (Ogorelec et al. 2006), could therefore provide ecological information about the controls of biogenic methane production in lakes. The aim of the current study was to evaluate the relative contribution of hydrogenotrophic and acetoclastic methanogenesis in the sediment profile of the dimictic eutrophic Lake Bled, using a combination of biogeochemical and molecular approaches. We tested whether the prevalence of hydrogenotrophic methane is reflected in the archaeal community composition as determined by terminal restriction fragment length polymorphism (T-RFLP) profiling and 16S ribosomal deoxyribonucleic acid (rDNA) cloning. In addition the quantity and quality of organic matter available for decomposition were estimated by lipid biomarker analysis.

Methods

Study site and sampling—Lake Bled (surface area 1.44 km^2, volume 26×10^6 m^3, maximum depth 30.5 m, mean depth 17.9 m) is a eutrophic, dimictic alpine lake, formed in the Würm glacial period and located in northwest Slovenia (Fig. 1). The surficial inflows are two small streams, Mišca and Solznik, in the western basin, while the water outflow proceeds through the Jezernica River into the Sava River. The lake is stratified, with an anoxic hypolimnion, except during early spring. The bottom temperature is stable (4–6°C) in the anoxic hypolimnion. In the shallower regions, oxidizing conditions prevail throughout the water column. Two amelioration projects have been carried out: freshwater inflow was diverted from the Radovna River in the early 1960s, and anoxic water from the eastern basin was pumped into the Jezernica River. The sediment of the lake is composed largely of clayey silt with a carbonate content ranging from 55% to 80% (Ogorelec et al. 2006). The highest sediment carbonate concentrations are localized in the central, deepest part of the lake and in the shallow littoral platform in the southwest part of the lake where calcite is precipitated. Most of the carbonates are made of low-magnesium calcite (up to 5 mole % of $MgCO_3$) of authigenic origin, but a minor percentage is composed of detrital dolomite and calcite from the Mesozoic carbonate rocks in the watershed (Ogorelec et al. 2006). The isotopic composition of carbonates ranges from −4.21‰ to −2.85‰, with an average value and standard deviation of −3.53 ± 0.68‰ (n = 36) (Ogrinc et al. 2002). Sedimentation rates in the western and eastern basins, based on ^{210}Pb distributions, are 2.4 and 1.2 mm yr^{-1}, respectively (Čermelj et al. 1996).

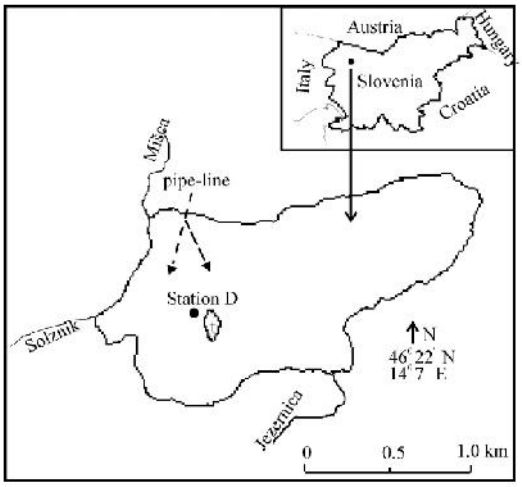

Fig. 1. Location of Lake Bled with Station D (the deepest Western Basin).

Sediment cores were collected at a fixed station D in the anoxic hypolimnion in the deepest part of the lake, at a depth of 30 m (Fig. 1), in December 2006 and December 2007. The water temperature was 6°C, and the concentration of dissolved oxygen was < 0.01 mg L^{-1}. Sediment cores, for pore-water extraction and geochemical analysis, were immediately transported to the laboratory, and

overlying water samples (~ 5 cm deep) were collected. Sediment cores were extruded and sectioned in 1-, 2-, or 4-cm intervals in a N_2-filled glove bag. Pore water was extracted by centrifugation at 3200 *g* followed by filtration through 0.45-μm Millipore HA membrane filters inside a glove bag. Sediment was analyzed immediately after extraction for pH, alkalinity (TA), methane, and isotopic composition of dissolved inorganic carbon ($\delta^{13}C_{DIC}$) and methane ($\delta^{13}C_{CH_4}$). The solid phase was freeze-dried and used for C and N elemental and isotopic analyses and lipid biomarker analysis. Tubes, cutting utensils, and glass jars were rinsed with distilled water, methanol, and hexane and wrapped with aluminum foil to prevent contamination. Samples were collected in glass jars, freeze-dried, homogenized, and stored until further analysis. Sediment samples for DNA extraction and molecular analysis were obtained from depths of 0–2 cm, 2–4 cm, 10–12 cm, and 18–20 cm, and separate layers were not mixed in order to preserve the spatial variability prior to collection of subsamples. Multiple subsamples were collected at each sediment depth and stored immediately at −80°C.

Pore-water analysis—The pH of pore water was measured inside the glove bag. Alkalinity was determined by the Gran titration method with a precision of ± 1%, and equilibrium equations for the carbonate system were used to calculate the concentrations of CO_2, HCO_3^-, and CO_3^{2-}, with subsequent recalculation for the in situ temperature. For methane analysis, 5-mL samples were injected into N_2-flushed ampoules and treated in an ultrasonic bath at 70°C for 20 min. Headspace gas samples (3–5 mL) were then analyzed by gas chromatography (HP 5840 A gas chromatography/flame ionization detector [GC/FID], Agilent Hewlett-Packard 6890N) with an analytical error of ± 3%. The $\delta^{13}C_{DIC}$ values were determined after extraction as CO_2 in glass septum tubes (Vacutainer Septum Tubes, Labco) using phosphoric acid. Next, 100–200-μL aliquots of 100% phosphoric acid were injected into the septum tube, capped, and purged with pure He to remove any air contamination. Five milliliters of water were then introduced by syringe. The isotope ratio of extracted CO_2 was determined directly from the headspace using a Europa Scientific PDZ Europa Ltd. 20-20 isotope-ratio mass spectrometer (IRMS) with an automated nitrogen carbon analyzer–trace gas (ANCA-TG) preparation module for trace gas samples equipped with a Gilson autosampler. The optimal extraction procedure for water samples was determined using a standard Na_2CO_3 solution with a known $\delta^{13}C$ value of −10.8‰ ± 0.2‰. The $\delta^{13}C_{CH_4}$ and $\delta^{13}C_{DIC}$ values were measured using the same device, with on-line chromatographic separation of the introduced gases and subsequent conversion of methane to CO_2 on CuO at 1000°C. The sample for $\delta^{13}C_{CH_4}$ determination was introduced into a CO_2-free glass vacutainer before analysis and heated in an ultrasonic bath at 70°C for 20 min.

Solid-phase analysis—Weight percentages of organic carbon (% OC) and total nitrogen (% TN) were determined (relative precision ± 2%) following acidification(1 M HCl) using a Carlo Erba model 1108 CHNS analyzer at a combustion temperature of 1020°C. Samples for measurement of isotopic composition of sedimentary organic carbon ($\delta^{13}C_{org}$) and total nitrogen ($\delta^{15}N_{TN}$) were prepared as for elemental composition measurements, which were performed using a Europa Scientific 20-20 IRMS with an ANCA solid/liquid (ANCA-SL) preparation module for solid and liquid samples. Analyses were calibrated against reference material: National Bureau of Standards 22 (NBS 22; oil) and International Atomic Energy Agency standard for carbon and hydrogen (IAEA-CH-7) were used for carbon, and IAEA–N-1, IAEA-N-2, and IAEA-NO-3 were used for nitrogen. All stable isotope results are reported using conventional delta (δ) notation in per mil (‰) relative to the Vienna Pee Dee Belemnite standard (VPDB) ($\delta^{13}C$) or air ($\delta^{15}N$). The precision of measurements was usually ± 0.2‰ for $\delta^{13}C_{DIC}$ and $\delta^{13}C_{org}$, ± 0.5‰ for $\delta^{13}C_{CH_4}$, and ± 0.3‰ for $\delta^{15}N_{TN}$.

For lipid biomarker analyses, sediment samples (5–7 g) were Soxhlet-extracted with dichloromethane for 8–10 h. Aliphatic hydrocarbons, aliphatic alcohols, sterols, and polar lipids were eluted, using 25 mL of hexane, 20 mL of 15% ethyl acetate in hexane, 20 mL of 20% ethyl acetate in hexane, and 20 mL of MeOH, respectively. Elemental sulfur was removed from the aliphatic hydrocarbons using activated copper. Aliphatic alcohols and sterols were derivatized using bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine to yield trimethylsilyl (TMS) ethers. Polar lipids were saponified with 0.5 mol L^{-1} KOH in MeOH, and released fatty acids were methylated using BF_3 : MeOH to yield fatty acid methyl esters (FAMEs). All fractions were dried with a stream of N_2, and residue was dissolved in iso-octane. Internal standards were used for quantification, with 5α-cholestane as a standard for aliphatic hydrocarbons, aliphatic alcohols, and sterols, and C_{19} FAME for fatty acids. Analysis of lipids (aliphatic hydrocarbons, aliphatic alcohols, sterols, and FAMEs) was performed using an Agilent model Hewlett-Packard 6890N gas chromatograph fitted with a DB 1 ms capillary column (60 m, 0.32 mm inner diameter [i.d.], 0.25 μm) fused by 100% dimethylpolysiloxane and an FID detector. Helium was used as the carrier gas with a flow rate of 1.5 mL min^{-1} and an injection volume of 1 μL. The injector and detector were set at 250°C. Components were identified with a Hewlett-Packard 6890 GC coupled to a mass spectrometer, using a DB-5MS (30 m × 0.25 mm i.d., 0.25 μm) fused phenyl-methylpolysiloxane capillary column. The oven temperature program for aliphatic hydrocarbons started at 40°C, followed by rapid heating to 300°C at 8°C min^{-1}, and a final holding time of 12 min. The oven temperature program for alcohols, sterols, and FAMEs started at 50°C for 1 min, followed by rapid heating to 220°C at 20°C min^{-1}, heating to 300°C at 10°C min^{-1}, and a final holding time of 13 min. The precision of the method was ± 6%.

Microbial community analysis—Archaeal and bacterial communities were characterized by T-RFLP analysis of 16S rDNA genes amplified from DNA extracted from four sediment layers (0–2 cm, 2–4 cm, 10–12 cm, and 18–20 cm)

sampled in December 2007. DNA was extracted from three 0.5-g sediment subsamples originating from each sediment layer using the UltraClean Soil DNA Isolation Kit (MO BIO Laboratories) according to manufacturer's protocol. Archaeal 16S rRNA genes were amplified using the primer pair Ar109f (5′-ACK-GCT-CAG-TAA-CAC-GT-3′) and 6-carboxyfluorescein (FAM)–labeled Ar915r (5′-GTG-CTC-CCC-CGC-CAA-TTC-CT-3′) (Egert et al. 2004). The standard reaction mixture contained, in a total volume of 25 μL, 2 μL of the environmental DNA extract, 5× Reaction Buffer (Promega), 1 U of GoTaq DNA polymerase (Promega), 1.5 mmol L^{-1} $MgCl_2$ (Promega), 200 μmol L^{-1} of each dATP, dTTP, dGTP, and dCTP (Fermentas, Life Sciences), 0.2 μmol L^{-1} of each primer (Invitrogen), and 0.4 mg mL^{-1} of bovine serum albumin (Sigma-Aldrich). All reactions were prepared at 4°C in 0.2-mL reaction tubes to avoid nonspecific priming. Polymerase chain reaction (PCR) was performed in a thermal cycler UNO-Thermoblock (Biometra) using the following program: initial denaturation for 90 s at 94°C, 35 cycles of denaturation (30 s, 94°C), annealing (45 s, 52°C), and extension (90 s, 72°C), and final extension for 5 min at 72°C. PCR amplification of bacterial 16S rRNA genes was performed as described by Kraigher et al. (2006) using 16S rRNA gene primers 27f (5′-AGAGTTTGATCCTGGCT-CAG-3′) labeled with 6-FAM (6-carboxyfluorescein) at the 5′ end and 927r (5′-CCGTCAATTCCTTTRAGTTT-3′) (Lane 1991). The 25-μL reaction mixtures contained 200 μmol L^{-1} of dNTP mixture, 0.2 μmol L^{-1} of each primer, 1% deionized formamide, 0.2 mg mL^{-1} of bovine serum albumin, 2 mmol L^{-1} of $MgCl_2$, 1× Mg-free reaction buffer B, 2 U Taq DNA polymerase (Promega), and 1 μL of template DNA solution. PCR was performed in the thermocycler with an initial denaturing step of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, annealing at 53°C, and 1.5 min extension at 72°C. Cycles were completed by extension for 10 min at 72°C. Aliquots of PCR products (10 μL for archaeal and 7 μL for bacterial) were digested with 10 U of enzyme HaeIII (Fermentas, Life Science) in 30-μL reactions of 1× Buffer R (Fermentas, Life Science) overnight at 37°C, and the enzymes were inactivated by incubation at 80°C for 20 min. Digests were purified by ethanol precipitation, and their sizes were analyzed on a capillary DNA sequencer 3130xl Genetic Analyzer (Applied Biosystems) using internal-lane standard Genescan 500 ROX (Applied Biosystems). The T-RFLP profiles were analyzed using Genescan analysis software (ABI). Terminal restriction fragments (T-RFs) that were less than 50 base pairs (bp) long were excluded from the analysis. The T-RFLP raw data sets were automatically converted to a digitized form using Bionumerics program version 3.0 (Applied Maths), and further analysis was performed as described by Stres et al. (2008). Intensity values and positions of detected fragments (T-RFs) were normalized and used for cluster analysis. Peaks that represented less than 1% of the total fluorescence of all peaks were excluded from the analysis. In total, less than 5% of all peaks were excluded. The Pearson correlation coefficient, which takes into account both fragment length and peak height, was used to calculate similarity coefficients. A dendrogram was constructed using the unweighted pair-group method with arithmetic means (UPGMA) algorithm.

Two clone libraries of archaeal 16S rDNA genes were prepared, one from each of two sediment depths: 0–2 cm and 10–12 cm. Three independent DNA preparations obtained from three 0.5-g subsamples from each depth were used as templates for PCR amplification as described previously. Products from three independent PCR amplifications were then purified using QIAquick PCR Purification Kit (QIAGEN) and QuickClean 5 M PCR Purification Kit (GenScript) and pooled. Amplicons were ligated into pGEM®-T Easy Vector and transformed into competent *Escherichia coli* JM109 cells according to the manufacturer's instructions (Promega). Individual white colonies were screened by direct PCR amplification using SP6 and T7 vector primers. Finally, 96 positive clones were selected from each library (0–2 cm and 10–12 cm) and partially sequenced (~ 740 bp) with the primer Ar109f on an ABI3730XL automated sequencer (Applied Biosystems). Sequences were checked manually and trimmed with program Chromas (version 2.33; http://www.technelysium.com.au/chromas.html). Sequences were submitted to the Bellerophon server (Huber et al. 2004) and Chimera Check at the Ribosomal Database Project (Cole et al. 2003) to detect the presence of chimeric artifacts. Of 192 cloned archaeal 16S rDNA sequences, 1 bacterial sequence and 9 chimeric sequences were identified and excluded from further analyses. Ninety-five sequences from the 10–12 cm depth and 84 sequences from the 0–2 cm depth were aligned with archaeal 16S rRNA sequences from GenBank using algorithm Clustal (Larkin et al. 2007). Evolutionary history was inferred using the neighbor-joining method (Saitou and Nei 1987). The percentages of replicate trees in which the associated taxa clustered in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using Kimura's two-parameter model (Kimura 1980), and units are the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

The Shannon-Weaver index (H′) with 95% confidence intervals was calculated with DOTUR (Schloss and Handelsman 2005), and the Shannon evenness (E) was calculated according to Blackwood et al. (2007). Operational taxonomic units (OTUs) were defined as groups of cloned sequences with ≥97% sequence similarity, representing the species level (Stackebrandt and Goebel 1994).

Calculations—The apparent isotopic fractionation factor for conversion of CO_2 to CH_4 is given by

$$\alpha_c = (\delta^{13}C_{CO_2} + 1000)/(\delta^{13}C_{CH_4} + 1000) \quad (1)$$

where $\delta^{13}C_{CO_2}$ and $\delta^{13}C_{CH_4}$ are the isotopic composition of CO_2 and CH_4, respectively. Relative contribution of hydrogenotrophically derived CH_4 to total CH_4 was determined by mass balance:

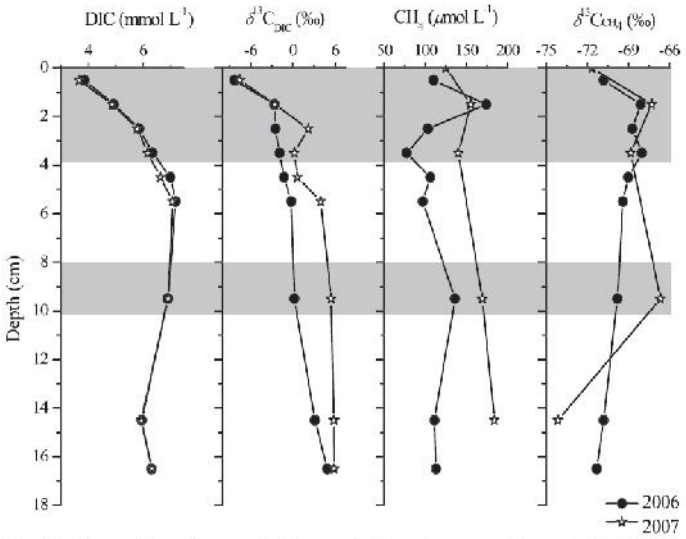

Fig. 2. Depth profiles of concentration and isotopic composition of dissolved inorganic carbon, (DIC, $\delta^{13}C_{DIC}$) and methane (CH_4, $\delta^{13}C_{CH_4}$) in pore water at station D of Lake Bled. Values were measured in years 2006 (black circles) and 2007 (white stars). Depths at which samples for microbial community structure were taken, are marked by gray color.

$$f_{CH_4,h} = (\delta^{13}C_{CH_4} - \delta^{13}C_{CH_4,h})/(\delta^{13}C_{CH_4,h} - \delta^{13}C_{CH_4,a}) \quad (2)$$

where $f_{CH_4,h}$ is the fraction of CH_4 formed by hydrogenotrophy, $\delta^{13}C_{CH_4}$ is the isotopic ratio of total produced CH_4, and $\delta^{13}C_{CH_4,h}$ and $\delta^{13}C_{CH_4,a}$ are the isotopic composition of CH_4 derived from hydrogenotrophic or acetoclastic methanogenesis, respectively. The $\delta^{13}C_{CH_4}$ and $\delta^{13}C_{CH_4,a}$ values were calculated using αc obtained by Whiticar et al. (1986) and $\delta^{13}C_{CO_2}$. In this calculation, two different α_c values were used, with values of 1.07 and 1.04 for hydrogenotrophy and acetotrophy, respectively. The $\delta^{13}C_{CO_2}$ values (about 9‰ more negative) were calculated from the measured $\delta^{13}C_{DIC}$ (Stumm and Morgan 1981). As Conrad (2005) pointed out, such deviation is helpful for rapid crude diagnosis of the predominant methanogenetic pathway.

The first approximation of OM source apportionment could be estimated from lipid composition and concentration (Muri and Wakeham 2006):

$$1 = \left(\sum C_{L,plan}/C_{TL}\right) + \left(\sum C_{L,bact}/C_{TL}\right) + \left(\sum C_{L,ter}/C_{TL}\right) \quad (3)$$

where $\Sigma C_{L,plan}$, $\Sigma C_{L,bact}$, and $\Sigma C_{L,ter}$ are the sum of the concentrations of lipids generally derived from plankton, bacteria, and vascular plant sources, respectively, while C_{TL} is the concentration of total extracted lipids present in sediment. Plankton and bacteria represent lipids of autochthonous origin, while lipids derived for vascular plants source are attributed to allochthonous sources of OM (Cranwell 1982; Meyers et al. 1984; Meyers 2003).

Results

Sediment pore water—Concentrations of DIC, CH_4, and their corresponding isotopic values in pore water were examined in 2006 and 2007 (Fig. 2). Concentrations of DIC were greater at greater depth in both years, ranging from 3.86 to 7.19 mmol L^{-1}. The $\delta^{13}C_{DIC}$ values displayed a high positive gradient (from −8.3‰ to +4.8‰ in 2006 and from −7.6‰ to +5.9‰ in 2007) from the bottom water layer to the sediment depth of 15 cm, which is typical of methanogenic sediments. The CH_4 concentrations were less variable, ranging between 80 and 180 μmol L^{-1}, and they showed no consistent pattern with depth. A slight increase in CH_4 concentration with depth was observed in 2007 and with higher concentrations than in 2006. However, $\delta^{13}C_{CH_4}$ values for methane dissolved in pore water were similar for the two sampling periods, with an average $\delta^{13}C_{CH_4}$ value of −69.5‰ ± 1.2‰.

C and N elemental and isotopic composition of sedimentary organic matter—The content of organic carbon decreased with sediment depth from 5.1 wt% at the surface to 2.1 wt%, while TN concentration decreased from 0.59 to 0.34 wt% (Fig. 3), but with a slight increase of organic carbon to 4.8 wt% at 12–13-cm depth. The average C : N ratio was 11.0 ± 0.7, indicating minor terrestrial OM input. The $\delta^{13}C$ values of the sedimentary OC ranged from −31.5‰ to −34.8‰, and $\delta^{15}N$ ranged from +3.6‰ to +8.1‰ (Fig. 3). Such low $\delta^{13}C$ values are often found in eutrophic lakes with anoxic hypolimnion and in strongly reductive sediments, probably due to sedimentation of anaerobic microbial biomass (Teranes and Bernasconi

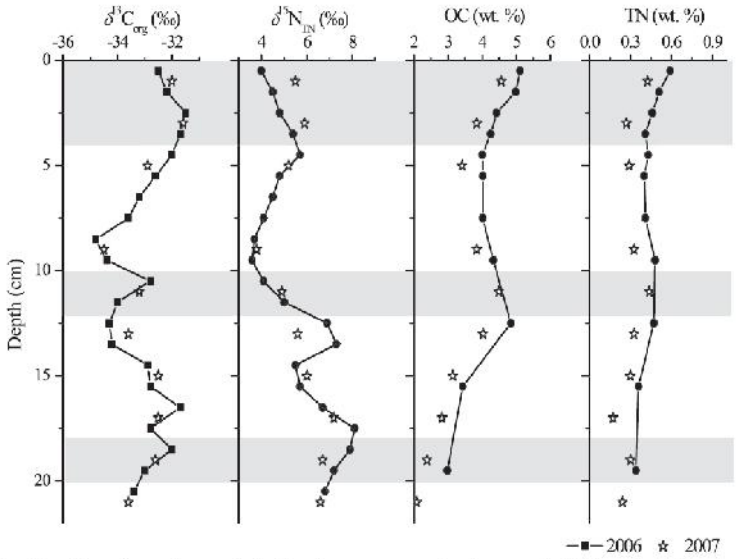

Fig. 3. Organic carbon and total nitrogen concentrations and $\delta^{13}C$ and $\delta^{15}N$ values of sedimentary organic matter as functions of depth at Station D in Lake Bled. Values were measured in years 2006 (black squares) and 2007 (white stars). Depths at which samples for microbial community structure were taken are marked by gray color.

2005; Conrad et al. 2010), while the $\delta^{15}N$ values indicate the prevalence of phytoplankton in sedimentary organic matter (Meyers 2003; Bratkič et al. 2012).

Lipid biomarkers—Acetate fermentation utilizes more labile organic matter, while CO_2 reduction is associated with more refractory organic matter (Whiticar et al. 1986; Sugimoto and Wada 1993). The distribution of more labile and more refractory organic matter in Lake Bled sediments was obtained by analysis of lipid biomarkers. The dominant lipids in sediments were *n*-alkanes, *n*-alkanols, fatty acids, and sterols, the latter being the most abundant. Their depth distribution is presented in Fig. 4. The concentration of total lipids (TL) decreased with depth down to 14 cm (from 13,820 to 3170 $\mu g\ g^{-1}$ OC in 2006 and from 12,671 to 6476 $\mu g\ g^{-1}$ OC in 2007), below which it increased to 10,027 and 11,702 $\mu g\ g^{-1}$ OC at a depth of 18 cm. At a depth of 22 cm, the lipid concentration reached a minimum at 2080 and 2712 $\mu g\ g^{-1}$ OC in 2006 and 2007, respectively (Fig. 4A). Lipid composition also changed with depth (Fig. 4B). In the surface sediment layer, sterols were the most abundant lipids, constituting 61% of the total lipid concentration. Total sterol concentration (TST) decreased steeply with depth, probably because they, together with fatty acids, are more susceptible to degradation. Total aliphatic hydrocarbons (THC) were also abundant, but their concentration was greatest at 18–20 cm. The concentration of total aliphatic alcohols (TCOH) ranged from 1% to 17% of total lipids. Total fatty acid (TFA) was only a small fraction of TL (3 to 9%), and their abundance (Fig. 4B) and composition (Fig. 4C) did not change significantly with depth.

Of the sterols, C_{27} sterols were dominant in the surface sediment layers, constituting 51% of TST (data not shown). Cholest-5-en-3β-ol (Δ^5 C_{27}, cholesterol) was the major C_{27} sterol, with a concentration of 1130 $\mu g\ g^{-1}$ OC at the sediment surface, followed by 5α-cholestan-3β-ol. C_{29} sterols dominated at the bottom of the sediment and constituted 77% of the TST concentration. Also, 24-ethylcholest-5-en-3β-ol (β-sitosterol) was the most abundant C_{29} sterol, comprising 31% of C_{29} sterols, followed by 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol; 28%) and 24-ethyl-5α(H)-cholestan-3β-ol (10%). The C_{28} sterols were the least abundant sterols in lake sediments and were dominated by 24-methylcholest-5-en-3β-ol ($C_{28}H_{46}O$, campesterol) at a concentration of 790 $\mu g\ g^{-1}$ OC, followed by 24-methylcholest-5,22-dien-3β-ol and ergosta-5-22-dien-3β-ol.

Aliphatic hydrocarbons showed different molecular distributions in the range *n*-C_{15} to *n*-C_{35} in sediments, with a significant odd-over-even carbon number predominance in most cases. The concentration of short-chain alkanes decreased sharply with depth, as exemplified by *n*-C_{17}, which decreased from 108 to 43 $\mu g\ g^{-1}$ OC. Longer-chain *n*-alkanes were more abundant in deeper sediments, especially C_{27}, C_{31}, and C_{29}, the last being the most abundant. The isoprenoid hydrocarbons, pristane and phytane, were detected throughout the sediment, and phytane concentration was greatest at the bottom of sediment core. Several hopanes were detected in the sediment, the most abundant being hop-17(21)-ene.

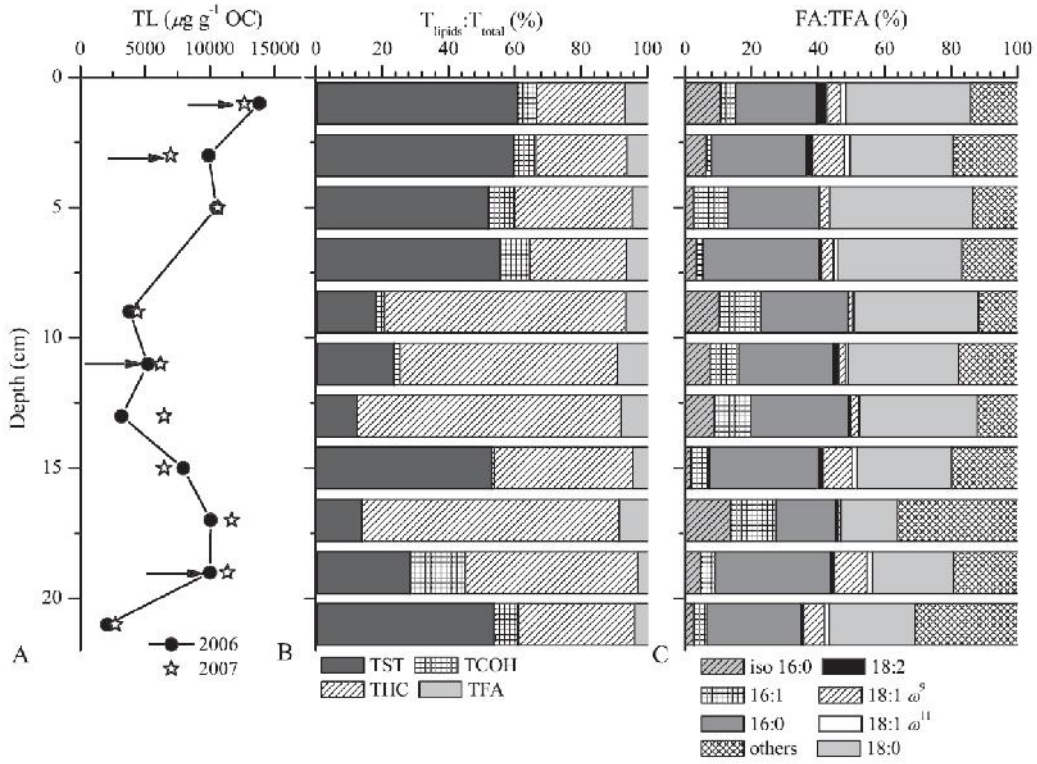

Fig. 4. (A) Distribution of lipids with depth in sediments at Station D. Depths at which the microbial community was determined are marked by arrows. (B) Distribution of dominant lipids in sediments - total sterols (TST), total aliphatic alcohols (TCOH), total aliphatic hydrocarbons (THC) and total fatty acids (TFA), and (C) Distribution of the branched methyl-substituted and unsaturated short chain C_{16} and C_{18} fatty acids in depth sediment profile.

The *n*-alkanols, consisting of even-numbered compounds, ranged from C_{22} to C_{30}, dominated by *n*-C_{24} with a concentration of 1490 μg g^{-1} OC at 24-cm depth. Longer chain *n*-alkanols comprised 83% of the total alkanol concentration (TOH).

The TFA profile consisted of saturated, monosaturated, branched-chain, and polyunsaturated fatty acids (FAs). Saturated *n*-alkanoic FAs displayed distributions from C_{12} to C_{32} with a strong even : odd preference and maximum occurrence at *n*-C_{16} and *n*-C_{18}. The relative abundances of different C_{16} and C_{18} FAs are presented in Fig. 4C. Concentrations of mono-unsaturated *n*-acids (*n*-$C_{16:1}$ and *n*-$C_{18:1}$; ω^9 and ω^{11} isomers) were relatively high, together comprising up to 20% of TFA. Low concentrations of branched methyl-substituted FAs were found in sediments, ranging from 3 to 40 μg g^{-1}, and contributing around 16% to TFA. These FAs were more abundant in deeper sediments. Concentrations of polyunsaturated acids were low and did not exceed 30 μg g^{-1} OC.

Archaeal and bacterial community structure—The depth-related structure of archaeal and bacterial communities in Lake Bled sediment was analyzed by T-RFLP analysis of 16S rRNA genes. The archaeal community formed two separate clusters, delineated at a Pearson's correlation of 68%, with one cluster containing the community from the upper layer, 0–2 cm (Pearson's correlation 86%), and the second the archaeal community from the remaining, deeper sediment layers (Pearson's correlation at 86%). The clustering of T-RFLP profiles in replicate samples from deep layers was tight, with the community structure from each layer forming a separate subgroup (Fig. 5A). Bacterial profiles of 16S rRNA genes also formed two groups, but one cluster included profiles from the two upper layers (0–2 cm and 2–4 cm), and the second represented communities from the two deeper sediment layers (10–12 cm and 18–20 cm). In the deeper layers, only bacterial profiles from 18–20-cm sediment depth formed a tight subgroup, while the rest were dispersed throughout the cluster. However, the Pearson's correlation within each cluster was 92%, suggesting high similarity between T-RFLP profiles (Fig. 5B).

Phylogenetic analysis of archaeal 16S rRNA genes—The main aim of this work was to clarify the origin of methane in sediment of Lake Bled. The T-RFLP clustering implied

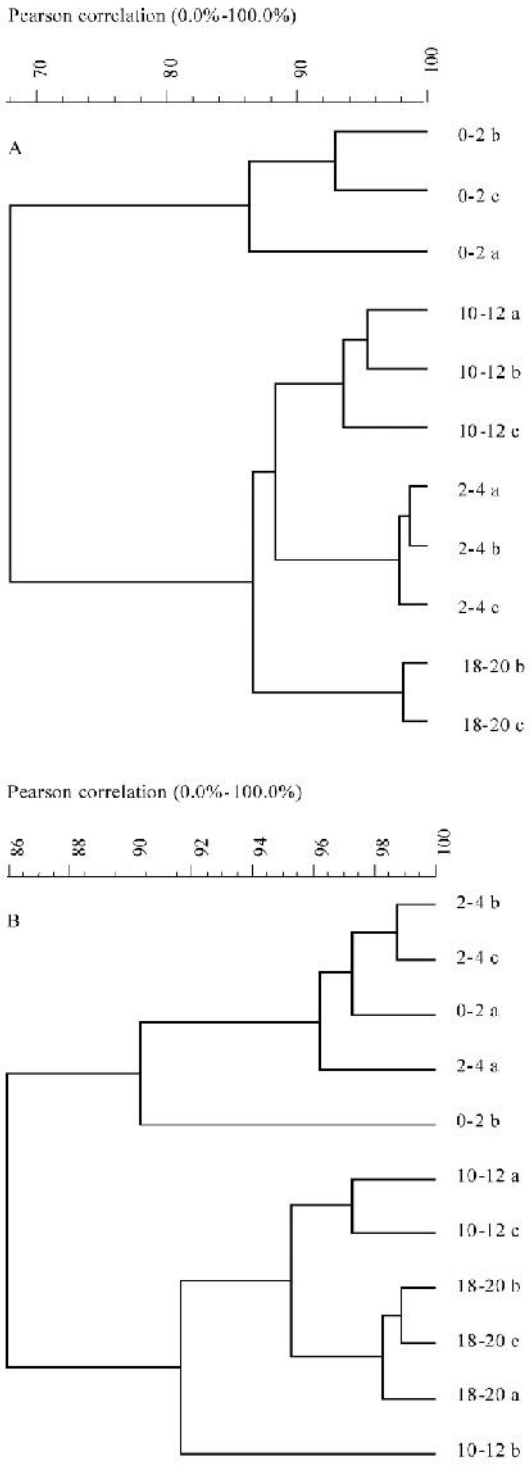

Table 1. Distribution of sequences isolated from sediment into phylogenetic groups.

Phylogenetic groups	0–2-cm sediment depth (% isolated sequences)	10–12-cm sediment depth (% isolated sequences)
Methanomicrobiaceae	38.9	19.0
Methanospirillaceae	15.8	2.4
Methanosaetaceae	17.9	16.7
Methanobacteriaceae	1.1	0.0
Thermoplasmatales and related lineages	3.2	23.8
RC V (rice cluster V)	8.4	7.1
Unclassified sequences	8.4	27.4
All methanogenic archaea	73.7	38.1

that the methanogenic community in the upper 2 cm differed from that in the deeper sediment layers. To gain a better insight into the phylogenetic composition of archaea, and therefore methanogens, clone libraries of archaeal 16S rRNA genes were prepared from two sediment depths (0–2 cm and 10–12 cm) (*see* Web Appendix, http://www.aslo.org/lo/toc/vol_57/issue_3/0868a.pdf). These depths were chosen to represent the upper and deeper layers, because each formed well-separated but tight clusters, suggesting different communities but low spatial variability at these depths (*see* Web Appendix). Almost all cloned 16S RNA gene sequences belonged to phylum Euryarchaeota, and only 6 out of 95 and 3 out of 84 sequences from 0–2-cm and 10–12-cm sediment depths were from phylum Crenarchaeota. Methanogenic archaea in the upper and lower sediment layers constituted 73.7% and 38.1% of all archaeal sequences (Table 1). In the upper layer, the majority of sequences were associated with hydrogenotrophic archaea: *Methanomicrobiaceae* (37 sequences), *Methanospirillaceae* (15 sequences), and *Methanobacteriaceae* (1 sequence). In the lower, 10–12-cm layer, hydrogenotrophic *Methanomicrobiaceae* (16 sequences) and *Methanospirillaceae* (2 sequences) were found to be less abundant. A similar relative abundance of acetoclastic archaea (*Methanosaetaceae*), with 17 sequences in the 0–2-cm layer and 14 sequences in the 10–12-cm layer and of rice cluster V representatives, with eight and six sequences in upper and lower sediment layers, was detected. All sequences that belonged to acetoclastic archaea were similar to *Methanosaeta concilii* and no representative of *Methanosarcinaceae* was detected among the cloned sequences. The

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Fig. 5. A dendrogram of T-RFLP profiles of PCR-amplified 16S rRNA gene fragments of (A) archaeal and (B) bacterial communities from four sediment depths (0–2, 2–4, 10–12, 18–20 cm). DNA was extracted from sediment layers sampled in December 2007. PCR products at each depth were obtained from three independent sediment samples (a, b, c). The cluster analysis was based on Pearson's correlation index and the unweighted pair-group method on arithmetic averages. Some data (18–20 cm for archaea and 0–2 cm for bacteria) were excluded from the analyses due to low DNA concentrations and poor resolution.

Table 2. Summary of sources of lipids used to calculate source distribution of sedimentary organic matter.

Compound	Plankton	Bacterial	Terrestrial plants	Reference
Long-chain linear compounds (*n*-alkanes, *n*-alkanols, *n*-fatty acid [FA])			X	Meyers et al. (1984); Meyers (2003)
Short-chain linear compounds (*n*-alkanes, *n*-alkanols, *n*-FA)	X			Cranwell et al. (1987); Stefanova and Disnar (2000); Meyers (2003)
C_{27} sterols	X			Weete (1976)
C_{28} sterols	X			Volkman (1986); Neunlist et al. (2002)
C_{29} sterols			X	Weete (1976); Volkman (1986)
Hopanoids		X		Ourisson and Rohmer (1992)
Iso- and anteiso-C_{15} and C_{17} FA		X		Wakeham et al. (2007); Bechtel and Schubert (2009)
C_{16} mono-unsaturated FA	X			Wakeham et al. (2007); Bechtel and Schubert (2009)
C_{18} mono-unsaturated FA		X		Wakeham et al. (2007); Bechtel and Schubert (2009)
16:1ω7 *n*-FA		X		Wakeham et al. (2007); Bechtel and Schubert (2009)
18:1ω9, ω11 *n*-FA		X		Wakeham et al. (2007); Bechtel and Schubert (2009)

percentage of sequences with no similarity to database sequences was higher in the deeper layer (27.4%) than in the upper 0–2-cm layer (8.4%). Sequences from *Thermoplasmatales* and related lineages were quite abundant (20 sequences) in the deeper, but not the upper sediment layer (three sequences).

The Shannon-Weaver (H′) indices with 95% confidence intervals were calculated on the basis of 16S RNA sequences. In the upper (0–2 cm) and lower (10–12 cm) sediment layers, the H′ indices were 3.04 ± 0.18 and 3.23 ± 0.24, suggesting similar diversity in both layers. Shannon evenness indices (0.89 ± 0.18 and 0.88 ± 0.24 for upper and deeper sediment layers) demonstrated that sequences were also evenly distributed at both sediment depths. Interestingly, when sequences from both libraries were combined, the Shannon-Weaver index was higher (3.64 ± 0.17) than when each was analyzed separately, suggesting that sequences from the two libraries differed phylogenetically, increasing total diversity of the archaeal community.

Discussion

Origin of methane—Methanogenesis in cold alpine lacustrine sediments has rarely been investigated, and the mechanisms influencing the relative contributions of acetoclastic versus hydrogenotrophic methanogenesis are not yet completely understood (Nozhevnikova et al. 2007). Acetoclastic methanogenesis has been recognized as the dominant methanogenic pathway in anaerobic lacustrine environments and other freshwater habitats (Oremland 1988). It was also hypothesized that at low temperature, typical of stratified alpine lakes such as Lake Bled, degradation of sedimentary organic matter may result from homoacetogenesis followed by acetate-dependent methanogenesis (Schulz and Conrad 1996; Nozhevnikova et al. 2007). However, our previous ^{13}C and ^{2}H isotopic studies of methane production in the stratified eutrophic Lake Bled (Lojen et al. 1999; Ogrinc et al. 2002) and in Lake Planina (Ogrinc et al. 2008) in the Julian Alps (west Slovenia) suggested that hydrogenotrophic methanogenesis should prevail. Hence, we tested this hypothesis using sedimentary geochemical analyses in combination with molecular analysis of microbial communities present in the sediment profile.

The prevalence of hydrogenotrophic methane production was further suggested by the extremely low acetate concentration (< 0.01 μmol L^{-1}) observed in lake pore waters (Lojen et al. 1999). However, low acetate levels in sediment could result from rapid acetate turnover, either by bacteria or by acetoclastic methanogens (Stams and Plugge 2009; Conrad et al. 2010). Furthermore, it was suggested recently that acetogenic CO_2 reduction, followed by acetoclastic methanogenesis, also produces CH_4 with $\delta^{13}C$ values similar to those observed from hydrogenotrophically produced CH_4 (Heuer et al. 2010).We therefore cannot rule out the possibility that the low $\delta^{13}CCH_4$ values in Lake Bled may be associated with acetoclastic methanogenesis preceding acetogenic CO_2 reduction to acetate, and that both hydrogenotrophs and acetoclastic methanogens may contribute to CH_4 production in the sediments. The calculated α_c values varied within 1.06 and 1.08 and increased with depth. These values were higher comparing to the α_c value of 1.055 that corresponds, according to Whiticar et al. (1986), to the rough boundary between hydrogenotrophy and acetotrophy. The calculated proportion of methane production from two sources using Eq. 2 showed that hydrogenotrophic formation of CH_4 prevailed in the lake sediment in both study periods. At the surface, the contribution of hydrogenotrophic methanogenesis was less pronounced, but it still accounted for more than 50% (51% in 2006 and 56% in 2007). Its contribution increased with depth, accounting for up to 90% at the depth of 15 cm. The higher contribution of acetotrophy observed at the surface compared to other sediment depths could be associated with the presence of more labile organic matter. The dominance of hydrogenotrophic methanogenesis has also been observed in ice-covered Lake Untersee, central Dronning Maud Land, East Antarctica (Wand et al. 2006) and in two Amazonian lakes (53% and 63%) in which the temperatures above the sediment were 31°C and 30°C (Conrad et al. 2010), respectively, indicating that temperature should not be the dominant factor influencing methanogenic pathways in lacustrine environments.

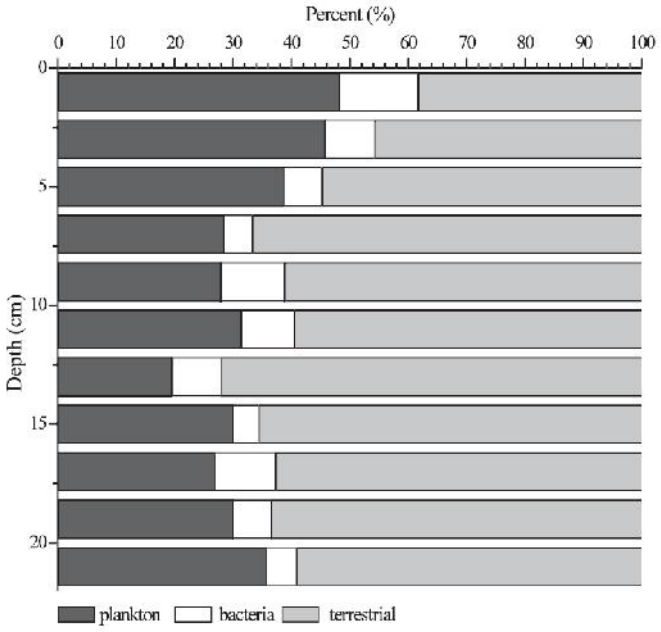

Fig. 6. Origin of lipids in sediment depth profile at Station D in Lake Bled. Calculations were based on measurements of two replicates.

Depth-related changes in archaeal and bacterial communities—Analysis of archaeal communities in Lake Bled sediment revealed the dominance of Euryarchaeota, with only a few crenarchaeal sequences in clone libraries, suggesting that methanogenesis is important in this anaerobic environment. Methanogens were represented mostly by the non-acetoclastic families *Methanomicrobiaceae*, *Methanospirillaceae*, and, to a lesser extent, *Methanobacteriaceae*, while representatives of the obligate acetoclastic *Methanosaetaceae* family were less abundant (< 18%). The abundance of acetoclastic *Methanosaetaceae* sequences was similar at the two sediment depths, as also reported for the Rotsee sediment profile from T-RFLP analysis (Zepp-Falz et al. 1999). In contrast, the abundance of hydrogenotrophic methanogens was higher in the upper sediment layer, which is consistent with T-RFLP analysis of 16S rRNA archaeal genes, which showed depth-related changes in Lake Bled sediment communities. *Methanosarcinaceae*, which are able to utilize both $H_2:CO_2$ and acetate (Krzycki et al. 1987), were absent from both clone libraries, in agreement with previous studies that also failed to detect representatives of this group in lacustrine sediments (Koizumy et al. 2004; Chan et al. 2005; Conrad et al. 2010). Furthermore, *Methanosarcinaceae* are favored by high hydrogen and acetate concentrations, and a diverse community that includes both *Methanosaetaceae* and $H_2:CO_2$ utilizing methanogens may have an advantage in low acetate environments (Jetten et al. 1992; Leybo et al. 2006) as observed in the Lake Bled sediment.

Sources of organic matter for methanogenesis—At the low temperatures typical of Lake Bled bottom waters (4–6°C), hydrogenotrophic methanogenesis is thermodynamically less favored than acetogenesis (Glissman et al. 2004), and alternative reasons for hydrogenotrophy in Lake Bled sediments should therefore be sought. Acetate fermentation appears to be associated with more labile organic matter, whereas CO_2 reduction to methane utilizes more refractory organic matter (Sugimoto and Wada 1993).

Previous reports of lipid distribution in Lake Bled sediments indicated a mixture of more labile autochthonous and more refractory allochthonous organic matter (Muri and Wakeham 2006). We estimated the contribution of these two sources by dividing lipids into three pools: plankton and bacterial as autochthonous pools and an allochthonous, terrestrial pool using Eq. 3. Lipid biomarkers and their suggested attribution to sources, based on literature data, are summarized in Table 2. The allochthonous, terrestrial pool includes long-chain lipids derived from allochthonous vascular plant sources, *n*-C_{20} to *n*-C_{35} aliphatic hydrocarbons, aliphatic alcohols, and saturated and unsaturated FAs. Of the sterols, which originate from a variety of eukaryotic organisms and vascular plants, C_{29} sterols were assigned primarily to higher terrestrial plants. The origin of short-chain lipids is often ambiguous, since several (*n*-C_{16} alcohols and *n*-$C_{16:0}$ FA) have multiple sources (Meyers et al. 1984). Nevertheless, short-chain lipids in lacustrine sediments are generally attributed to autochthonous sources (Cranwell 1982; Meyers 2003).

Thus, short-chain lipids in the range n-C_{14} to n-C_{19} and saturated, unsaturated, and methyl-substituted FAs were considered as an autochthonous source. Saturated n-alkanoic FAs have a maximum peak at n-C_{16} and n-C_{18}, characteristic of autochthonous organic matter production in lakes (Cranwell et al. 1987; Stefanova and Disnar 2000). The C_{27} and C_{28} sterols are considered to be derived from an autochthonous source. The source of C_{28} sterols is less specific, since they are relatively abundant in both algae and terrestrial higher plants (Volkman 1986). The isotopic composition of C_{28} sterols (average $\delta^{13}C$ value of −36.1‰) indicates a relatively constant microalgal source in Lake Bled (Neunlist et al. 2002), and C_{28} is therefore considered to be of autochthonous origin.

The bacterial contribution to the autochthonous pool was calculated based on the hopanoid distribution in the neutral lipid fraction and on the concentrations of branched chain FAs. Hop-17(21)-ene was the most abundant throughout the sediment column, and it has been suggested (Farimond et al. 2000) that bacteriohopanoids in sediments are derived largely from type I methanotrophs. The most specific FA biomarkers for type I methanotrophs are also mono-unsaturated FAs (n-$C_{16:1}$ and n-$C_{18:1}$; ω^9 and ω^{11} isomers) (Ratledge and Wilkinson 1988), which were the most abundant branched FAs in the sediments (Fig. 4C). However, in addition to methanotrophs, mono-saturated C_{16}-FAs are also abundant in manganese-, iron-, and sulfate-reducing bacteria (Wakeham et al. 2007; Bechtel and Schubert 2009), which are common in anaerobic environments. Other FAs of bacterial origin present in our sediments were *iso*- and *anteiso*-methyl-FAs C_{15} and C_{17}, probably derived from cyanobacteria and sulfate-reducing bacteria and other gram-positive bacteria (Wakeham et al. 2003).

We estimated (Fig. 6) that the autochthonous contribution changed from 62% at the surface to 41% at a depth of 20 cm, where lipids of terrestrial origin, which are less susceptible to degradation, prevailed. Lipids of bacterial origin were more evenly distributed. Their contribution was more pronounced at the surface, where they comprised 13% of total lipids, while their contribution in deeper sediments ranged from 4% to 11%. In summary, lipid biomarker analysis indicates that sedimentary organic matter is mostly autochthonous and derived from deposited plankton and bacteria. It is thus more labile and should be more prone to acetate production. The lipid-based assessment generally agrees with the source appointment based on C : N ratios. Its mainly plankton origin (> 70%) is further supported by $\delta^{13}C$ values of remineralized C ($DIC + CH_4$), with a mean $\delta^{13}C$ value of −28.4‰ determined by Ogrinc et al. (2002).

In conclusion, biogeochemical and archaeal community analyses support the hypothesis that hydrogenotrophic methanogenesis is the dominant pathway of CH_4 production in sediments of the alpine Lake Bled, despite its low temperature and the prevalence of autochthonous, and therefore more labile, organic carbon. Acetoclastic methanogenesis contributes to the biogenic methane, but the extremely low pore-water acetate concentration suggests that part of the acetate produced is consumed in other competitive processes, among which sulfate reduction appears likely. This is supported by the presence of framboidal pyrite (Lojen et al. 1999) and lipid biomarkers belonging to sulfate-reducers in Lake Bled sediments. This study also showed that the processes and methanogenic archaeal communities were in many ways similar to those in other midaltitude lakes, and even to tropical lakes, indicating that temperature could not be the main factor controlling the methanogenetic pathway.

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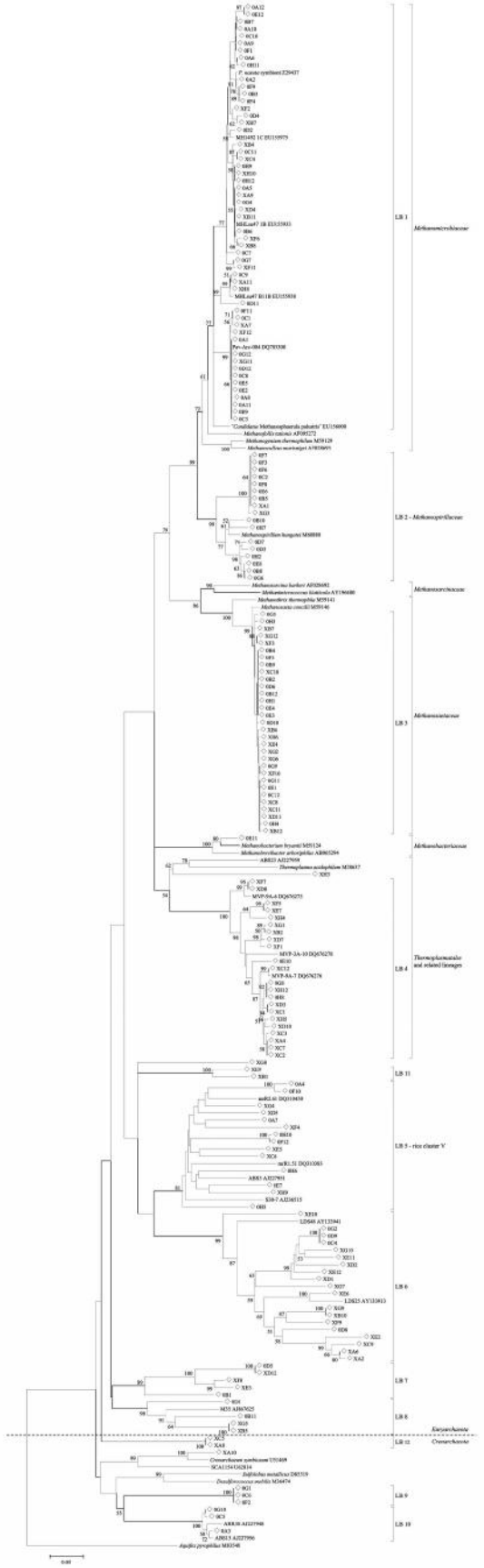
Methanogenesis pathways in a stratified eutrophic alpine lake (Lake Bled, Slovenia)

Mandic-Mulec, Ines, Katja Gorenc, Marinka Gunn Petrišic, Jadran Faganeli, and Nives Ogrinc

Figure A1. Neighbor-joining tree showing phylogenetic relationships of 16S rRNA archaeal gene sequences cloned from the anoxic sediment of Lake Bled from depths of 0-2 cm (depicted by white diamonds) and 10-12 cm (depicted by black diamonds). PCR products from each depth were amplified from three sediment subsamples which were pooled prior to cloning. Cloned sequences are clustered in eleven Lake Bled (LB) groups. The phylogenetic tree was reconstructed using the neighbor-joining method with 1000 bootstrap replicates and the Kimura-2-parameter evolutionary model within the MEGA version 4 (Tamura et al. 2007). Bootstrap values of >50 are shown. Scale bar represents the number of estimated substitutions per nucleotide position.



3.3 Scientific paper: »Stable isotopes and source identification of lipids in oxic and anoxic sediments of Lake Bled (NW Slovenia)«

In this chapter, the paper entitled “Stable isotopes and source identification of lipids in oxic and anoxic sediments of Lake Bled (NW Slovenia)” by Marinka Gams Petrišič, Ester Heath and Nives Ogrinc is presented. The paper is under review in Organic Geochemistry.

Organic matter diagenesis is an important part of local and global C cycles, acts as a food source for benthic animals and microbes, and provides information about the paleolimnologic record preserved in lake sediments that can be used for interpreting past environmental change. The relative lack of the use of stable isotopes of biomarkers in lacustrine sediment studies prompted us to investigate the distribution and isotope composition of OM in sediments from an alpine, dimictic lake, Lake Bled (NW Slovenia). The distribution of lipids in the sediments has been already described (Muri and Wakeham, 2006), thus our research was focused on the determination of the isotopic composition of lipids with the aim of allowing a more detailed reconstruction of the sources of these compounds. Two different depositional environments were studied, under anoxic conditions (western basin) and permanently oxic (Zaka Bay).

Redox conditions appear to be a key factor governing the preservation, formation and degradation of the OM and thus, not surprisingly, influenced the lipid distribution. The isotope data indicated that, in both environments, OM was derived from mixed sources, plankton and terrestrial and an anaerobic-microbial. The similarity of $\delta^{13}C$ values for long-chain *n*-alkanes, short - and long-chain *n*-alcohols and short-chain FAs in sediments obtained from the two sites indicated that these compounds had similar sources and were not dependent on different depositional regimes. The highest $\delta^{13}C$ values were observed for short-chain FAs in both environments, indicating higher proportions of terrestrial OM. The increased, more refractory, terrestrial material brought by the Solznik stream could be also seen in the higher $\delta^{13}C$ values for short-chain *n*-alkanes in Zaka Bay sediments. On the other hand the average $\delta^{13}C$ values for long-chain FAs, and especially those of sterols, were lower in the anoxic western basin, indicating a more pronounced contribution of anaerobic (methanotrophic) microbial origin than for Zaka Bay.

It has been suggested that source assignment based on lipid composition was in sharp disagreement with the origin of bulk OM (Muri and Wakeham, 2006) probably due to selective degradation of lipids. On the contrary our results are in a good agreement with a source allocation based on bulk $\delta^{13}C$ values and OC/TN values. It was found that, in both environments, autochthonous OM was the main source of lipid compounds. This was evident in the low $\delta^{13}C$ values for long-chain *n*-alkanes, *n*-alcohols and FAs and, especially, in those determined in C_{29} sterols, indicating that these compounds were derived mainly from microalgae and bacteria and not from higher plants or other terrigenous OM, as previously reported. Our results indicated that statements regarding the source of OM based only on lipid distribution have to be treated with caution and should not be used without the input from stable isotope geochemistry.

Stable isotopes and source identification of lipids in oxic and anoxic sediments of Lake Bled (NW Slovenia)

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Abstract

Sources of lipid biomarkers in two Lake Bled sediment cores deposited under oxic (Zaka Bay) and anoxic (western basin) conditions were determined using stable carbon isotopes. Lipid biomarkers were of mixed origin including, terrestrial, planktonic and microbial sources. $\delta^{13}C$ values for long -chain (LC) *n*-alkanes, short -chain (SC) and LC *n*-alcohols, SC *n*-fatty acids (FAs) were similar for both sediments showing that identification of their sources was not connected to different depositional regimes. A larger contribution of more refractory terrestrial organic matter (OM) was observed in $\delta^{13}C$ values obtained from SC *n*-FAs in both environments and from SC *n*-alkanes in Zaka Bay. In contrast, the $\delta^{13}C$ values observed in LC *n*-FAs, and especially in sterols, were lower in the western basin than in Zaka Bay, indicating the pronounced contribution of an anaerobic, bacterial origin. Overall, the data indicated that the main source of lipids was autochthonous in both sediment environments including, LC *n*-alkanes, *n*-alcohols, *n*-FAs and C_{29} sterols previously assigned to an allochthonous higher plant source. It further follows that source assignment in such situations should be supported using isotope geochemistry.

Keywords: biomarkers, aliphatic hydrocarbons, sterols, fatty acids, stable isotopes, oxic-anoxic sediments, Lake Bled

Introduction

The organic matter (OM) content of lakes is influenced greatly, not only by biological and chemical reactions occurring within the lake, but also by the characteristics of the terrestrial and stream components of the surrounding catchment areas and of the sediment underlying the lake water. Sediment biogeochemistry plays an active role regulating carbon cycling in aquatic environments - the final sink for all descending particulate matter that escapes degradation within the water column (Jones and Bowser, 1978; Meyers and Ishiwatari, 1993; Bechtel and Schubert, 2009). OM in sediments is usually exposed to additional (often rapid) diagenetic changes regulated by microbial oxidation, using various electron acceptors (Froelich et al., 1979). The flux of reduced C -species

(e.g. methane) from sediment to the water column can control redox conditions in a lake and the distribution of metals and other nutrients. Thus OM diagenesis is an important part of local and global C cycles, acts as a food source for benthic animals and microbes, and provides information about the paleolimnologic record preserved in lake sediments that can be used for interpreting past environmental change.

OM in lake sediments is a complex mixture of which lipid biomarkers constitute only a minor part. Such biomarkers derive from various sources with a variable degree of preservation (Meyers, 2003). Aquatic and watershed organisms constitute the primary sources of sedimentary OM, while microbes provide secondary biomarkers during the early diagenetic reworking of the primary OM (Meyers, 2003). Further, some compounds are more susceptible to microbial degradation than others or may be transformed to more stable chemical structures (Volkman et al., 1998; Sauer et al., 2001; Schouten et al., 2003; Killops and Killops, 2005). Studies of individual biomarker compounds have focused mainly on the more long-lived lipid groups such as hydrocarbons, alcohols, sterols and fatty acids – (FAs). To understand the processes influencing the distribution of lipid biomarkers, it is necessary not only to characterize and quantify them but also to identify their major sources. Compound -specific stable isotope ratio mass spectrometry is receiving increasing attention as a tool that can yield information about the origin of OM in sediments. It is particularly useful in aquatic settings for distinguishing between lake and continental plants of sedimentary OM, and identifying OM from different types of land plants. In addition, isotope analyses of biomarkers provides information about microbial community structure, since they link directly (Boscher and Middelburg, 2002) microbial identity (biomarker identity), biomass (biomarker concentration) and activity (isotope assimilation).

The relative lack of the use of stable isotopes of biomarkers in lacustrine sediment studies prompted us to investigate the distribution and isotope composition of OM in sediments from an alpine, dimictic lake, Lake Bled (NW Slovenia). The distribution of lipids in the sediments has been described (Muri and Wakeham, 2006), together with the methanogenesis pathway, using a combination of biogeochemical and molecular approaches (Mandić-Mulec et al., 2012). We report here the ^{13}C -composition of lipids with the aim of allowing a more detailed reconstruction of the sources of these compounds. Two different depositional environments were studied, under anoxic conditions (western basin) and permanently oxic (Zaka Bay).

Materials and methods

Study site

Lake Bled is an urban, sub-alpine, dimictic lake of glacial-tectonic origin and located in NW Slovenia (46° 23' N, 14° 07' E). It occupies an area of 1.44 km^2 and has a maximum depth of 30.1 m (avg. 17.9 m). Additional hydromorphological data are given in Table 1. The lake is divided into two basins separated by an island - a deeper western basin (depth 30.5 m), and a 24 m deep eastern basin. Two small streams, Mišca and Solznik, constitute the surficial inflows in the W basin, with water outflow through the Jezernica stream into the River Sava. The lake is stratified most of the year, except during early spring, and the hypolimnion (< ca. 15 m) is anoxic during most of the year. In the shallower parts, O_2 excess down to the bottom is typical for the water column, due to biological activity. The general lack of wind circulation and the poor natural inflow have promoted the speed and intensity of eutrophication. For these reasons two amelioration projects were undertaken – introduction of a fresh water inflow from the river Radovna, and pumping of anoxic water from the eastern basin into the Jezernica. In addition, Bled's sewage system was renewed in 1985, resulting in a decreased inflow of wastewater into the lake. These improvements have contributed to the increase in water quality and Lake Bled is now classified as a mesotrophic lake according to OECD criteria (OECD, 1982). The predominant phytoplankton community in the water column is cyanobacteria (Cyanophyta) for most of the year, except in spring when golden algae (Chrysophyta) are the most abundant.

The sediment is composed largely of clayey silt with a carbonate content ranging from 55 to 80% and the OM content ranges from 5 to 10%. The highest sediment carbonate concentration is localized in the central, deepest part of the lake and in the shallow littoral platform in the SW, where calcite (chalk) is precipitated (Ogrinc et al., 2002; Ogorelec et al., 2006). Mineralogically, low -Mg calcite prevails, followed by dolomite, quartz (partially of diatomaceous origin) and feldspar. The clay minerals are composed of muscovite/illite and chlorite. The pyrite and low -Mg calcite are of authigenic origin. Sedimentation rate in the western and eastern basins, based on ^{210}Pb distributions, is 2.4 and 1.2 mm yr^{-1} (Čermelj et al. 1996).

Sampling

The samples were collected from the western part of the lake in November 2011 at two locations: (i) at a depth of 12 m in the thermocline (Zaka Bay) and in (ii) the anoxic

hypolimnion in the deepest part of the lake at a depth of 30 m (western basin). In Zaka Bay the water column is supersaturated with O_2 down to the bottom of the lake during most of the year. Undisturbed cores to a depth of 40 cm were taken using a gravity core sampler equipped with a Plexiglas tube (6 cm i.d.). Samples were cut from the cores and stored in glass jars, freeze-dried, homogenized and stored frozen until analysis.

Solid phase analysis

Organic carbon (OC, wt. %) and total nitrogen (TN, wt.%) were determined (relative precision ± 2%) following acidification (1M HCl) using a Elemental Vario MicroTube analyser at a combustion temperature of 1150 °C. Samples for $\delta^{13}C_{OC}$ and $\delta^{15}N_{TN}$ measurements were prepared as for elemental composition measurements, with a Europa Scientific 20-20 isotope ratio mass spectrometery (IRMS) instrument equipped with an ANCA-SL preparation module for solid and liquid samples. Analyses were calibrated against reference material: NBS 22 (oil), IAEA-CH6 and IAEA-CH7 for C, and IAEA–N1, IAEA-N2 and IAEA-NO3 for N. Values are reported relative to Vienna Peedee Belemnite (VPDB, $\delta^{13}C$) or AIR ($\delta^{15}N$). The precision of measurements was usually ± 0.2‰ for $\delta^{13}C_{OC}$ and ± 0.3‰ for $\delta^{15}N_{TN}$.

Lipid extraction and fractionation

All solvents and reagents (JT Baker, Mallinckrodt Baker) were of analytical grade. All glassware was rinsed with distilled water and solvent to prevent contamination. Dry sediment samples were weighed in cellulose extraction thimbles and extracted using the procedure described by Muri et al. (2004). In short, dry sediment (5-7 g) were extracted with CH_2Cl_2 for 8-10 h in a Soxhlet extractor. The extract was concentrated by a evaporator, dissolved in hexane, and fractionated on a glass silica gel column packed with 5 % deactivated silica (Sigma-Aldrich, 70-230 mesh). Aliphatic hydrocarbons, aliphatic alcohols, sterols and polar fatty acids (FAs) were eluted using 25 ml hexane, 20 ml 15 % EtOA in hexane, 20 ml 20 % EtOA in hexane and 20 ml MeOH. Elemental S was removed from the aliphatic hydrocarbon using activated Cu. Aliphatic alcohols and sterols were derivatized using BSTFA [bis(trimethylsilyl)trifluoroacetamide] and pyridine to yield the trimethylsilyl (TMS) ethers. Polar lipids were saponified with 0.5 M KOH in MeOH and the FAs released were methylated BF_3-MeOH to yield FA methyl esters (FAMEs). Each fraction was dried in a stream of N_2 and the residue dissolved in iso-

octane. Internal standards were used for quantification, with 5α-cholestane for aliphatic hydrocarbons, aliphatic alcohols and sterols, and C_{19} FAME for FAMEs.

Gas chromatography (GC) and GC-mass spectrometry (GC-MS)

A Hewlett-Packard 6890N gas chromatograph fitted with a DB 1 MS capillary column (60 m, 0.32 mm i.d., 0.25 µm) and a flame ionization detector (FID) were used for lipid analysis. Injector and detector temperature was 250 °C and 280 °C, respectively. The oven temperature was programmed from 120 °C to 300 °C (held 20 min) at 3 °C min^{-1}. Peak identity was confirmed using a Hewlett-Packard 6890 GC-MS instrument (Hewlett-Packard, Waldbron, Germany) equipped with a DB-5MS (30 m×0.25 mm i.d., 0.25 µm) column. He was used as carrier gas (36 cm s^{-1}). Samples (1 µl) were injected at 250 °C in splitless mode with the interface at 280 °C. The oven temperature program for hydrocarbon analysis was 40 °C to 300 °C (held 10 min) at 8 °C min^{-1}. That for alcohols, sterols and FAMEs was 50 °C (1 min) to 220 °C at 20 °C min^{-1} and then to 300 °C at 10°C min^{-1}. The MS instrument was operated in electron ionization (EI) mode over *m/z* 50 to 500. The precision of the method was 5-10%. The concentration of lipid biomarkers was normalized to sedimentary OC.

GC-Combustion (C) -IRMS

Isotopic composition of lipids was determined using an Isoprime GV GC-C-IRMS system. The gas chromatograph was fitted with a DB 1MS column (60 m, 0.32 mm i.d., 0.25 µm) and He was used as carrier gas. Samples in isooctane were injected at 120 °C and the oven was programmed to 300 °C (held 20 min) at 3 °C min^{-1}. Reproducibility and accuracy were evaluated routinely using reference material of known $\delta^{13}C$ values (*n*-alkanes mixed C1 and ^{13}C-enriched C_{16} FAME from Indiana University, USA). In addition C_{19} FAME was used as internal laboratory standard. Its $\delta^{13}C$ value was previously determined by elemental analyzer and isotope ratio mass spectrometer (EA-IRMS) Europa Scientific 20-20 equipped with ANCA-SL preparartion module. Typically, one injection of reference material was performed for every eight sample analyses. The precision of the GC-C-IRMS measurements ranged from 0.3 to 0.5‰, while the accuracy ranged from 0.2 to 1.0‰. Corrections for the isotope change introduced in the derivatization of sterols and FA were determined through derivatization of standards of known isotope composition and by applying the equation of Jones et al. (1991).

Results and discussion

Bulk organic parameters

The concentrations of OC and OC/TN, $\delta^{13}C_{OC}$ and $\delta^{15}N_{TN}$ values for the western basin and Zaka Bay are presented in Fig. 1. OC ranged from 3.0 to 5.5 wt.% (total dry sediment) in the western basin, and OC/TN from 10.4 to 16.7. The highest OC/TN values were in the sediments below 25 cm suggesting a higher proportion of terrestrial input and/or variation in N degradation. The $\delta^{13}C_{OC}$ values ranged from −34.8 to −31.5‰. Such low values are probably a consequence of the sedimentation of anaerobic microbial biomass often found in eutrophic lakes in strongly reductive sediments (Teranes and Bernasconi, 2005; Conrad et al. 2010). Biomass from methanotrophic bacteria had very low $\delta^{13}C$ values, because the CO_2 utilized by bacteria is strongly depleted in ^{13}C following CH_4 oxidation (−70 ± 15‰; Lehmann et al., 2002; Mandić-Mulec et al., 2012). The $\delta^{15}N_{TN}$ values ranged from +3.6 to +7.6‰ (Fig. 1) indicating the prevalence of phytoplankton in the sedimentary OM (Bratkič et al. 2012). In Zaka Bay the OC content was lower vs. the western basin, ranging from 2.4 to 4.8 wt.%. OC/TN values ranged from 11.1 to 14.7, with the highest value at a depth of 30-32 cm. The $\delta^{13}C_{OC}$ values were higher than those in the western basin, ranging from −31.3 to −29.4‰; $\delta^{15}N_{TN}$ values were less variable, with an average of +5.8 ± 0.4‰.

The correlation between OC and TN at both locations was high and could be described with the following linear equations: OC (wt. %) = 7.41 · TN (wt. %) + 1.05; r^2 = 0.62 in the western basin and OC (wt. %) = 7.47 · TN(wt. %) + 1.18; r^2 = 0.83 in Zaka Bay. This indicated a close relationship between the two parameters. The intercept values of 1.05 and 1.18 indicate that there could be some inorganic N in the samples and that a slope-derived OC/TN value would not be representative of the bulk OM in these samples. Poor correlation between both $\delta^{13}C_{OC}$ and $\delta^{15}N_{TN}$ and OC/TN means that OC/TN values could not be used to evaluate the sources of OM. The latter could be better estimated using $\delta^{13}C_{OC}$. A $\delta^{13}C$ value of −26‰ could be taken for terrestrial OM and, for autochthonous OM, a value of −38‰ (Bratkič et al., 2012). Based on an isotope mass balance it was estimated that OM was more than 70 % autochthonous in the western basin whereas, in Zaka Bay, terrestrial OM was more pronounced and autochthonous OM was < 40%.

Lipid composition

The concentration of total lipids (TLs) comprising total aliphatic hydrocarbons (THCs), total aliphatic alcohols (TOHs), total sterols (TSTs) and total fatty acids (TFAs) in the western basin decreased with depth to 14 cm (from 12,002 μg g^{-1} OC to 2,785 μg g^{-1} OC), below which it increased to 10,027 μg g^{-1} OC at 18 cm. At 31 cm depth the concentration reached a minimum at 2,101 μg g^{-1} OC. The lipid composition (Fig. 2) also changed with depth. In the surface sediment layer TSTs were the most abundant lipids, constituting 49% of the total lipid concentration. TSTs decreased steeply with depth. THCs were also abundant but the concentration was highest at 2-3 cm. A higher concentration was also determined at 20-22 cm. THC abundances did not change significantly downcore (25% to 13%), while the TOHs increased slightly with depth, constituting from 9% to 14% of TLs. TFAs constituted the smallest portion of the TLs (6% to 0.7%) and their abundance and composition did not differ significantly with depth. The distribution of lipids matched well the data presented by Muri and Wakeham (2006). A different distribution of lipids was found in sediments in the eutrophic Lake Lugano (Switzerland) in which TFAs constituted the main fraction of TLs (Bechtel and Schubert, 2009).

The distribution of TLs concentration in Zaka Bay differed from that in the western basin. TLs concentrations were quite uniform, with the highest concentration of 4,601 μg g^{-1} OC determined at the surface of sediments. Here TOHs, at up to 68%, constituted the largest fraction of TLs. The second most abundant class of lipids were the TSTs, followed by THCs and TFAs. TSTs concentrations ranged from 470 to 1284 μg g^{-1} OC, contributing, on average, 40% of TL.s THCs and TFAs constituted ca. 12% and 6%, respectively. Sources of lipids were determined using the stable isotope composition, which was highly variable, ranging from −71.9 to −21.5‰. All the results are presented in Tables S1 and S2 (Supplementary material), while the major sources of individual compounds are described below.

n-Aliphatic hydrocarbons

The aliphatic hydrocarbons in sediments from the western basin ranged from C_{17} to C_{35}, with significant odd predominance in most cases. The most abundant was *n*-C_{17}, which is usually produced by cyanobacteria and other eukaryotic algae (Meyers and Ishiwatari, 1993). This assumption was supported by the $\delta^{13}C$ values, which ranged from −50.7 to −35.8‰ (Fig. 3) in the sediment depth profile, indicating a variety of source origins. At

depths of 14-16 cm the influence of methanotrophic archaea could not be excluded. The short -chain *n*-alkanes (C_{13}-C_{20}) are generally of autochthonous origins, but the $\delta^{13}C$ values, which ranged from −33.8 to −25.2‰, showed that here they were of mixed origin. The highest proportion of an autochthonous origin was found in *n*-C_{19}, while all other *n*-alkanes contain more allochthonous OM. Longer chain *n*-alkanes were more abundant deeper in sediments, especially *n*-C_{27}, *n*-C_{29} and *n*-C_{31}, which are generally derived from a terrestrial source of higher plant wax (Doskey, 2001; Meyers, 2003). However $\delta^{13}C$ values were more negative than for the short chain *n*-alkanes and, on average, lower than typical terrestrial OM $\delta^{13}C$ value of −26‰. These data thus indicate that the long -chain *n*-alkanes were mainly of autochthonous origin. Published compositions of hydrocarbons in cultures of microalgae are rare, so it is not clear whether or not such compounds also have a bacterial or microalgal origin. Available evidence suggests that microalgae, including diatoms, are one possible source (Volkman et al., 1983). Long -chain C_{24}-C_{35} *n*-alkanes with no odd predominance have also been found in the sulfate-reducing bacterium *Desulfovibrio desulfuricans* (Davis, 1968). Despite these occurrences, few studies report the presence of algal-derived long -chain *n*-alkanes in sediments (Wakeham et al., 1991). Hopanoids are among the most diagnostic biomarkers for bacteria originating from diverse taxa including cyanobacteria, purple non-sulfur bacteria, methano- and methylotrophs, and nitrifying, anammox and sulfate reducing bacteria (Cvejic et al., 2000; Thiel et al., 2003; Sinninghe Damsté et al., 2004; Blumenberg et al., 2006; 2007). In the Bled sediments, hopanoids with $\delta^{13}C$ values from −71.9 to −53.1‰ can be considered a product of methanotrophic bacteria, since the $\delta^{13}C$ of methane determined in the anoxic part of the lake was about −70‰ (Lojen et al., 1999; Ogrinc et al., 2002; Mandić-Mulec et al., 2012). A similar range of hopanoid $\delta^{13}C$ values was also observed by Neunlist et al. (2002). Another important ^{13}C depleted alkane in the surface sediment layer is 7-methyl-C_{17}, usually considered to be of cyanobacterial origin (Shiea et al. 1990); however its low $\delta^{13}C$ values of −61.5 and −66.9‰ reflect a methanotrophic source. Higher $\delta^{13}C$ values were observed deeper in the sediments (Table S1), clearly showing that this compound had a different source, probably chemoautotrophic bacteria. Our previous study showed a pronounced change in microbial community at this depth. Archaeal sequences were detected, comprising mostly those of unknown affiliation with Euryarchaeota, Thermoplasmatales and related linkages, with only 38% of methanogenic archaea (Mandić-Mulec et al. 2012). Isoprenoid pristane was found only in the surface sediments, while phytane was observed deeper down. Pristane is produced primarily in

the digestive tract of copepods from the phytol derived from the side chain of chlorophyll (Shi et al. 2001), while phytane is produced by anaerobic microorganisms (Risatti et al. 1984). These two isoprenoid alkanes have multiple sources, including petroleum; however $\delta^{13}C$ values of −28.3 and −27.0‰ indicate that pristane was mainly of mixed origin. On the other hand, phytane exhibited lower $\delta^{13}C$ values, with average of −37.1 ± 0.7‰, showing that a contribution from anaerobic bacterial origin cannot be ignored.

Long -chain *n*-alkanes also prevailed (C_{25}-C_{33}) in Zaka Bay, with *n*-C_{27} the most abundant. The $\delta^{13}C$ values of the short -chain *n*-alkanes range from −32.9 to −26.3‰ (Fig. 4) and were higher than for the western basin. A more pronounced influence of terrestrial OM was indicated, which could be transported to sediments with soil OM delivered by the Solznik stream. The $\delta^{13}C$ values did not vary significantly with sediment depth and were diagenetically resistant, since they had already been partially degraded in the lake surroundings and inflows (Meyers and Ishiwatari, 1993). The long -chain *n*-alkanes exhibited lower $\delta^{13}C$ values than the short -chain members, ranging from −39.4 to −29.5‰ (Fig. 4), similar to those found in the western basin, indicating the pronounced influence of autochthonous OM. The lowest values were for the *n*-C_{33} alkane. For Zaka Bay sediments, it was possible to determine the values for *n*-C_{26}, which had a relatively high average $\delta^{13}C$ value of −24.2 ± 0.6‰, indicating a possible macrophyte origin. A relatively low concentration of 7-methyl-C_{17} was observed in Zaka Bay sediments; so only for the surface sediments $\delta^{13}C$ values could be determined. They were lower than for the western basin, ranging from −48.0 to −44.3‰, and may be considered to be of cyanobacterial origin. The only biomarkers originating from methanotrophic bacteria were hopanoids, with a abundances below 10 cm, where anoxic conditions started to prevail (Ogrinc et al., 2002). Their $\delta^{13}C$ values ranged from −69.4 to −61.1‰. The two isoprenoid alkanes were also present in sediments, but their abundances were too low for determination of $\delta^{13}C$ values.

n-Alkanols and steroidal alcohols

In the western basin sediments long-chain components predominated strongly (*n*-C_{22} *to n*-C_{30}), peaking at *n*-C_{28}, while in surface sediments *n*-C_{26} prevailed. While short -chain *n*-alkanols (C_{14}-C_{20}) are usually indicative of algal or bacterial inputs (Albro, 1976; Weete, 1976), long -chain ones are generally derived from an allochthonous sources. However, the $\delta^{13}C$ values from −38.6 to −26.6‰, did not support this assumption but indicated a

mixed autochthonous and allochthonous origin. The lower $\delta^{13}C$ values for the long -chain *n*-alcohols (*n*-C_{28} lowest) suggested that bacteria could be the source. Saturated *n*-C_{22} *to* *n*-C_{28} even *n*-alcohols have been identified in the heterocyst glycolipids of cyanobacteria (Abreu-Grobois et al., 1977). On the other hand, zooplankton are a potential source of *n*-C_{16} to *n*-C_{20} saturated and monosaturated alcohols in sediments and could also contribute to our sediments. The finding of higher $\delta^{13}C$ values for the short -chain components supports this assumption. Higher values, averaging –28.5 ± 1.2‰, were also observed in *n*-C_{26}. Phytol was the only branched alkanol present in amounts sufficient for isotope determination. The values ranged from –41.6 to –39.6‰, indicating multiple sources. Such low values suggested that the chlorophylls of cyanobacteria or purple sulfur bacteria were the main source for phytol. However, it cannot be excluded that the algae in the lower water column were also depleted in ^{13}C and that their chlorophyll was also a source of the sedimentary phytol (Bratkič et al., 2012). A similar observation has been made for Lake Cisó where phytol was inferred to derive from purple sulfur bacteria (Hartgers et al., 2000).

The *n*-alcohols in Zaka Bay sediments exhibited a similar range of $\delta^{13}C$ values, from –39.7 to –26.3‰, with the highest values being in short -chain ones. These data indicate mixed sources with a more pronounced autochthonous contribution. The highest $\delta^{13}C$ values were for *n*-C_{26} and with greater variability than in the western basin sediments (avg. –29.9 ± 3.2‰). The concentration of phytol in Zaka Bay sediments was too low for determination of its isotope composition.

The sterol $\delta^{13}C$ values (–57.7 to –29.4‰) clearly indicate their in-situ formation and a significant diversity and variability in the source organisms. The most depleted compounds were 5α-cholestan-3β-ol and 24-ethyl-5α-cholestan-3β-ol with values ranging from –57.7 to –41.0‰. The western basin, with its anoxic conditions, is an ideal environment for reduction of Δ-stenols to 5α –stanols (Wakeham, 1995), as is also evident from the high stanol/stenol ratio in trap material from 28 m depth (Gams Petrišič and Ogrinc, 2013). In addition, 24-ethyl-5α-cholestan-3β-ol was the most ^{13}C-depleted sterol with a value of –58.0‰ for this sample. Such low values further indicated a significant influence of microbial reworking, such as the reduction of Δ^5 bond (Nishimura, 1978; Gagosian et al., 1980). C_{27} sterols were the most abundant in surface sediments. They exhibited $\delta^{13}C$ values of around –40‰ and over a wide range ($\Delta\delta^{13}C$ of 6‰), reflecting their mixed origin. This was not really surprising since C_{27} sterols have poor specificity and can be found in many organisms including diatoms (Mackenzie et al.,

1982; Volkman et al. 1998; 1999, Volkman, 2003). Further high ^{13}C depletion was also observed for C_{29} sterols, which is not compatible with the generally accepted origin from higher plants and/or marsh grasses (Canuel et al., 1997), so multiple sources including bacteria, have been suggested. The highest values were for 24-ethylcholest-5-en-3β-ol (avg. −31.6 ± 1.1‰). All these data indicated that the C_{29} sterols were mainly of autochthonous origin and that algae and bacteria played an important role in its production. There are a number of examples from microalgae and cyanobacteria in which the main sterol is 24-ethylcholesterol or 24-ethylcholesta-5,22E-dien-3β-ol (Barett et al., 1995; Volkman, 1986). C_{28} sterols gave values around −38‰ and probably arose from the constant algal source. Sterols used as sewage indicators, such as 5β-cholestan-3β-ol (coprostanol) and 24-ethyl coprostanol (Tolosa et al., 2008), also exhibited low values ranging from −44.1 to −38.5‰, indicating a mixed origin in which a microbial contribution has to be considered.

A wide range of $\delta^{13}C$ values (−46.4 to −29.1‰) was also found in the sterols from Zaka Bay sediments. As in the western basin sediments, the lowest values were also found for 5α-cholestan-3β-ol and 24-ethyl-5α-cholestan-3β-ol, but at a sediment depth below 10 cm where anoxic conditions prevailed (Ogrinc et al., 2002). Similar values were also obtained for 24-ethylcholest-5-en-3β-ol (avg. −33.0 ± 0.9‰). The average $\delta^{13}C$ values found for C_{27}, C_{28} and C_{29} sterols were: −37.9 ± 1.8‰, −36.6 ± 2.7‰ and −37.0± 3.2‰ – higher, on average, than the $\delta^{13}C$ values observed in the western basin sediments. These data indicate that the sterols in Zaka Bay had a similar source, mainly from algae.

n-Fatty acids

FAs constituted only a minor fraction of the TLs, so $\delta^{13}C$ values were obtained mainly for saturated FAs. $C_{16:0}$ and $C_{26:0}$ were the most abundant and differed in their average $\delta^{13}C$ values: −29.5 ± 0.9 for $C_{16:0}$ and −41.5 ± 1.0‰ for $C_{26:0}$. The data therefore indicated different sources – algae for $C_{16:0}$ and bacteria for $C_{26:0}$. Indeed, 16:0 and 18:0 have both been assigned prevalently to microalgae (Cranwell et al. 1987) and the results were also comparable with those obtained from anoxic areas in the Black Sea (Wakeham et al., 2007). Short -chain FAs had higher $\delta^{13}C$ values than long -chain FAs, the highest values being observed for $C_{14:0}$ (−23.5 ± 1.2‰). The significant ^{13}C-depletion for the higher homologues, from 22:0 to 26:0, was unexpected since these are frequently assigned to terrestrial plants (Rieley et al. 1991). The results indicated that they were derived from

bacteria (chemoautotrophic or cyanobacteria). A relatively high concentration of mono-unsaturated *n*-acids was observed in the western basin sediment, where 16:1*n-7* and 18:1 (*n*JT and *n*JV isomers) were the most abundant. On the other hand, a low level of branched methyl-substituted FAs was detected. Determination of the isotope composition of 16:1*n-7* and 18:1 (*n*JT and *n*JV isomers), *iso*-$C_{14:0}$ and *iso*-$C_{15:0}$ was only possible for the surface sediments. The lowest values were for the 16:1*n-7* FAs (–44.8 and –51.4‰), indicating a more pronounced influence of methanotrophic bacteria. The latter can be differentiated between Type I (rich in 16:1*n-7*) and Type II (rich in 18:1*n-7* and 18:1*n-8*) based on the FAs distribution (Wakeham et al., 1997; Niggemann and Schubert, 2006). However the present data were not sufficiently adequate for distinguishing between Type I and Type II methanotroph contributions. The values for 18:1*n-7* and 18:1*n-9* (–40.6‰ and –42.9‰) were higher than those for 16:1*n-7*, showing the less pronounced influence of methanotrophic bacteria and more pronounced influence of cyanobacteria (Hartgers et al., 2000). *iso*-$C_{15:0}$ has been found in cultures of cyanobacteria and sulfate-reducing bacteria (Wakeham et al. 2003) and here had a value of –38.7‰. Culture experiments have shown that this particular lipid is ca. 15‰ depleted vs. the substrate (Mather et al. 1997), suggesting that in Lake Bled the isotope composition of living substrate was ca. –24‰ using terrestrial or possibly algal cell material.

In Zaka Bay sediments, $\delta^{13}C$ values were determined only for saturated FAs, which ranged from –38.3 to –23.6‰. The highest values were for $C_{14:0}$ (avg. –25.6 ± 1.4‰), suggesting an important influence of terrestrial OM. Short -chain FAs were present only in the upper part of the sediments and were ^{13}C-enriched relative to long -chain FAs. It is interesting to note that the values for 16:0 and 18:0 (avg. –26.5 ± 10.9‰ and –28.4 ± 1.4‰) were comparable to those for the western basin. Thus, the sources of these FAs were similar and not indicative of different depositional regimes. The presence and isotope composition of long -chain FAs deeper in the sediments indicates their in-situ formation from algae and bacteria.

Conclusions

The sources of lipid compounds in Lake Bled sediments with different oxic (Zaka Bay) and anoxic (western basin) depositional regimes have been determined from their carbon isotope compositions. Redox conditions appear to be a key factor governing the preservation, formation and degradation of the OM and thus, not surprisingly, influenced

the lipid distribution. The isotope data indicated that, in both environments, OM was derived from mixed sources, plankton and terrestrial and an anaerobic-microbial. The similarity of $\delta^{13}C$ values for long -chain *n*-alkanes, short - and long -chain *n*-alcohols and short -chain FAs in sediments obtained from the two sites indicated that these compounds had similar sources and were not dependent on different depositional regimes. The highest $\delta^{13}C$ values were observed for short -chain FAs in both environments, indicating higher proportions of terrestrial OM. The increased, more refractory, terrestrial material brought by the Solznik stream could be also seen in the higher $\delta^{13}C$ values for short -chain *n*-alkanes in Zaka Bay sediments. On the other hand the average $\delta^{13}C$ values for long -chain FAs, and especially those of sterols, were lower in the anoxic western basin, indicating a more pronounced contribution of anaerobic (methanotrophic) microbial origin than for Zaka Bay.

It has been suggested that source assignment based on lipid composition was in sharp disagreement with the origin of bulk OM (Muri and Wakeham, 2006) probably due to selective degradation of lipids. On the contrary our results are in a good agreement with a source allocation based on bulk $\delta^{13}C$ values and OC/TN values. It was found that, in both environments, autochthonous OM was the main source of lipid compounds. This was evident in the low $\delta^{13}C$ values for long -chain *n*-alkanes, *n*-alcohols and FAs and, especially, in those determined in C_{29} sterols, indicating that these compounds were derived mainly from microalgae and bacteria and not from higher plants or other terrigenous OM, as previously reported. Our results indicated that statements regarding the source of OM based only on lipid distribution have to be treated with caution and should not be used without the input from stable isotope geochemistry.

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Table 1. Hydromorphological characteristics of Lake Bled

Characteristic	Values
Latitude	46°22´30˝N
Longitude	14°07´30˝E
Area (km^2)	1.44
Maximum depth (m)	30.1
Mean depth (m)	17.9
Volume (m^3)	26×10^6
Catchment area	8.1 m^2
Water renewal (yr)	1.5 (since 1964)
	3.6 (before 1964)
Deoxygeneation	Yes
Freezing	Yes
Total phosphorous (μg/l)	20

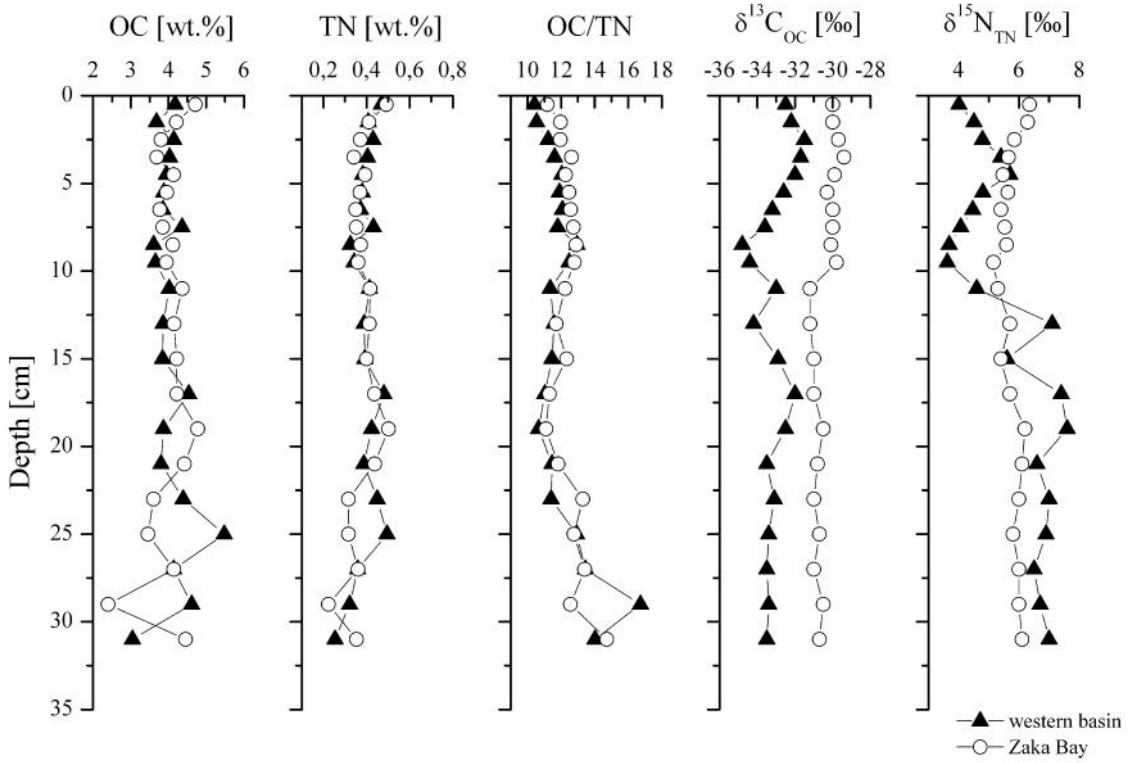

Fig. 1. Depth profiles of concentrations of organic carbon (OC) and total nitrogen (TN), and OC/TN and $\delta^{13}C_{OC}$ and $\delta^{15}N_{TN}$ in the western basin and Zaka Bay sediments from Lake Bled.

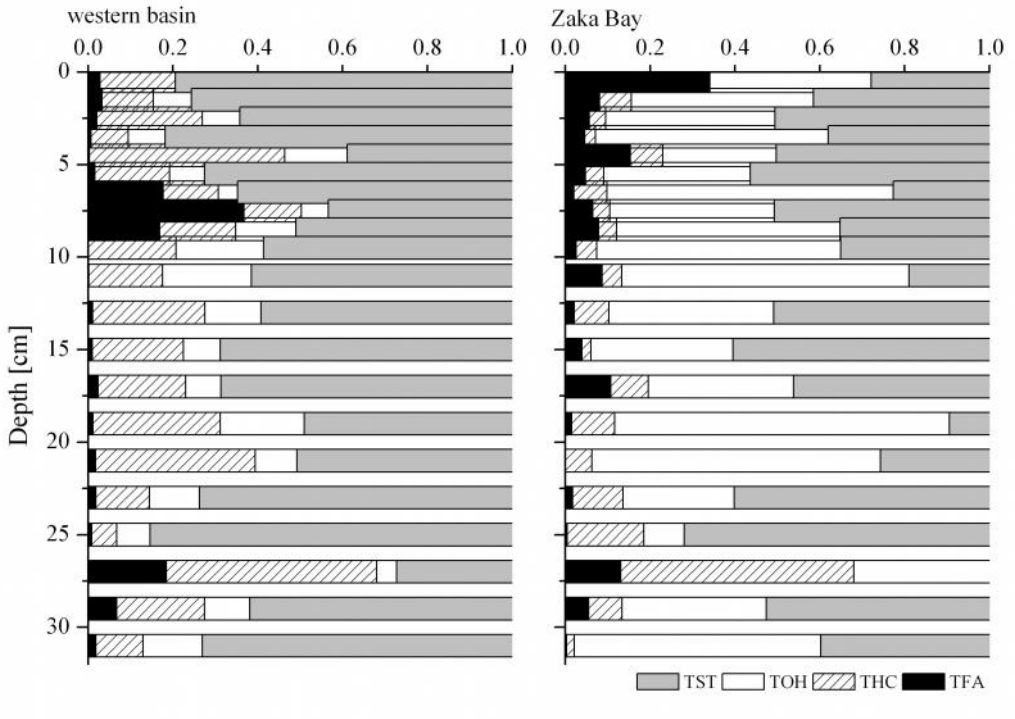

Fig. 2. Composition of lipids in western basin and Zaka Bay sediments (THC, total aliphatic hydrocarbons; TOH, total aliphatic alcohols; TST, total sterols; and TFA, total fatty acids).

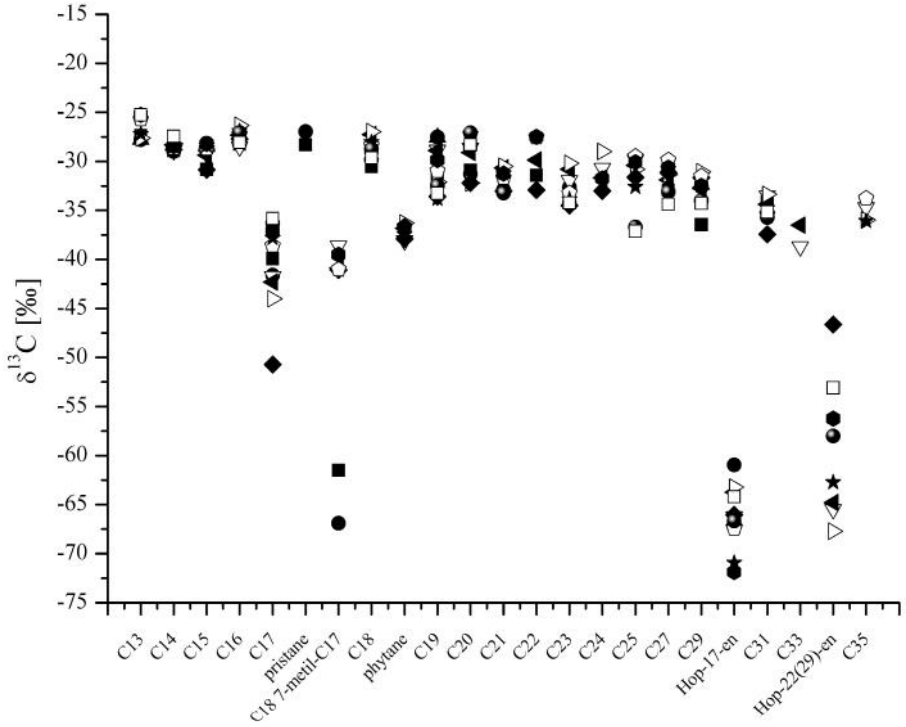

Fig. 3. Carbon isotopic composition of *n*-alkanes in sediments in western basin from Lake Bled.

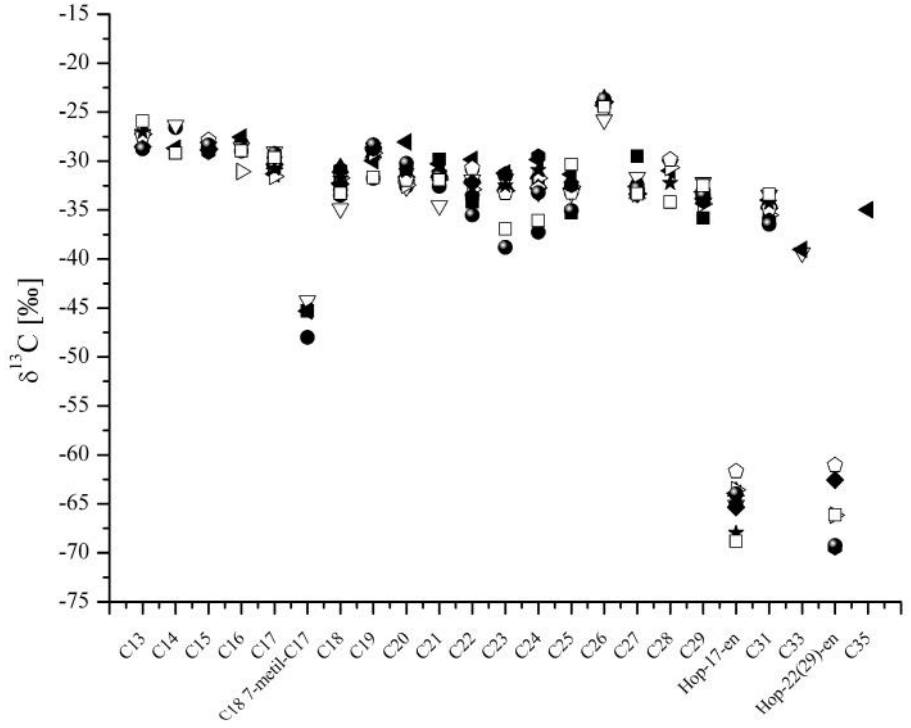

Fig. 4. Carbon isotopic composition of *n*-alkanes in Zaka Bay sediments from Lake Bled.

Table S1. Isotopic composition of lipid compounds in anoxic lake sediments in western basin of Lake Bled.

Compound	**¢^{13}C (‰)**											
	Sediment depth (cm)											
	0-1	**1-2**	**3-4**	**5-6**	**9-10**	**10-12**	**14-16**	**16-18**	**18-20**	**20-22**	**24-26**	**30-32**
Alkanes												
C13	-27.4	-27.8	-27.8	-	-	-27.6	-	-25.7	-25.2	-27.2	-	-25.3
C14	-28.9	-29.0	-	-28.9	-28.3	-28.2	-28.8	-28.6	-28.4	-28.5	-	-27.4
C15	-30.8	-28.1	-28.5	-29.2	-29.4		-30.8	-28.7	-28.2	-28.4	-	-
C16	-28.3	-28.2	-	-28.5	-27.3	-26.3	-27.7	-27.2	-27.6	-26.9	-27.1	-28.1
C17	-39.9	-41.6	-	-41.7	-42.3	-44.0	-50.7	-38.6	-37.1	-37.8	-36.3	-35.8
pristane	-28.3	-27.0	-	-	-	-	-	-	-	-	-	-
C18 7-metil-C17	-61.5	-66.9	-	-38.6	-40.9	-	-41.1	-41.0	-39.5	-	-	-
C18	-30.5	-29.8	-	-	-27.3	-27.0	-	-28.4	-28.9	-28.1	-28.7	-29.6
phytane	-	-	-	-38.1	-36.8	-36.3	-37.9	-36.7	-36.7	-37.5	-	-
C19	-31.9	-27.5	-27.6	-28.9	-28.9	-32.1	-33.6	-31.0	-29.9	-33.8	-32.5	-33.2
C20	-30.9	-31.3	-	-28.7	-29.1	-32.2	-32.2	-27.2	-28.5	-27.3	-27.1	-28.3
C21	-	-32.9	-	-	-30.7	-30.5	-33.0	-31.7	-31.2	-31.2	-33.2	
C22	-31.4	-	-	-	-29.9	-	-32.9	-27.5	-27.5	-	-	-
C23	-	-32.7	-	-31.9	-30.8	-30.2	-34.5	-33.2	-34.4	-33.9	-34.4	-34.2
C24	-	-	-	-30.7	-31.7	-29.0	-33.0	-	-31.7	-	-	-
C25	-	-	-	-29.8	-30.4	-30.8	-31.6	-29.5	-30.1	-32.6	-36.7	-37.1
C27	-32.9	-	-	-	-31.9	-31.3	-31.2	-29.9	-30.6	-30.8	-33.1	-34.4
C29	-36.5	-32.6	-32.4	-32.1	-32.7	-31.1	-31.7	-31.5	-32.5		-34.0	-34.3
Hop-17-en	-	-61.0	-66.6	-66.2	-63.7	-63.2	-66.0	-67.4	-71.9	-70.9	-66.7	-64.2
C31	-34.2	-	-	-33.5	-34.4	-33.4	-37.4	-35.4	-35.4	-34.8	-35.7	-35.2
C33	-	-	-	-38.7	-36.5	-	-	-	-	-	-	-
Hop-22(29)-en	-	-	-	-65.5	-64.8	-67.7	-46.6		-56.2	-62.7	-58.0	-53.1

C35	-	-	-	-34.7	-	-36.0	-	-33.8	-	-36.1	-	-
Alcohols												
14-ol	-29.7	-30.2	-30.4	-29.9	-29.5	-29.8	-	-	-28.8	-	-	-
16-ol	-31.1	-30.0	-30.0	-29.7	-29.1	-29.6	-29.0	-28.4	-28.6	-29.6	-29.4	-30.9
18-ol	-29.7	-29.9	-30.0	-29.3	-29.5	-29.7	-29.5	-29.2	-29.5	-29.9	-30.0	-30.7
phytol	-39.9	-41.4	-40.4	-39.6	-	-	-42.5	-40.0	-41.3	-41.6	-40.5	-
20-ol	-35.3	-34.9	-34.9	-35.3	-34.3	-35.0	-35.4	-33.8	-35.2	-37.2	-	-
22-ol	-35.4	-35.3	-35.0	-33.9	-34.5	-32.3	-32.9	-31.1	-31.4	-33.8	-37.6	-35.6
24-ol	-33.5	-33.1	-33.0	-33.3	-32.3	-32.6	-32.3	-32.2	-31.9	-33.2	-35.6	-37.2
26-ol	-28.0	-28.0	-27.6	-27.7	-28.0	-26.6	-28.4	-28.4	-28.4	-31.1	-29.1	-30.2
28-ol	-36.8	-37.4	-37.1	-38.0	-37.2	-37.8	-38.0	-37.6	-37.8	-37.9	-38.1	-38.6
Sterols												
cholest-7-ene 5α ($C_{27}H_{46}O$)	-39.8	-34.8	-	-34.5	-38.6	-43.8	-44.7	-44.4	-45.5	-45.2	-46.5	-45.8
5α-cholest-3α-ol	-	-32.2	-	-33.1	-34.8	-39.0	-39.4	-40.0	-45.3	-45.9	-39.2	-
cholesta-5,22E-dien-3b-ol	-46.0	-39.4	-37.5	-37.5	-	-	-	-	-	-	-	-
cholest-5-en-3β-ol	-40.9	-37.4	-38.8	-37.8	-39.0	-37.8	-38.4	-38.9	-38.9	-39.0	-38.4	-
5a(H)-cholestan-3β-ol	-	-45.7	-47.3	-49.2	-46.1	-51.9	-50.9	-57.7	-56.3	-56.4	-53.1	-
5b-cholestan-3β-ol	-41.4	-41.8	-42.4	-42.3	-40.9	-42.3	-41.8	-40.9	-39.4		-38.5	-38.5
24-methylcholesta-5,22E-dien-3β-ol	-37.7	-43.1	-41.7	-41.7	-	-30.7	-30.8	-31.6	-29.9	-32.7	-43.5	-42.2
24-methyl-5a-cholestan-3b-ol	-40.8	-	-40.2	-	-	-35.8	-35.4	-37.4	-	-	-38.1	-
24-methylcholest-5-en-3β-ol	-39.1	-39.8	-40.7	-39.1	-39.5	-	-	-	-	-	-	-38.5
24-ethylcholesta-5,22E-dien-3β-ol	-41.9	-40.7	-41.3	-41.1	-39.6	-	-	-	-	-	-	-
24-ethyl-5β-cholestan-3β-ol	-41.0	-43.5	-44.1	-42.0	-43.9	-41.9	-41.6	-41.7	-41.6	-	-42.7	-41.5
24-ethylcholest-5-en-3β-ol	-29.4	-31.7	-32.0	-32.4	-32.6	-	-	-	-	-	-31.5	-
24-ethyl-5α(H)-cholestan-3β-ol	-41.0	-43.5	-42.4	-43.9	-42.7	-	-44.3	-44.9	-44.4	-46.4	-54.5	-
Fatty acids												
12:0	-30.3	-29.5	-	-27.1	-	-	-	-	-	-	-	-
iso-14:0	-32.0	-29.1	-	-28.0	-	-	-29.5	-31.1	-	-34.3	-32.8	-
14:0	-23.2	-21.5	-23.5	-23.3	-	-25.5	-	-23.7	-23.5	-	-24.8	-
iso-C15:0	-38.7			-	-	-	-	-	-	-	-	-

15:0	-26.4	-26.3	-26.8	-	-	-26.4	-	-	-	-	-	-
16:1n-7	-44.8	-51.4		-	-	-	-	-	-	-	-	-
16:0	-27.3	-26.3	-28.3	-26.8	-	-27.3	-27.2	-26.9	-26.7	-28.6	-29.1	-27.6
17:0	-38.3	-37.8	-	-	-	-	-	-	-	-	-	-
C18:1n-7	-40.6	-	-	-	-	-	-	-	-	-	-	-
C18:1n-9	-42.8	-	-	-	-	-	-	-	-	-	-	-
18:0	-27.8	-26.9	-26.4	-26.7	-	-27.7	-27.6	-28.1	-28.0	-30.8	-29.4	-28.6
21:0	-	-30.9			-	-	-	-	-	-	-	-
22:0	-29.5	-30.7	-28.0	-28.1	-	-	-	-28.1	-28.5	-	-	-
23:0	-	-	-	-28.5	-	-	-	-	-	-27.6	-	-
24:0	-37.6	-36.3	-42.6	-	-	-	-36.6	-	-37.7	-36.9	-39.0	-
25:0	-38.0	-29.4	-	-	-	-	-	-	-	-	-	-
26:0	-	-42.7	-41.6	-	-	-	-	-	-41.5	-40.0	-41.6	-

Table S2. Isotopic composition of lipid compounds in oxic lake sediments in Zaka Bay of Lake Bled.

Compound	**¢^{13}C (‰)**											
	Sediment depth (cm)											
	2-3	**3-4**	**4-5**	**5-6**	**8-9**	**9-10**	**12-14**	**14-16**	**16-18**	**18-20**	**22-24**	**30-32**
Alkanes												
C13	-	-	-26.8	-27.3	-	-27.2	-28.5	-27.4	-28.7	-27.0	-28.8	-25.9
C14	-	-26.6	-	-26.3	-28.7	-	-	-	-	-	-	-29.2
C15	-	-29.0	-28.4	-28.5	-	-28.8	-28.9	-27.9	-29.1	-28.8	-28.4	-
C16	-	-28.2	-	-28.6	-27.5	-31.1	-	-	-	-	-29.0	-29.0
C17	-30.8	-30.1	-30.3	-29.0	-31.3	-31.5	-30.3	-29.8	-29.6	-30.8	-29.2	-29.6
C18 7-metil-C17	-45.3	-48.0	-	-44.3	-45.3	-	-	-	-	-	-	-
C18	-31.5	-32.9	-30.7	-34.9	-32.3	-31.7	-32.3	-31.3	-30.8	-31.8	-33.5	-33.3
C19	-29.3	-31.8	-29.2	-29.3	-30.0	-29.2	-28.6	-28.4	-28.7	-28.8	-28.3	-31.7
C20	-31.1	-32.6	-31.5	-32.6	-28.1	-32.4	-31.6	-31.9	-30.7	-31.0	-30.2	
C21	-29.8	-32.6	-31.0	-34.6	-30.3	-31.9	-31.6	-31.3	-31.3	-32.2	-31.6	-31.9

C22	-34.2	-30.8	-31.6	-31.9	-29.8	-32.9	-32.2	-30.7	-33.6	-33.7	-35.5	
C23	-33.1	-31.5	-32.5	-31.8	-31.2	-33.0	-33.0	-33.2	-31.3	-32.5	-38.8	-36.9
C24	-32.5	-37.2	-31.3	-33.2	-29.9	-31.7	-32.7	-32.8	-29.5	-30.9	-33.2	-36.1
C25	-35.3	-33.2	-32.4	-33.2	-31.3	-32.6	-32.8	-33.2	-32.4	-32.4	-35.0	-30.3
C26	-	-	-23.6	-25.8	-24.3	-23.9	-24.0	-24.1	-24.2	-24.1	-23.7	-24.5
C27	-29.5	-32.4	-33.3	-31.7	-32.6	-33.3	-33.4	-33.2	-32.9	-33.0	-32.8	-33.3
C28	-	-	-	-30.4	-31.0	-30.7	-	-29.9	-	-32.2	-	-34.2
C29	-35.8	-33.0	-33.9	-32.2	-33.1	-34.4	-33.8	-33.7	-34.1	-33.7	-33.5	-32.5
Hop-17-en	-	-65.2	-63.7	-65.1	-63.9	-63.6	-65.3	-61.7	-65.2	-68.0	-64.0	-68.8
C31	-35.4	-34.5	-34.2	-34.5	-34.0	-35.5	-34.8	-34.9	-36.1	-34.3	-36.4	-33.4
C33	-	-	-	-39.4	-39.0	-	-	-	-	-	-	-
Hop-22(29)-en	-	-	-	-	-	-66.2	-62.6	-61.1	-69.4	-	-69.3	-66.1
C35	-	-	-	-	-35.0	-	-	-	-	-	-	-
Alcohols												
14-ol	-29.6	-29.8	-	-	-	-	-	-	-	-	-	-
16-ol	-29.3	-29.3	-28.8	-29.3	-29.3	-	-	-29.6	-29.1	-	-	-
18-ol	-29.1	-29.4	-29.1	-32.4	-33.2	-31.2	-33.9	-33.1	-33.0	-33.1	-33.2	-33.6
20-ol	-	-	-	-32.2	-31.7	-31.0	-32.3	-32.6	-32.3	-32.7	-33.1	-32.0
22-ol	-33.9	-	-	-37.7	-36.7	-	-36.2	-39.3	-39.7	-39.0	-39.2	-38.7
24-ol	-34.9	-	-32.6	-31.0	-30.2	-30.6	-	-32.2	-30.1	-31.6	-31.2	-
26-ol	-33.3	-33.5	-33.2	-33.7	-33.5	-29.6	-27.6	-28.0	-26.6	-26.3	-26.6	-26.4
28-ol	-29.5	-29.4	-30.2	-30.8	-31.0	-36.1	-36.3	-36.5	-35.9	-36.5	-36.1	-36.4
Sterols												
cholest-7-ene 5α ($C_{27}H_{46}O$)	-	-	-	-	-	-	-	-	-	-38.0	-40.8	-38.5
cholesta-5,22E-dien-3b-ol	-	-	-	-36.8	-	-36.0	-	-	-	-	-38.9	-
cholest-5-en-3β-ol	-37.1	-36.8	-36.8	-37.1	-36.9	-36.7	-35.9	-36.7	-36.7	-35.5	-35.6	-36.0
5a(H)-cholestan-3β-ol	-	-	-	-	-44.3	-44.2	-43.4	-44.1	-44.4	-45.4	-44.3	-43.1
5b-cholestan-3β-ol	-41.7	-	-	-38.8	-38.4	-39.0	-38.3	-38.3	-40.4	-38.0	-42.0	-38.9
24-methylcholesta-5,22E-dien-3β-ol	-	-	-	-37.9	-	-39.7	-38.4	-37.2	-40.0	-35.7		-35.1
24-methyl-5a-cholestan-3b-ol	-34.6	-29.1	-36.3	-37.4	-33.9	-37.2	-	-33.0	-34.1	-	-33.7	-

24-methylcholest-5-en-3β-ol	-	-	-	-38.1	-	-39.2	-37.7	-37.5	-38.0	-	-41.6	-
24-ethylcholesta-5,22E-dien-3β-ol	-	-39.1	-39.4	-38.8	-38.2	-39.7	-38.9	-38.9	-38.5	-38.2	-38.9	-38.6
24-ethyl-5β-cholestan-3β-ol	-38.7		-40.1	-38.5	-40.6	-40.9	-40.9	-	-41.3	-	-39.2	-40.3
24-ethylcholest-5-en-3β-ol	-32.7	-32.5	-34.4	-34.4	-31.8	-33.4	-32.9	-32.1	-32.0	-33.8	-33.2	-32.9
24-ethyl-5α(H)-cholestan-3β-ol	-	-	-	-	-42.4	-44.6	-46.1	-45.4	-46.4	-42.2	-45.6	-44.2
Fatty acids												
12:0	-27.2	-27.2	-27.8	-27.1	-	-	-	-	-	-	-	-
14:0	-24.7	-23.6	-25.0	-27.1	-26.7	-26.5	-	-	-	-	-	-
15:0	-26.4	-26.3	-26.8	-	-	-26.4	-	-	-	-	-	-
16:0	-26.1	-25.2	-25.6	-26.9	-26.8	-27.6	-27.0	-26.3	-27.6	-26.2	-	-
17:0	-38.3	-37.8	-	-	-	-	-	-	-	-	-	-
18:0	-27.0	-26.8	-27.7	-27.6	-27.4	-27.7	-27.6	-29.9	-30.8	-29.7	-30.1	-
21:0	-	-29.1	-	-	-	-	-	-	-	-	-	-
22:0	-29.5	-30.7	-28.0	-28.1	-	-	-30.4	-30.8	-30.9	-32.4	-32.4	-
23:0	-	-	-	-28.5	-	-	-	-	-	-	-	-
24:0	-	-	-34.0	-33.8		-36.4	-36.5	-33.8	-36.8	-36.5	-37.8	-
25:0	-	-28.2	-28.5		-35.9				-	-	-	-
26:0	-	-36.2	-35.8	-36.2	-	-37.0	-37.0	-38.3	-	-	-	-

3.4 Scientific paper: »Source identification of polycyclic aromatic hydrocarbons in Lake Bled (NW Slovenia) sediments using stable carbon isotopes«

In this chapter, the paper entitled “Source identification of polycyclic aromatic hydrocarbons in Lake Bled (NW Slovenia) sediments using stable carbon isotopes” by Marinka Gams Petrišič, Gregor Muri and Nives Ogrinc is presented. The paper was published in Environmental Science & Technology, 2013.

This paper described the use of stable isotopes to assign the sources of pollutants such as polycyclic aromatic hydrocarbons (PAH) in the environment. PAHs are a large group of compounds, which consist of two or more fused aromatic rings made entirely from carbon and hydrogen. They are one of the most widespread organic pollutants and in many cases, their derivate can be highly mutagenic and cancerogenic. The main route which PAH and their derivatives enter the environment is a result of natural and anthropogenic emission. Input of PAH in a natural way is very small, while the contribution of anthropogenic PAH increases every day. One of the ways to predict sources of organic compounds isolated from various environmental samples is a measurement of stable isotope compositions of individual PAHs. Concentrations of PAH can be related to their origins by comparing their composition with those of potential sources. We have used a combined molecular and isotopic approach to trace and identify the sources of PAH in lacustrine sediments of Lake Bled (NW Slovenia). In addition, traditional methods of identifying contaminant sources by compositional information have been compared with the stable carbon isotope composition approach to show the utility of both approaches for source apportionment for past deposition of PAH in sediment.

First the precision and accuracy of the $^{13}C/^{12}C$ ratio of individual compounds in PAH standards was determined using two different techniques. First $^{13}C/^{12}C$ ratios of individual PAH were determined using an elemental analyzer and isotope ratio mass spectrometer (EA-IRMS). Second PAH standards were prepared by weighing approximately 20 mg of each individual compound and dissolved with dichloromethane. Measurements of individual compound and a PAHs mixed standard were measured by GC-C-IRMS. The PAHs exhibit $\delta^{13}C$ values in the range from −27.3 to −22.7‰ with GC-C-IRMS values always higher comparing to values obtained by EA-IRMS. The isotopic composition of the EA-IRMS measurements is taken as a ‘true values’ of the standard PAH and thus the accuracy of the GC-C-IRMS determinations ranged between 0.5 and 1.0‰. With the exception of dibenzo[a,h]anthracene, there is no notable difference among isotopic values obtained for the PAH using both methods. GC-C-IRMS measurements in sediment samples were performed with a precision of 0.3‰ for well separated PAH compounds and up to 1.0‰ for some co-eluting isomers.

It was found that the dominant signatures identified in sediments of Lake Bled were mainly attributed to a coal/wood burning source but PAH from carsoot could also contributed to the overall isotope signatures. Retene (Re) and Perylene (Per) in Zaka Bay exhibit profiles and isotopic composition that are distinct from those of other PAHs suggesting their natural origin. However $\delta^{13}C$ values of Re determined at station D linked Re to both natural and pyrolytic origin. Alpine Lake Bled (NW Slovenia), with its anoxic hypolimnion, could therefore provide valuable information about the transport and fate of PAHs in the lacustrine environment. At the same time it was demonstrated that PAH were resistant to weathering reactions in anoxic sediments and thus useful in identification of paleo-environmental pollution activities.

Part of this work was also presented and published at scientific conference; 19th Young Investigators' Seminar on Analytical Chemistry, June 27-30, 2012, Nova Gorica, Slovenia and at Slovenski kemijski dnevi, September 12-14, 2012, Portorož, Slovenia.

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Source Identification and Sedimentary Record of Polycyclic Aromatic Hydrocarbons in Lake Bled (NW Slovenia) Using Stable Carbon Isotopes

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Supporting Information

ABSTRACT: A combination of molecular and stable isotope analyses was used to trace and identify the sources of polycyclic aromatic hydrocarbons (PAH) in sediments of Lake Bled (NW Slovenia). Sediment samples were taken from two locations with contrasting depositional regimes: Zaka Bay, with permanently oxic bottom and station D, where anoxic conditions prevail throughout the year. The concentrations of PAH in surface sediments at the two locations were comparable and higher than in previous studies, reaching 4230 and 4380 ng g^{-1}, respectively. It was found that retene (Re) and perylene (Per) are both mainly of natural origin in Zaka Bay while, at station D, the value of $\delta^{13}C$ determined at a depth of 12−14 cm in the 1950s indicated that Re was of pyrolytic origin. The distribution of $\delta^{13}C$ values of other individual PAH showed that PAH input to lake sediments was of pyrolytic origin, likely dominated by coal and later in 1950s also by wood burning. PAH from vehicular emissions could also contribute to the overall isotope signatures at the depth of 12−14 cm at station D and Zaka Bay corresponding to the period 1953−1961.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) constitute a large group of Persistent Organic Pollutants (POP) containing from two to six fused benzene rings. Certain PAH are among the most carcinogenic substances known and can be acutely toxic or genotoxic depending on the number and configuration of the benzene rings and the presence and position of their substituents.[1] PAH exhibit different molecular distribution according to their origin formed during incomplete organic matter combustion (pyrolytic origin) or natural and anthropogenic fossil fuel combustibles (petrogenic origin). Source apportionment techniques are of particular interest motivated by environmental remediation, prevention of future contamination, and evidence in litigation.

Compound-specific isotope analysis (CSIA) is a useful tool for studying the carbon cycle on the molecular scale and is important in identifying sources of PAH. This is possible because compound specific isotope composition does not appear to be modified by chemical, physical, or biological changes, as are the traditional molecular compositions.[2,3] Recently, this technique was successful in identifying sources of PAH in many environmental systems including sediments.[2,4−7] The sedimentary records of PAH concentrations have frequently been studied to develop historical records of combustion,[8−12] but studies of stable carbon isotope composition of PAH in sediment cores are limited.[13,14] Studies in aquatic systems focused mainly on surface sediments.[4,6,7] McRae et al.[14] have shown that PAH from other anthropogenic sources in urban lakes are more enriched in ^{13}C than combustion-derived PAH. Various sources of PAH have also been distinguished in sediments along the St. Lawrence River.[6] Localized ^{13}C-enrichment in the stable isotope signature of PAH was shown to be due to petroleum-related and aluminum smelter contributions against the regional backdrop of combustion-dominated PAH sources.

We have used a combined molecular and isotopic approach to trace and identify the sources of PAH in lacustrine sediments of Lake Bled (NW Slovenia). In addition, traditional methods of identifying contaminant sources by compositional information have been compared with the stable carbon isotope composition approach to show the utility of both approaches for source apportionment for past deposition of PAH in sediment.

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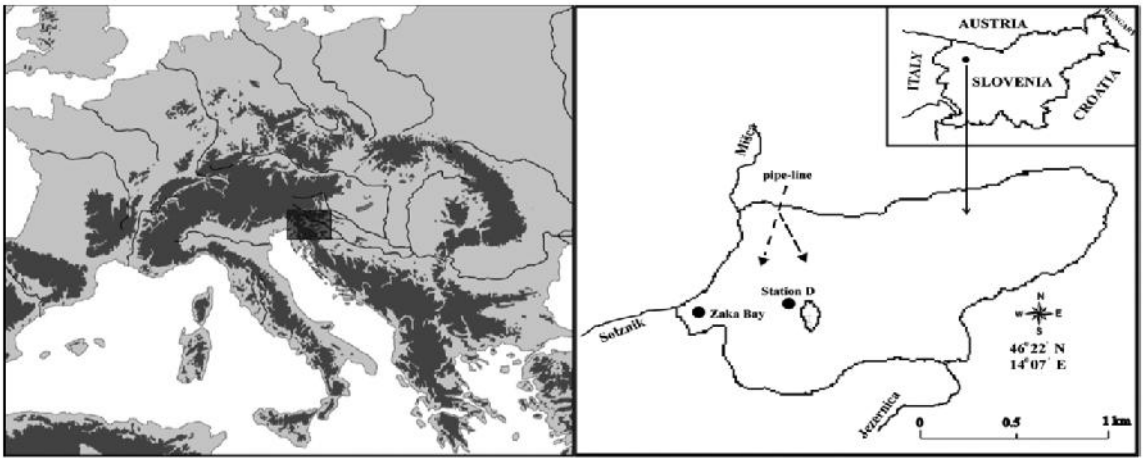

Figure 1. Map showing the location of Lake Bled, NW Slovenia with sampling sites Zaka Bay and station D in the West Basin.

EXPERIMENTAL SECTION

Study site description. Lake Bled is an urban subalpine lake located in NW Slovenia (Figure 1) having a surface area of 1.44 km^2 and an elevation of 475 m above sea level. The lake is divided into two basins: the deepest, western basin with a depth of 30.5 m and the eastern basin with a depth of 24 m. The surficial inflows are two small streams, Mišca and Solznik, in the western basin, whereas water outflow proceeds through the Jezernica stream into the River Sava. The lake is stratified with an anoxic hypolimnion for most of the year, except during early spring and late autumn. In the shallower regions, oxidizing conditions prevail throughout the water column. Lake Bled is located in an industrially, touristically, and agriculturally developed area. To improve the quality of lake water two amelioration projects have been undertaken: freshwater inflow, diverted in the early 1960s from the River Radovna, and pumping of anoxic water from the western basin into the Jezernica. The residence time in Lake Bled was shortened from 4 years to 1.1 year after the amelioration projects took place. According to OECD[15] criteria, the lake is now classified as mesotrophic. Surficial sediment of Lake Bled is composed of dark gyttya type clayey silt with 5%−10% of organic matter. The sediment below is fine laminated and composed of homogeneous silt and clayey silt. Mineralogically, low-Mg calcite prevails, followed by dolomite, quartz, partially of diatomaceous origin, and feldspar. Clay minerals are composed of muscovite/Illite and chlorite. Authigenic minerals are pyrite and low-Mg calcite deposited as lake chalk in restricted area south of Zaka Bay.[16,17]

Sampling. Samples of sediments were collected in November 2011 at two locations at the western part of the lake − in the shallow Zaka Bay in the thermocline at a depth of 12 m, and in the anoxic hypolimnion at Station D in the deepest part of the lake, at a depth of 30 m (Figure 1). In Zaka Bay the water column is supersaturated with oxygen down to the bottom during most of the year. Undisturbed sediment cores to a depth of 40 cm were taken with a gravity core sampler equipped with a Plexiglas tube (6 cm i.d.). Sediment samples were extruded, cut, and stored in glass jars. All utensils and glassware were prerinsed with distilled water and organic solvents to prevent contamination. In laboratory sediment samples were freeze-dried, homogenized, and stored frozen until analyzed.

Analyses. All solvents and reagents (JT Baker, Mallinckrodt Baker) were of analytical grade or higher. Dry sediments were weighed into cellulose extraction thimbles and spiked with deuterated PAH (naphthalene-d_8, acenaphthene-d_{10}, phenanthrene-d_{10}, chrysene-d_{12}, and perylene-d_{12}; Dr. Ehrenstorfer) for the correction of the mass spectrometer. This step was followed by the extraction procedure described in detailed by Muri and Wakeham.[18] After Soxhlet extraction with dichloromethane, extracts were concentrated by rotary evaporator, solvent-exchanged to hexane, and fractionated on a glass silicagel column. After eluting aliphatic hydrocarbon with hexane, PAH were eluted using additional 25 mL of hexane and 20 mL of hexane/toluene (3:1). Both PAH fractions were combined and concentrated with a rotary evaporator. Fractions were dried using nitrogen and redissolved in iso-octane for instrumental analysis. Eighteen PAH compounds, that is acenaphthene, acenaphthylene, phenanthrene, anthracene, fluoranthene, fluorene, pyrene, benzo[a]anthracene, chrysene, benzofluoranthenes [b and k isomers], benzo[a]pyrene, indeno[1,2,3-cd]pyrene, benzo[ghi]perylene, dibenzo[a,h]anthracenes, naphthalene, and 1-methyl and 2-methyl naphthalene were determined in the samples. The individual compounds were analyzed and identified with an Agilent Technologies 6890 GC coupled to a MSD using a DB-5 ms (30 m × 0.25 mm i.d., 0.25 μm) fused phenylmethylpolysiloxane capillary column. The PAH were determined in selected ion−monitoring mode with an ionization energy of 70 eV, whereas their identification was based on the m/z peaks corresponding to the molecular weights of the individual PAH. Concentrations of PAH were calculated using response factors; the peak area of the individual PAH was normalized with the peak area of the corresponding deuterated surrogate. In addition, procedural blanks were run with each set of six samples to monitor background contamination.

Compound-specific stable isotope ratios were determined using a gas chromatography-isotope ratio mass spectrometer, which are connected together by a combustion furnace (GC-C-IRMS; Isoprime GV, UK). The gas chromatograph was fitted with a DB 1 ms capillary column (60 m, 0.32 mm i.d., 0.25 μm) fused by 100% dimethylpolysiloxane, using helium as carrier

gas. Carbon isotope measurements were reported in delta notation (δ) relative to the Vienna-Pee Dee Belemnite (V-PDB) standard as follows:

$$\delta^{13}C(‰) = [(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard} - 1] \times 10^3$$

$\delta^{13}C$ values were calibrated against reference CO_2, which was introduced directly into the source two times at the beginning and end of every GC isotope determination. The precision and accuracy of $\delta^{13}C$ values from GC-C-IRMS were determined using individual PAH compounds initially measured with an elemental analyzer (EA) coupled to IRMS (Europa Scientific 20−20 with ANCA-SL solid−liquid preparation module). The isotope values of EA-IRMS were taken as a true values of the standards and compared with GC-C-IRMS measurements. In addition, multiple compound-specific isotope measurements were performed on a standard mixture of PAH. It was found that the precision of the GC-C-IRMS measurements ranged between 0.3 and 0.5‰, whereas accuracy ranged between 0.2 and 1.0‰. Samples dissolved in isooctane were injected and stable carbon isotope composition determined by duplicate analyses with a precision of 0.3‰ for well separated PAH compounds and up to 1.0‰ for some high molecular weight coeluting isomers.

Weight percentages of organic carbon (% OC) in sediments were determined following acidification with 1 M HCl using a Carlo Erba model 1108 CHNS analyzer at a combustion temperature of 1020 °C with precision expressed in terms of relative standard deviation ±2%. This analysis was performed at Marine Biological Station, National Institute of Biology, Piran, Slovenia. Isotope compositions of sedimentary organic carbon ($\delta^{13}C_{OC}$) were measured with a Europa Scientific 20−20 IRMS with an ANCA-SL preparation module for solid and liquid samples. Results were calibrated against reference materials: National Bureau of Standards 22 (NBS 22, oil) and International Atomic Energy Agency standard for carbon and hydrogen (IAEA-CH-7). The precision of measurements was ±0.2‰.

Sediments had previously been dated radiometrically using a γ-ray spectrometer equipped with a high-purity Ge well-type detector. ^{210}Pb and ^{137}Cs activities in each sediment slice were measured. Sedimentation rate was obtained based on unsupported ^{210}Pb activities levels using the constant rate of supply (CRS) model. The dating was additionally confirmed by ^{137}Cs. An average sedimentation rate of 4.5 mm yr^{-1} was used for age assessment at Zaka Bay[18] and of 2.4 mm yr^{-1} at station D.[17]

■ RESULTS AND DISCUSSION

Organic carbon contents in sediments at station D ranged from 3.6 to 5.8 wt.% (average = 4.1 ± 0.4%, n = 18), the highest value being at a layer dated to 1902−1911 (24−26 cm). $\delta^{13}C_{OC}$ values ranged from −34.8 to −31.5 ‰ (Figure 2). Such low $\delta^{13}C$ values are often found in eutrophic lakes with an anoxic hypolimnion and in strongly reductive sediments.[19−21] The OC content in Zaka Bay ranged from 3.5 to 4.8 wt.%. Values of $\delta^{13}C_{OC}$ ranged from −31.3 to −29.4‰ and were higher than those at station D. These results indicate a greater influence of terrestrial OC at this station (Figure 2).

Sedimentary total PAH concentrations were measured at both sampling locations, together with the concentrations and isotope composition of OC (Figure 2). Concentrations at the surface at station D were 4380 ng g^{-1} and decreased with

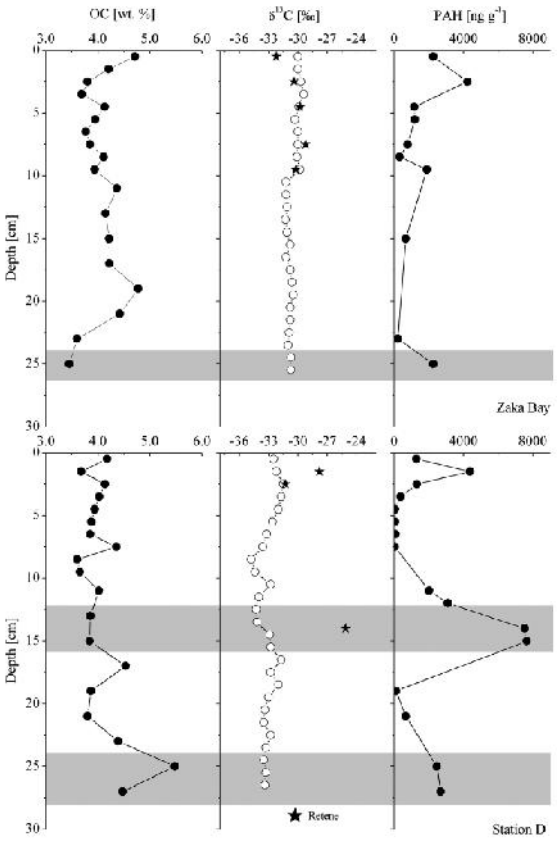

Figure 2. Depth profiles of organic carbon content (OC), $\delta^{13}C$ values, and concentrations of total polycyclic aromatic hydrocarbons (PAH) from the sediments in Zaka Bay and Station D. $\delta^{13}C$ values for retene (Re) are also included. Depths at which $\delta^{13}C$ values of PAH were determined are marked by gray color.

depth. The maximum concentrations up to 7650 ng g^{-1} were reached at depths of 12−14 and 14−16 cm. These depths had been dated previously as about 1944−1961.[18] The concentrations were lower below 16 cm, being 2470 ng g^{-1} at 25 cm (deposited around 1907). Concentrations of individual PAH species ranged from 26 to 250 ng g^{-1}, whereas at the depths of 12−16 cm where the maximum of total PAH concentrations was observed they ranged from 87 ng g^{-1} to 703 ng g^{-1} (Table S1 of the Supporting Information). Most PAH, that is fluoranthene (Fl), pyrene (Py), benzo[*a*]anthracene (BaA), chrysene (Chy), benzo[*e*]pyrene (BePy), benzo[*a*]pyrene (BaPy), indeno[1,2,3-cd]pyrene (IPy), and benzo[*ghi*]perylene (BghiPer) exhibited a similar depth profile, although their abundances differed. Perylene (Per) and retene (Re) had different depth patterns. Concentrations of Per were quite uniform in the first 10 cm, with an average concentration of 53 ng g^{-1}, but reached 324 ng g^{-1} deeper in the sediments. Re concentrations were low at the surface, but slightly higher, up to 87 ng g^{-1}, at depths corresponding to the period from 1953 to 1961. The concentrations again decreased deeper in the sediment to 8 ng g^{-1}. The depth profile of total PAH concentrations in Zaka Bay differed significantly from that at sampling station D (Figure 2). In addition, the deepest sample

analyzed in Zaka Bay was relatively young (~56 years) compared to the deepest sample at station D (~100 years). Total PAH concentrations were the highest at the surface but decreased to a depth of 24–26 cm, where the next maximum, corresponding to around the 1955s, was observed. At this depth, concentrations of individual PAH ranged from 65 ng g^{-1} (BaA) to 431 ng g^{-1} (Fl) (Table S2 of the Supporting Information). Concentrations of Per and Re were higher in Zaka Bay than at station D. Concentrations of contemporary Per were ~170 ng g^{-1} and increased with depth to 284 ng g^{-1}. Re concentrations was ~47 ng g^{-1} at the surface and decreased with depth to 6 ng g^{-1}. The highest concentration of 163 ng g^{-1} was observed at the depth of 24–26 cm (Table S2 of the Supporting Information).

The depth profiles of PAH are in a good agreement with those from our previous studies.[18,22] However, at the top 4 cm, much higher concentrations, up to 4380 ng g^{-1}, were observed at both sampling locations. Because most of the historical records of PAH recorded in the literature were generated before the 1990s, the assumption that PAH concentrations would continue to decline persisted for over 2 decades. However in 2000, Van Meter[23] reported that PAH emissions were increasing in certain areas of the United States. The rise in PAH paralleled increased automobile usage, implying a link between PAH input and urban sprawl. Increased PAH concentrations were also observed in a recent study conducted on the anoxic sediments of the Pettaquamscutt River basin, Rhode Island.[11] Consumption of diesel was suggested to be the most probable source of this increase. For the European Union, contrasting trends for PAH emissions were reported between 1990 and 2010.[24] In most countries including Slovenia, PAH emissions decreased in this period. The reduction in Slovenia amounted to 14%, but the emissions tend to increase slightly in the last years. However, PAH emissions increased in several countries in the same period. In Italy, a country that borders Slovenia and contributes substantial amounts of PAH emissions in the EU, a 54% increase was reported between 1990 and 2010, whereas the most remarkable rise was observed in the mid 2000s. It was explained by increasing use of wood for residential heating. Lake Bled is subject to both long-range pollution, through atmospheric deposition, and local pollution, due to its urban watershed, channelled mainly by stormwater runoff. The PAH in Lake Bled sediments have been suggested to be of pyrolytic origin, derived mainly from coal combustion; however, the presence of Re and Per in sediments also suggested minor contribution of diagenetically derived compounds.[18,22] In addition, loss of PAH by degradation was shown to be unlikely in these sediments.[22] Therefore, the source identification of PAH in these sediments was further investigated using stable isotope approach.

The isotope composition of different PAH at station D could only be measured at the depths where PAH concentrations were higher: at the surface, 12–14, 14–16, 24–26, and 26–28 cm. These depths correspond to the periods 1944–1961 and 1894–1911. In addition, two isomers were difficult to be analyzed separately due to poor peak quality and size and are thus presented as a summed (benzo[b]fluoranthene + benzo[k]fluoranthene, BFl). $\delta^{13}C$ values of individual PAH at these depths ranged from −31.3‰ to −21.7‰ (Table S1 of the Supporting Information). The lowest $\delta^{13}C$ value of −31.3‰ was found in Re at the surface, whereas Py exhibited the highest $\delta^{13}C$ value of −21.7‰ at a depth of 12–14 cm. Although Per was present in sediments, its concentrations were insufficient for determination of its isotope composition at station D. However, $\delta^{13}C$ values for Re were determined at three depths at the surface (0–1, 2–3, and 3–4 cm) and at a depth of 12–14 cm. The values in the upper part of the sediment ranged from −31.3‰ to −27.8‰, whereas a value of −25.1‰ was recorded deeper in the sediment (Table S1 of the Supporting Information). $\delta^{13}C$ values in PAH determined at the surface and a depth of 24–26 cm in Zaka Bay ranged from −32.2‰ to −21.2‰ (Table S2 of the Supporting Information). The lowest $\delta^{13}C$ value was recorded for Re and the highest for Fl near the surface. Values of $\delta^{13}C$ for Re ranged from −32.2‰ to −28.2‰ and for Per from −29.3‰ to −26.1‰.

Per and Re can have both anthropogenic and natural origins. Ret has been proposed as a tracer for the combustion of coniferous wood.[25] It is a derivative of abietic acid present in coniferous resins[26,27] and is present in extracts of algal and bacterial organic matter.[28] The concentration depth profile of Ret at station D follows those of the pyrolytic PAHs suggesting that Re at this location probably originated from a pyrolytic source such as coal-burning emissions. This suggestion is supported by the $\delta^{13}C$ value of −25.1‰, determined at a depth of 12–14 cm for Re, which falls within the range of $\delta^{13}C$ values of pyrogenic PAHs (Table S1 of the Supporting Information). However, in the upper part of the sediment, lower $\delta^{13}C$ values of Re (from −31.3‰ to −27.8‰) indicate a predominantly natural origin (Figure 2). The $\delta^{13}C$ values of Re in Zaka Bay, ranging from −32.2‰ to −28.2‰, coincide with the bulk $\delta^{13}C_{OC}$ values in sediments (Figure 2) suggesting that Re can originate from similar organic precursors and has a mainly diagenetic origin. At this location, the percent of allochthonous organic material, which is delivered by the small stream Solznik, is higher than that at station D. The stream Solznik drains the partially coniferous Lake Bled watershed.

The origin of Per is less specific and has been the subject of considerable debate. Emissions from automobiles[29] and municipal incinerators[30] and in situ diagenesis of marine and terrestrial organic matter[31] have been cited as potential sources of Per. The concentration profiles of Per in Lake Bled sediments differ markedly from those of the pyrogenic PAHs. Postdepositional, in situ diagenetic formation has been suggested as explaining the increase of Per with depth at station D.[22] This assumption was further supported by Py/Per ratios ~1.0 found in the older western basin sediments. The Py/Per for diagenesis was suggested to be <0.5.[12] A similar range of $\delta^{13}C$ values for Per was observed in surficial sediments of Lake Erie.[13] Throughout the Zaka Bay core the Py/Per ratios averaged ~1.0 indicating that Per in the whole Zaka Bay sediments is mainly of natural origin. A part of Per in Zaka Bay may be washed in with soil organic matter delivered by Solznik. The delivery of soil organic matter could not effect Per distribution in the western basin (station D) because the water circulation in this part of the lake is limited. The $\delta^{13}C$ values of Per in Zaka Bay ranged from −29.3‰ to −26.1‰ with lower values observed deeper in sediments. These data show that Per originates from both terrigenous and aquatic sources of organic matter because the $\delta^{13}C$ value for terrestrial plants near Lake Bled was found to be −26‰, whereas for aquatic organic matter $\delta^{13}C$ value of −28‰ was accepted.[33] A similar conclusion was reached from sediments from the Saanich Inlet fjord.[32] The study showed that Per originated from microbial aromatization of constituents from both aquatic and terrestrial sources and its formation could be controlled by the

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rate of microbial degradation rather than by the presence of a specific precursor.

The $\delta^{13}C$ values for pyrolytic PAHs at different depths, related to the age of deposition, are collected in Tables S1 and S2 of the Supporting Information. The isotopic composition of PAHs generated by wood burning varies with ring size with 3- and 5-ring PAHs being more ^{13}C-depleted than 4-ring compounds.[2] The $\delta^{13}C$ results obtained from the sediments of Lake Bled for individual PAHs show no clear correlation with ring size. However, pyrogenic PAHs (except for A and Cry at both locations and BagiPer in Zaka Bay) were consistently ^{13}C-enriched relative to Per (−29.3‰ to −26.1‰) and sedimentary OC (Figure 2).

High-temperature combustion is probably the major source of PAH in Lake Bled sediments.[18] The influence of temperature of formation on $\delta^{13}C$ values of PAHs was addressed by McRae et al.,[4] who determined the isotope composition of PAHs derived from coals of different ranks under various process conditions. The $\delta^{13}C$ values of individual PAHs were observed to correspond to more ^{13}C-depletion with increased temperature of formation. PAHs released by low temperature combustion processes exhibited $\delta^{13}C$ values similar to those of the parent coal (−24‰ to −25‰). However, combustion of different fuels can yield similar mixtures of pyrogenic PAHs, and the ranges of $\delta^{13}C$ values of the main energy sources (coal, petroleum, and wood) greatly overlap in the range of −30 to −20‰. Hence, the difficulty in relying solely on $\delta^{13}C$ values to separate the contributions of PAHs derived from two combustion processes. The highest $\delta^{13}C$ values in PAHs were found in gas exhaust particles ranging from −13.3 ‰ to −26.8‰, whereas those in diesel exhaust particles ranged from −21.7‰ to −26.3‰.[34] The carsoot fingerprint is dominated by isotopically heavy 4- and 5-ring species, particularly Py, whereas woodburning sources are dominated by isotopically heavy BaA.[2,35] Chy and BF are ^{13}C-depleted in both sources, whereas BaPy is ^{13}C-enriched relative to BF.[2]

Even though the trend in $\delta^{13}C$ values in our study could be related mainly to a high-temperature combustion source, the actual $\delta^{13}C$ values are quite different. Higher variations were observed for 3-ring PAH in sediments than for the high-molecular weight species at different sediment depth. This was observed in most of sediment samples and could indicate that other processes such as weathering may have influenced the isotopic composition. Thus the further discussion will focus mainly on the 4- and 5-ring PAH because they are more stable in the environment.[2] The $\delta^{13}C$ values were higher for 4- and 5-ring PAHs, particularly for Py with $\delta^{13}C$ values of −21.7‰ and −22.8‰ at all investigated depths in sediments at station D and in Zaka Bay at the depth of 24−26 cm (Tables S1 and S2 of the Supporting Information, Figure 3). The difference between $\delta^{13}C$ values at the depth of 12−14 and 14−16 cm at station D was observed for 4-ring PAHs, whereas 5-ring PAHs have similar $\delta^{13}C$ values. $\delta^{13}C$ values increased from F to Py from −28.2 to −21.7‰ at the depth of 12−14 cm. A similar trend in $\delta^{13}C$ values were typically observed in carsoot fingerprint.[2,34,35] Unlike the carsoot, the wood burning source is characterized by ^{13}C-enriched BaA relative to Fl and Py and ^{13}C-depletion in Chy and BFl.[2,35] Our data showed higher $\delta^{13}C$ values for BFl and lower $\delta^{13}C$ values for BaA and Chy at both depths. In addition, the measured $\delta^{13}C$ value of −28.4‰ for A at the depth of 14−16 cm is outside the range reported by O'Malley et al.[2] A similar observation was also found in the study performed by Stark et al.[6] in St. Lawrence River

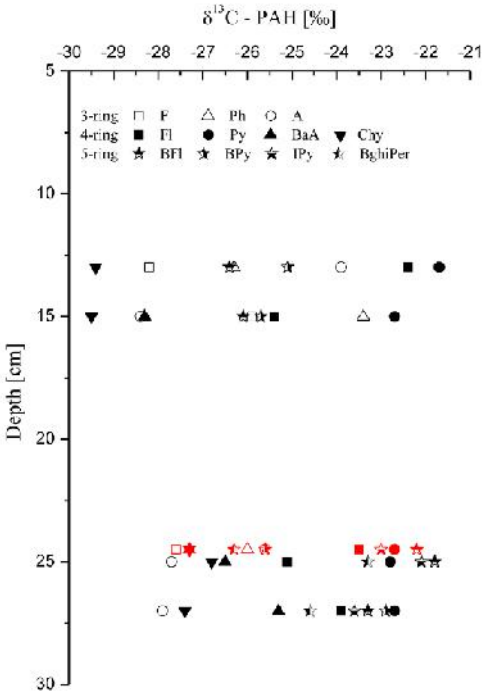

Figure 3. $\delta^{13}C$ values of PAH obtained from different depths in Lake Bled sediments at Zaka Bay and station D: fluorine, F; phenanthrene, Ph; anthrancene, A; fluoranthene, Fl; pyrene, Py; benz[a]anthracene, BaA; chrysene, Chy; benzofluoranthene, BFl; benzopyrene, BPy; indeno[123-cd]pyrene, IPy; benzo[ghi]perylene, BghiPer. $\delta^{13}C$ values of PAH in Zaka Bay are marked red.

sediments suggesting that type of wood could influence the $\delta^{13}C$ values of PAHs. The type of burning material could be also the reason for the differences in the PAH $\delta^{13}C$ values observed in our study. $\delta^{13}C$ values for BaA and 5-ring PAH were higher at the depth of 24−28 cm comparing to PAH at the depth of 14−16 cm (Figure 3). In addition higher $\delta^{13}C$ values were also found for IPy and BghiPer at the depth of 24−28 cm (Table S1 of the Supporting Information, Figure 3). It seems that at these depths which correspond to years 1894 to 1911 coal burning was the main source of PAH. This assumption could be further supported by the Fl/(Fl+Py) ratio of 0.6 and 0.7 ratio and IPy/(IPy + BghiPer) ratio of 0.51 and 0.43 determined at the depth of 24−26 and 26−28 cm, respectively. Fl/(Fl + Py) ratios between 0.5 and 0.8 and IPy/(IPy + BghiPer) ratio of 0.56 are common for coal combustion.[36,37] Fl/(Fl + Py) ratios were 0.7 and 0.9 at the depth of 12−14 and 14−16 cm, respectively. At these two depths, isotopically lighter signatures of high molecular mass PAH were also observed suggesting another input of PAH in sediments. The most likely source is wood burning, in view of the industrial history of the area.

In the Zaka Bay, the increase in $\delta^{13}C$ values of F, Ph, Fl, and Py was observed and followed the trend seen at the depth of 12−14 cm at station D except for A. In addition the same Fl/(Fl + Py) ratio of 0.7 was determined. However $\delta^{13}C$ values of higher molecular mass PAH generally followed the trend observed at the depth of 26−28 cm at station D (Figure 3).

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These data indicated that the sources of PAH in sediments in Zaka Bay were coal burning and vehicular emissions.

The results of our study indicated that together molecular and isotope composition of PAHs are useful tools for obtaining more detailed identification about sources of PAH inputs into lacustrine sedimentary environments. It was found that the dominant signatures identified in sediments of Lake Bled were mainly attributed to a coal/wood burning source but PAH from carsoot could also contributed to the overall isotope signatures. Re and Per in Zaka Bay exhibit profiles and isotopic composition that are distinct from those of other PAHs suggesting their natural origin. However $\delta^{13}C$ values of Re determined at station D linked Re to both natural and pyrolytic origin. Alpine Lake Bled (NW Slovenia), with its anoxic hypolimnion, could therefore provide valuable information about the transport and fate of PAHs in the lacustrine environment. At the same time it was demonstrated that PAH were resistant to weathering reactions in anoxic sediments and thus useful in identification of paleo-environmental pollution activities.

■ ASSOCIATED CONTENT

Supporting Information

Tables of isotopic composition and concentration of selected PAHs from different depth in sediments at sampling location D together with the corresponding deposition age, and isotopic composition and concentration of selected PAHs from different depth in sediments at Zaka Bay together with the corresponding deposition age. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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Source identification and sedimentary record of polycyclic aromatic hydrocarbons in Lake Bled (NW Slovenia) using stable carbon isotopes

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The manuscript includes: 21 pages, 3 figures.

Table S1. Isotopic composition and concentration of selected PAHs from different depth in sediments at sampling location D together with the corresponding deposition age.

	2011		1953-1961		1944-1953		1902-1911		1894-1902	
Station D	0-1 cm		12-14 cm		14-16 cm		24-26 cm		26-28 cm	
PAH	$\delta^{13}C$ (‰)	ng/g	$\delta^{13}C$ (‰)	ng/g	$\delta^{13}C$ (‰)	ng/g	$\delta^{13}C$ (‰)	ng/g	$\delta^{13}C$ (‰)	ng/g
Fluorene (F)	–24.0	67	–28.2	98	–25.9	106	–	–	–	–
Phenanthrene (Ph)	–25.8	73	–26.3	232	–23.4	324	–	–	–	–
Anthracene (A)	–28.1	76	–23.9	175	–28.4	169	–27.7	121	–27.9	135
Fluoranthene (Fl)	–	–	–22.4	486	–25.4	703	–25.1	89	–23.9	187
Pyrene (Py)	–	–	–21.7	231	–22.7	35	–22.8	57	–22.7	76
Benzo(a)anthracene (BaA)	–	–	–	–	–28.3	31	–26.5	49	–25.3	35
Retene (Re)	–31.3	52	–25.1	87	–	23	–	16	–	8
Chrysene (Chy)	–	–	–29.4	248	–29.5	57	–26.8	46	–27.4	31
Benzofluoranthenes (BFl)	–28.9	291	–26.4	325	–26.1	689	–21.8	111	–23.3	143
Benzo(a)pyrene (BaPy)	–23.9	71	–25.1	170	–25.7	124	–	22	–22.9	48
Indeno(123-cd)pyrene (IPy)	–	–	–	–	–	–	–22.1	97	–23.6	79
Benzo(ghi)perylene (BghiPer)	–	–	–	–	–	–	–23.3	94	–24.6	103

Table S2. Isotopic composition and concentration of selected PAHs from different depth in sediments at Zaka Bay together with the corresponding deposition age.

Zaka Bay	2011		1953-1957	
	0-1		24-26 cm	
PAH	$\delta^{13}C$ (‰)	ng/g	$\delta^{13}C$ (‰)	ng/g
Fluorene (F)	–28.6	285	–27.6	192
Phenanthrene (Ph)	–29.6	133	–25.6	133
Anthracene (A)	–27.8	189	–26.0	202
Fluoranthene (Fl)	–21.2	243	–23.5	431
Pyrene (Py)	–		–22.7	224
Benzo(a)anthracene (BaA)			–27.3	87
Retene (Re)	–29.8	47	–30.1	163
Chrysene (Chy)	–	–	–27.3	226
Benzofluoranthenes (BFl)	–27.6	141	–22.2	286
Benzo(a)pyrene (BaPy)	–23.2	114	–25.6	152
Indeno(123-cd)pyrene (IPy)	–	–	–23.0	147
Benzo(ghi)perylene (BghiPer)	–	–	–26.3	152
Perylene (Per)	–26.6	38	-27.0	82

4 Conclusions

The findings of this study indicate that isotope geochemistry is a complementary tool with which to address the fate and source identification of organic compounds and pollutants in the environment, and should be used more generally. We suggest that, in the absence of this tool, several possible conclusions could have been erroneous. The novel contribution to the science performed within this study has resulted in five published papers in SCI international journals and two papers submitted for publication. The work has been presented at eighteen international conferences with eleven oral presentations (Appendix I). The main conclusions of the thesis can be summarized as follows:

1. The main benefits have been the development of new methods on the molecular level for studying organic compounds and associated pollutants in the environment. Three characteristic features have been explored: molecular structure, absolute and relative abundances and stable isotope composition. The examination of individual lipid compounds has been focused mainly on the more persistent lipid groups such as hydrocarbons, alcohols, sterols, and fatty acids, and on polycyclic aromatic hydrocarbons (PAHs) as pollutants. Measurements were performed in different environmental matrices (particulate organic matter and sediment trap material, sediments).
   - The fractions of aliphatic hydrocarbons, PAHs, fatty acids, alcohols and sterols obtained after extraction with Soxhlet were quantified by gas chromatography (GC), and individual compounds identified by gas chromatography-mass spectrometry (GC-MS). GC-MS identification was performed by interpretation of mass spectra and comparison with authentic standards and/ or literature data.
   - Isotope compositions of lipids were determined using an Isoprime GV GC–combustion-isotope ratio mass spectrometer (GC-C-IRMS). Reproducibility and accuracy for isotope composition were evaluated routinely using reference materials of known $\delta^{13}C$ values, though there are no reference materials for determining the isotope composition of PAHs. $^{13}C/^{12}C$ ratios of each individual PAH were first determined using an elemental analyzer and isotope ratio mass spectrometer (EA-IRMS) and taken as a ‘true values’. Measurements of individual PAHs were then performed by GC-C-IRMS and corrected according to ‘true values’ determined by EA-IRMS.

2. Our first investigation used molecular and stable carbon isotope ratios of specific lipid biomarkers **to evaluate their sources and to explore variations** in the biogeochemistry of the **particulate organic matter (POM) and sediment trap material** located at three depths in the water column at the deepest part of the subalpine Lake Bled, NW Slovenia.
   - The results indicate that the abundance of lipid biomarkers in trap material was two to four times higher than in POM, indicating that, during sinking, OM had been extensively reprocessed by microbes, which was more intensive under thermocline.

- Fatty acids were more abundant in POM while, in trap material, the contribution of *n*-alkanes to the particulate organic carbon (POC) was greater than that of FAs.
- Autochthonous lipid material accounted for the major part of POM and trap material. When supported by $\delta^{13}C$ values, shorter chain saturated *n*-alkenes and *n*-alcohols from *n*-C_{14} to *n*-C_{19}, saturated and unsaturated C_{27} sterols and shorter chain saturated and unsaturated FAs from *n*-C_{14} to *n*-C_{19} were found to be prevalent.
- The predominance of $C_{18:0}$ *n*-FA, short chain, even carbon (*n*-C_{14}, *n*-C_{16} and *n*-C_{18}) *n*-alkenols, the high proportion of cholesterol, higher cholesterol/phytosterols ratio and $\delta^{15}N_{PN}$ values all indicate zooplankton grazing at 12 m in POM and trap material.
- In the hypolimnion we observed more active reprocessing induced by bacteria. The distribution and composition of fatty acids were influenced by three processes: (1) algal production in the oxic epilimnion, (2) preferential degradation of labile algal components and presence of zooplankton in the metalimnion, and (3) production of bacterial fatty acids in the anoxic hypolimnion. The lowest $\delta^{13}C$ value, −51.7 ‰, was observed in 18:1*n*-7 FA in trap material at 28 m, the only FA that could be linked to methanotrophic bacteria. It was estimated that methanotrophic bacteria contributed 58% to 18:1*n*-7 FA.
- The observed sterol distribution reflects a primarily plankton source. It was suggested that much of the 24-ethylcholest-5-en-3β-ol and cholest-5-en-3β-ol in trap material at 28 m had derived from phytoplankton and not from higher plants and zooplankton, respectively. 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol) and 24-methylcholest-5-en-3β-ol (campesterol) were both of allochthonous origin, coming from terrestrial plants, while 24-methylcholest-5,22(E)-dien-3β-ol, the most abundant C_{28} sterol, originated from constant microalgal sources.

3. In the second phase of our study, lipid compositions of Lake Bled sediments were evaluated to establish the proportions of autochthonous and allochthonous OM contributions. The source was first identified based on the **lipid distribution in the sediments** and then using isotope composition of individual lipid compounds. The sources of lipid biomarkers were identified in **two different depositional environments**, **anoxic** (western basin) and **permanently oxic** (Zaka Bay).
   - The isotope composition of lipid biomarkers in sediments in both oxic and anoxic environments indicated that the source of OM was of mixed origin, being derived from autochthonous origin (plankton, microbial) and allochthonous, terrestrial origin.
   - The similarity of $\delta^{13}C$ values, obtained from sediments at the two sites, for long-chain *n*-alkanes, short- and long-chain *n*-alcohols and short-chain FAs, indicates that these compounds came from similar sources and were not dependent on different depositional regimes. The higher $\delta^{13}C$ values for short-chain *n*-alkanes in Zaka Bay sediments indicate a higher proportion of more refractory, terrestrial material brought by the Solznik stream. On the other hand the average $\delta^{13}C$ values for long-chain FAs, and especially those of sterols, were lower in the anoxic western basin, indicating a more pronounced contribution of anaerobic (methanotrophic) microbial origin than in Zaka Bay.
   - The use of compound-specific carbon isotope analysis of sedimentary lipids to study recent anoxic sediments indicates that, despite the fact that biomarker analysis revealed mostly plankton and terrestrial sources of lipids, an important fraction of sedimentary lipids, especially sterols, are autochthonous and of anaerobic microbial origin. This consideration is strongly supported by the

differences in ^{13}C lipid composition between settling particles, collected about 2 metres above the bottom, and surface sediments. Basically, lipid compounds were more ^{13}C-depleted in sediments than in settling particles, indicating diverse and differing source organisms. These results show that even the same **compounds** found in both compartments may have **different sources,** as indicated by their isotope composition. Thus, **lipid source signatures** in anoxic environments based solely on biomarker composition have to be treated with caution and should not be used without input from **stable isotope geochemistry**. This is further proved by examination of the results of Muri and Wakeham (2006) where source identification was based only on lipid distribution in Lake Bled sediments. They suggested that the source assignment based on lipid composition was in disagreement with the origin of bulk OM. In their estimation **C_{29} sterols** contributed to the allochthonous pool of OM. However, our results indicate that these compounds were derived mainly from **microalgae and bacteria**.

4. The prevalence of "fresh" autochthonous derived OM in sediments was also thought to be a key parameter in determining the pathway of methanogenesis. Acetate fermentation appears to be associated with the more labile autochthonous organic matter, whereas CO_2 reduction to CH_4 utilizes the more refractory allochthonous organic matter (Sugimoto and Wada 1993). The average $\delta^{13}C_{CH4}$ value of −69.5 ± 1.2‰, associated with low acetate concentrations, suggested that CH_4 should be formed, predominantly, hydrogenotrophically. The majority of archaeal sequences belonged to *Euryarchaeota*. The methanogenic population accounted for 73% and 38% of the archaeal community at depths of 0-2 cm and 10-12 cm. In the upper 2 cm, hydrogenotrophs, mostly *Methanomicrobiaceae*, were dominant. In the deeper sediment, archaeal sequences comprised mostly those of unknown affiliation with *Euryarchaeota, Thermoplasmatales* and related linkages, and only 21% of the hydrogenotrophic methanogenic archaea were detected. These results indicate that hydrogenotrophic methanogenesis is the dominant pathway in the sediments of alpine Lake Bled. It was also shown that the processes and methanogenic archaeal communities were in many ways similar to those in other mid-altitude lakes, and even in tropical lakes, indicating that temperature could not be the main factor controlling the methanogenic pathway.

5. Results of molecular and isotope composition of PAHs in lake sediments show that they were **resistant to weathering reactions** in **anoxic sediments** and thus useful in identifying paleoenvironmental pollution activities.
   - Perylene (Per) and retene (Re) can have both anthropogenic and natural origins. The concentration depth profile of Re from the western basin in Lake Bled follows those of the pyrolytic PAHs, suggesting that Re at this location probably originated from a pyrolytic source such as coal-burning emissions. This suggestion is supported by the $\delta^{13}C$ value of −25.1‰, determined at a depth of 12−14 cm for Re, which falls within the range of $\delta^{13}C$ values of pyrogenic PAHs. However, in the upper part of the sediment, lower $\delta^{13}C$ values of Re (from −31.3‰ to −27.8‰) indicate a predominantly **natural origin**. Re in Zaka Bay was also found to be of natural origin, since $\delta^{13}C$ values ranged from −32.2‰ to −28.2‰ and coincide with the bulk $\delta^{13}C_{OC}$ values in sediments. At this location, the percentage of allochthonous organic material, which is delivered by the small stream Solznik, is higher than at station D in western basin. The stream Solznik drains the partially coniferous Lake Bled watershed which could be the source of Re in Zaka Bay sediments.

- The concentration profiles of Per in Lake Bled sediments, that differ markedly from those of the pyrogenic PAHs, and the isotope composition of Per suggest post-depositional, *in situ* diagenetic formation.
- The distribution of $\delta^{13}C$ values of other individual PAHs showed that PAH input to lake sediments was of **pyrolytic origin**, probably dominated by coal and later, in the 1950s, also by wood burning. PAH from vehicle emissions could also contribute to the overall isotope signatures at a depth of 12-14 cm at station D in western basin and Zaka Bay, corresponding to the period 1953-1961.

This thesis indicates that the alpine Lake Bled (NW Slovenia), with its anoxic hypolimnion, could provide valuable information about the transport and fate of OM and PAHs in the lacustrine environment. PAHs were found to be resistant to weathering reactions in anoxic sediments and thus useful in identifying paleo-environmental pollution activities. Artificially induced or promoted natural eutrophication of lakes by anthropogenic pollution is only one of the processes that, in turn, can have many ecological and socio-economic effects on the local scale. On the global scale, results of the proposed research will enable a better understanding of natural processes of formation, emission and transformation of greenhouse gases (CO_2 and CH_4) in eutrophic aquatic environments, affecting global environmental and climatic changes.

Stable isotopes of biomarkers could be further used in different environments such as marine and riverine systems to determine the sources of organic matter and other field of investigation – for example controlling the authenticity of food products. Source identification using isotopic composition of PAHs could be used in environmental pollution studies. PAHs in atmospheric particles resulting from natural sources could be distinguishing from various anthropogenic combustion processes.

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Appendix

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MARINKA GAMS PETRIŠIČ [28478]

ARTICLES AND OTHER COMPONENT PARTS

1.01 Original scientific article

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3.25 Other performed works

Mlekuž, D.; Budja, M.; Žibrat Gašparič, A.; Horvat, M.; Ogrinc, N.; Gams Petrišič, M.; Žigon, D. *From landscape to lipids: multiscalar intepretation of Maharski prekop site from Ljubljana marshes: predavanje na 19. Neolitskem seminarju "Changing Paradigms: Interdisciplinary Studies of Eurasian Prehistory"*. (Mestni muzej, Ljubljana, 2012).