BACTERIAL RECYCLING OF ARCHAEAL BIOMASS AS A NEW STRATEGY FOR EXTREME LIFE IN THE DEAD SEA DEEP SEDIMENT

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Abstract

Archaea and Bacteria that inhabit the deep subsurface (known as the deep biosphere) play a prevalent role in the recycling of sedimentary organic carbon. In such extreme environment, this process can occur over millions of years[1] and requires microbial communities to cope with limited sources of energy. Because of this scarcity, metabolic processes come at a high energetic cost, but the ways heterotrophic microbial communities develop to enable the least energy expenses for a maximized yield remain unclear. Here, we report molecular biomarker evidence for the recycling of archaeal cell wall constituents by bacteria in extreme evaporitic facies of the Dead Sea deep sediments. Isoprenoid wax esters (WE) derived from the recombination of hydrolyzed products of archaeal membrane lipids were retrieved in gypsum and/or halite sedimentary deposits down to 243 meters below the lake floor (mblf), implying the reutilization of archaeal necromass by deep subsurface bacteria. By recycling the building blocks of allegedly better adapted archaea, heterotrophic bacteria build up intracellular carbon stocks and gain access to free water in this deprived environment. This strategy illustrates a new pathway of carbon transformation in the subsurface and how life is maintained in extreme environments experiencing long-term isolation and minimal energetic resources.

In extreme environments, any metabolic process that significantly lowers the energetic cost of life is favored[2]. In such settings, growth rates decrease, and most energy is diverted to maintenance functions[3] such as osmotic equilibration, O_2 stress defense, motility or shifts to sustainable metabolic pathways. These selective conditions of life promote the dominance of prokaryotes and generally favor Archaea relative to Bacteria[2]. This is mostly due to the reduced membrane permeability of Archaea, which requires less maintenance energy with respect to bacterial membranes[4]. This advantage is particularly striking in environments characterized by high osmotic stress such as hypersaline environments. There, Bacteria may use alternative strategies that allow competition with allegedly better adapted Archaea, for example by recycling available organic molecules as osmotic solutes[5]. The intracellular accumulation of available organic carbon is a common way of economizing energy in harsh environments. Under stressing conditions of growth, some bacteria are known to accumulate intracellular lipid droplets[6,7] in the form of polyhydroxyalkanoates, triglycerides or wax esters. The latter can be biosynthesized by condensation of long-chain alkyl lipids present in the sediment or formed during organic matter degradation[8]. However, in environments such as the deep subsurface that are deprived of labile organic carbon[9], this mechanism may not be a satisfactory option and Bacteria must resort to other strategies to survive.

We here provide unprecedented evidence for an original strategy developed by Bacteria to survive in the deep hypersaline sediment of the Dead Sea. The lipid composition of different sedimentary facies obtained through the Dead Sea Deep Drilling Project showed the presence of significant amounts of isoprenoid WE (up to 0.2 $\mu g.g^{-1}$ TOC) in halite and gypsum samples retrieved between 90 and 250 mblf (Fig. 1). Wax esters are a type of energy storage molecules that can be formed and accumulated by Eukarya and Bacteria, particularly in conditions of stress, but that have not been reported in the archaeal domain[10]. The formation of WE arises

from the condensation of linear or isoprenoid alkyl chains available in the environment and provides easily accessible (intracellular) sources of carbon[7]. The WE detected in the Dead Sea halite/gypsum samples mostly consist of lipid subunits derived from the cell walls of Archaea, the dominant organisms of the lake[11,12]. The major WE detected was phytanyl phytanate (3,7,11,15-tetramethylhexadecyl-3,7,11,15-tetramethylhexadecanoate; iC_{20}-iC_{20}, Fig. 2) which was accompanied by series of other isoprenoid WE composed of a C_{20} or C_{25} isoprenoid alcohol esterified to a linear, methyl-branched or isoprenoid acyl chain (Fig. 2a and Table S2). The isoprenoid WE were systematically observed with significant amounts of membrane lipids of halophilic archaea -archaeol (2,3-*O*-diphytanyl-*sn*-glycerol) and extended archaeol (2,3-*O*-phytanyl-*O*-sesterterpanyl-*sn*-glycerol)[13,14]- along with several of their hydrolyzed (and eventually oxidized) products (Figs 1; 2b). Lipids of halophilic archaea are specifically composed of C_{20} and C_{25} isoprenoid alkyl chains which allow for a better control on membrane permeability under strong osmotic conditions compared to bacterial fatty acid membranes[4,15]. Archaeol was the most abundant isoprenoid alcohol in most of the investigated sediment samples (Fig. 1). Extended archaeol, a membrane lipid specific of halophilic archaea of the Halobacteria class[13] that dominate the Dead Sea halite and gypsum sediment[16], was also found enriched in halite and gypsum facies. In most of the sedimentary intervals where WE occurred, the core lipids of halophilic archaea (archaeol and extended archaeol) were found in lower abundance than in the other halite/gypsum samples (Fig. 1). Conversely, the degradation products of these archaeal membrane lipids were preferentially observed in the WE-rich intervals (Fig. 1), and mainly consisted of C_{20} and C_{25} isoprenoid alcohols and acids (phytanol, phytanic acid and C_{25} homologues), and of *sn*-1 and *sn*-2 isoprenoid C_{20} and C_{25} monoalkyl glycerols (Fig 2b and supplementary data).

The report of WE with isoprenoid C_{25} carbon chains (iC_{20}-iC_{25} and iC_{25}-iC_{20}) in natural samples (Fig. 2a and Table S2) is unprecedented and testifies for the use of subunits of core

lipids from halophilic archaea as a source for the isoprenoid alkyl and acyl chains present in the WE. The formation of isoprenoid WE has been previously demonstrated during growth of bacteria on free isoprenoid compounds such as phytane, phytol or squalene [8,17,18]. However, such isoprenoid compounds were not observed together with the isoprenoid WE from the Dead Sea. Together, our results constitute an indisputable proof for the reutilization of archaeal membrane lipids to form isoprenoid WE.

The characterization of isoprenoid WE has been shown to constitute a useful tool to decipher the metabolic pathways employed by microorganisms for the degradation of isoprenoid substrates[8,17,18]. The condensation of isoprenoid compounds with themselves or with their degradation products to form isoprenoid WE indeed allows the trapping of transient metabolites which, under their free form, would not necessarily be preserved. The structural analysis of the different WE present in the Dead Sea halite samples allowed to decipher the metabolic pathways involved in the degradation of archaeal core lipids. Following the hydrolysis of the ether linkages present in archaeol and extended-archaeol, part of the released isoprenoid C_{20} and C_{25} alcohol moieties is being oxidized to the corresponding isoprenoid fatty acids, providing the building blocks for the biosynthesis of iC_{20}-iC_{20}, iC_{25}-iC_{20} and iC_{20}-iC_{25} WE. The isoprenoid fatty acids may then be further degraded to shorter chain acids via successive α-oxidation, β-oxidation and β-decarboxymethylation sequences[8,18] (Fig. S2). The identification of iC_5-iC_{20} WE in some samples demonstrates the almost complete degradation of the isoprenoid alkyl chains released from the hydrolysis of archaeol and extended archaeol to provide carbon and energy to the deep biosphere.

Current knowledge suggests that the ability to form and accumulate WE has only arisen in bacterial and eukaryal domains[10]. Eukaryotic life in the Dead Sea has been constrained to humid intervals of the glacial periods and occasional blooms triggered by high precipitation

106 during interglacial stages[16,19]. Hence, isoprenoid WE present in the most arid intervals of the
107 Dead Sea sediment (halite/gypsum) cannot originate from eukaryotes. The occurrence of
108 specific bacterial methyl-branched C_{17} fatty acids in the sedimentary intervals where WE
109 were preferentially produced (Fig. 1) rather supports a bacterial origin for the isoprenoid
110 WE[20]. Such methyl-branched C-odd fatty acids are common in sulfate-reducing bacteria[21].
111 Our data therefore support the development of bacteria upon archaeal necromass in the
112 extreme environment of the Dead Sea subsurface.
113 The concentration of intracellular WE in Bacteria has been shown to rise particularly in
114 situations of nutrient starvation, especially when nitrogen is limiting[22]. Additionally, the
115 recycling of archaeal biomass by Archaea themselves was suggested to minimize the energy
116 cost of life in nutrient- and carbon-limited environments[23]. We here suggest that the bacterial
117 recycling of archaeal necromass as storage lipids like WE constitutes a way to save and store
118 energy in nutrient- and energy-demanding hypersaline environment of the Dead Sea. The
119 presence of WE with mixed linear and isoprenoid chains alongside the major isoprenoid WE
120 indicates that the WE-forming bacterial population also recycled bacterial and/or eukaryal
121 lipids, in addition to the archaeal ones (Figs. 1, 2 and 3). As a result, bacteria create easily
122 accessible carbon stocks from the necromass, in a lake with very little allochthonous inputs
123 and rare primary production[24].
124 The accumulation of WE has been previously evidenced in temporarily emerged microbial
125 mats from hypersaline environments[25], and suggested to allow for a better survival of
126 microbial cells during periods of desiccation, due to the release of H_2O from esterification.
127 The Dead Sea sediments that bear WE originate from the deepest part of the lake, where no
128 trace of desiccation has been observed. These sedimentary levels correspond, however, to
129 periods where salinity was the highest in the deep brine, as supported by the bromide
130 concentration curve (Fig. 1). The latter is interpreted as a good estimate of the degree of

131 dilution/concentration of the Dead Sea water column during the Quaternary[26]. The WE levels
132 generally correspond to the highest concentrations of this conservative element (Fig. 1; Table
133 S1) observed during the driest climatic intervals (Marine Isotopic Stages 5E, early 5A and 2/1
134 transition). Water availability is a major issue not only in dry environments, but also in
135 hypersaline settings due to high concentrations of salts. By recombining hydrolyzed moieties
136 of core lipids originating from the buried archaeal necromass, the WE-forming bacterial
137 population creates accessible water molecules that favor their survival.
138
139 The conditions under which the early Holocene or Pleistocene halite have deposited are
140 expected to be similar to the present-day environment that allows halite precipitation from the
141 Dead Sea water column[26]. If such conditions were favorable to the formation of WE, the
142 occurrence of these lipids would also be expected in the most surficial halite sediment. Since
143 this is not the case, the WE biosynthesis likely occurred deeper in the sedimentary column.
144 The formation of WE has been essentially documented for aerobic microorganisms and
145 environments[8,27,28], but their biosynthesis by anaerobic microorganisms has already been
146 recognized[29,30]. Hence, the unique occurrence of isoprenoid WE in deep halite/gypsum
147 deposits (dated from the late Marine Isotopic Stage 5 to the early Holocene) is suggestive of
148 their production within the anoxic sediment.
149 Wax esters are generally poorly preserved in the sedimentary record[27], although some of these
150 compounds have been reported in ca. 40 ka-old lacustrine sediments[31]. The age of the Dead
151 Sea sediments containing the isoprenoid WE has been estimated between 120 and 11.4 ka
152 (Table S1) based on U-Th on aragonite[32] and ^{14}C dating[33]. This supports either the
153 extraordinary preservation of labile organic matter in the Dead Sea subsurface, or the post-
154 depositional biosynthesis of these compounds by active bacterial communities until 243 m
155 below lake floor.

156 The present investigation illustrates the high plasticity of Bacteria and their ability to use
157 varied strategies for energy production and preservation under harsh conditions. By studying
158 an environment that pushes life to its limits, we catch a glimpse of the processes that fuel life
159 in the deep subsurface, and add a new loop to the sedimentary carbon cycle.
160
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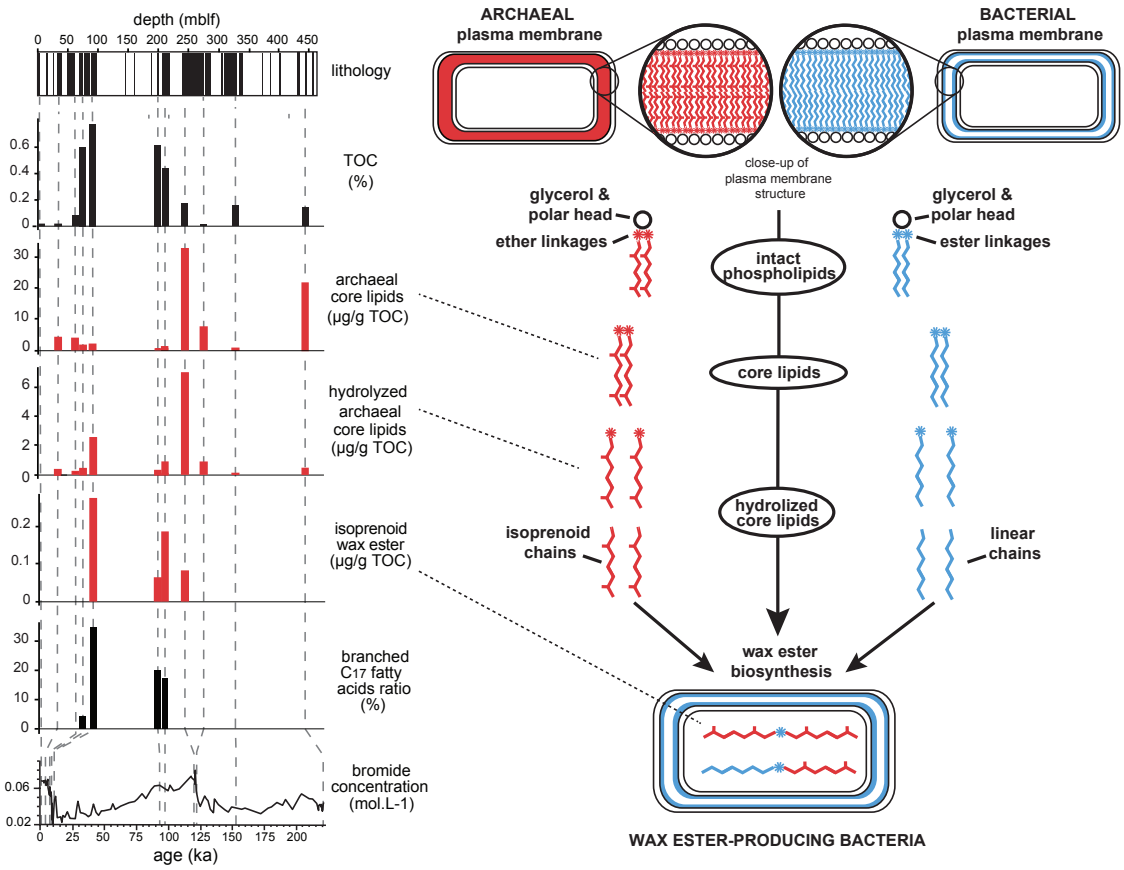

Fig. 1: Occurrence of wax esters (WE) and other lipid biomarkers along the Dead Sea core (gypsum and halite intervals are blacked, broken lines show the measured samples). The formation of the building blocks of WE are schematized on the right hand side of the panel and linked to their quantitative profiles. The branched C_{17} fatty acids ratio indicates the presence of specific bacteria likely involved in WE-production. The sampled layers are compared to bromide concentrations in the pore water of the Dead Sea [used as a proxy for lake water dilution/concentration[26]] and fitted to ^{14}C ages[34] and U-ages[32]. Red items indicate an archaeal origin, and blue items a bacterial origin.

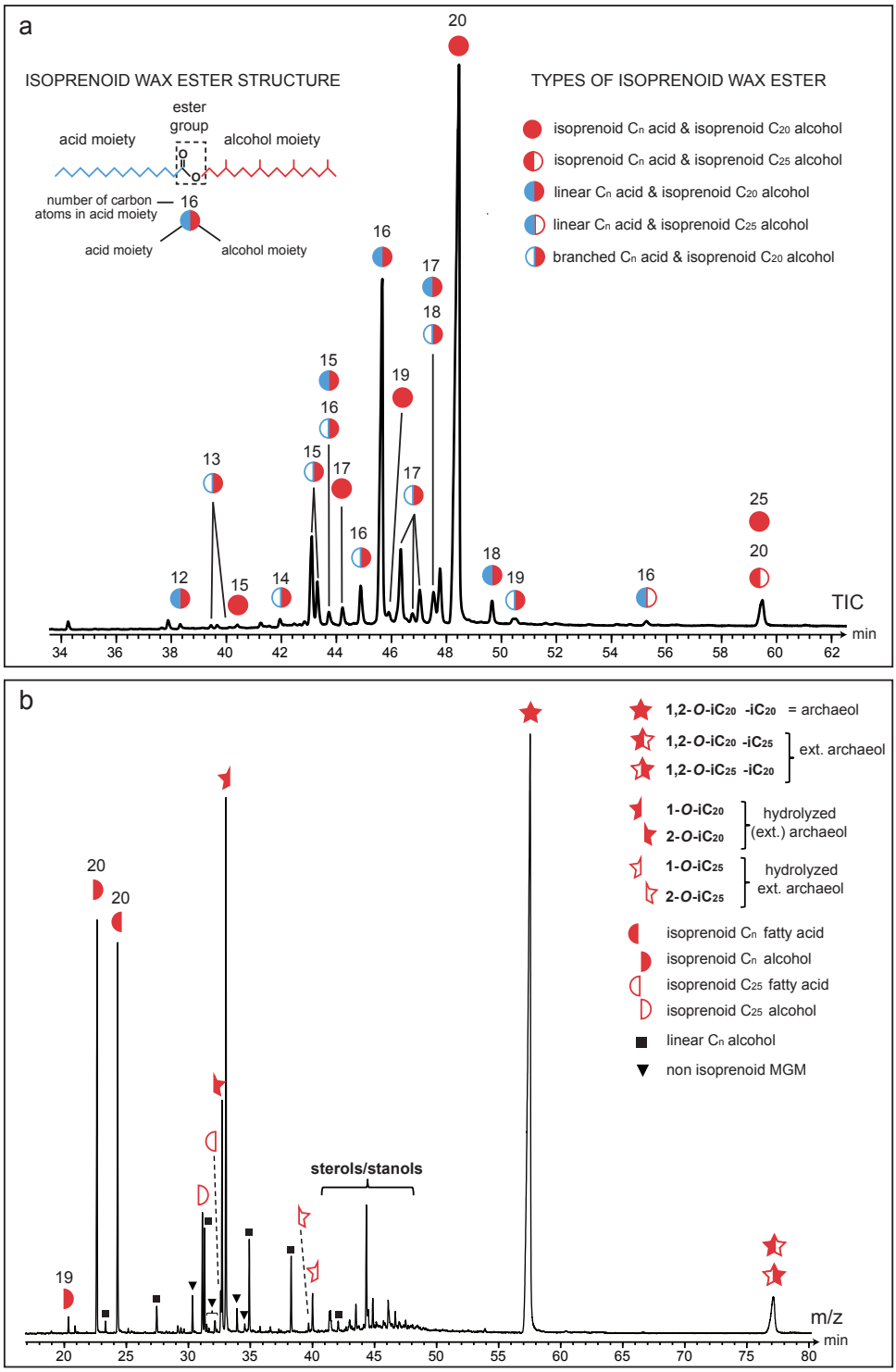

Fig. 2: (a) Total ion chromatogram (TIC) of the wax ester fraction from the gypsum sample at 91.04 mblf and (b) selected ions chromatogram of a polar lipid fraction from the gypsum sample at 243.22 mblf. These intervals were selected as they show the highest concentrations of isoprenoid WE and hydrolyzed core lipids, respectively. MGM: monoalkyl glycerol monoethers. Red symbols indicate an archaeal origin and blue symbols a bacterial and/or eukaryal origin. The numbers on top of symbols shown in (b) correspond to the number of carbon atoms in the chain.

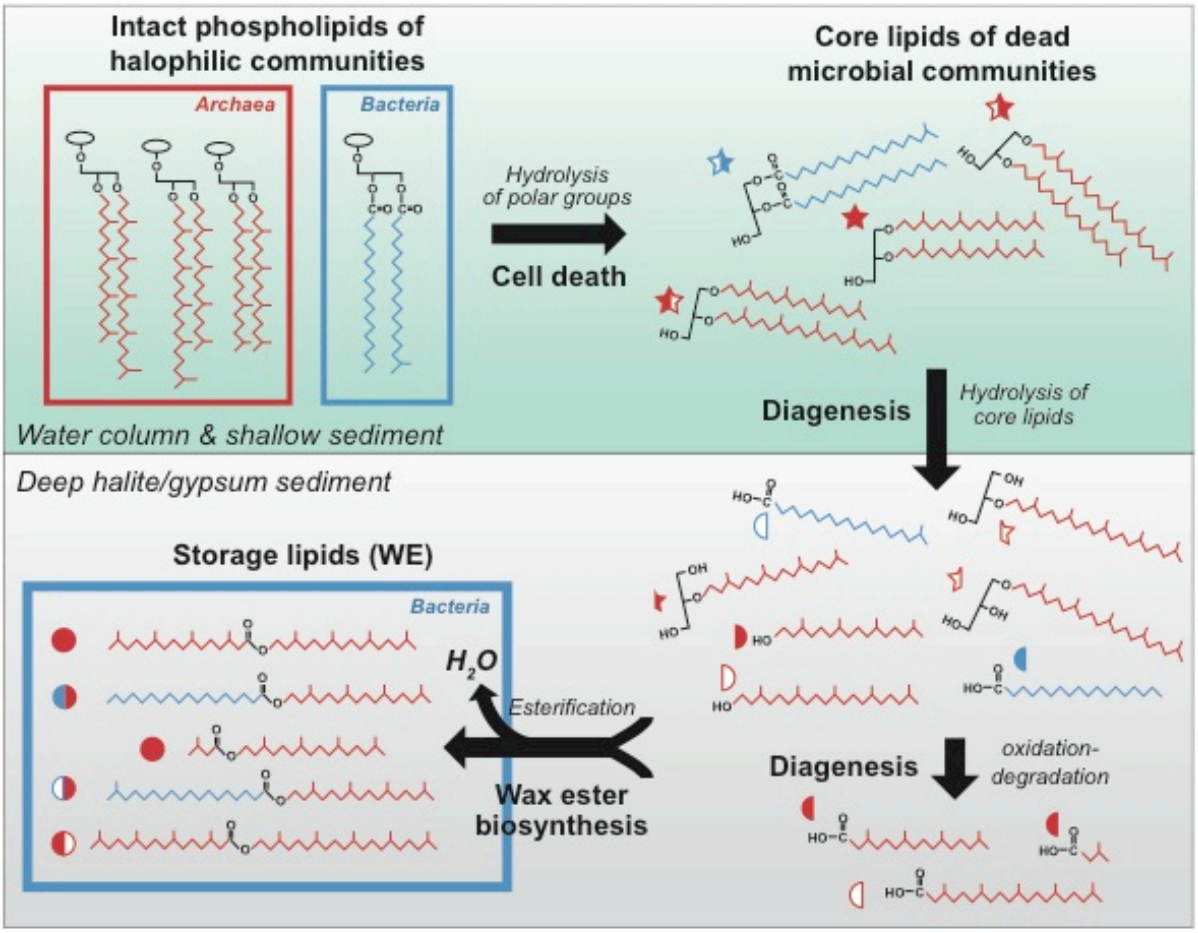

Fig. 3: Schematic pathway for the formation of isoprenoid wax esters by bacteria recycling membrane lipids of halophilic organisms in extreme sediments from the Dead Sea. Intact phospholipids from archaeal and bacterial communities are degraded and some of the degradation products are transformed into WE by bacterial communities of the deep halite/gypsum sediment. Symbols refer to molecules identified in Fig. 2. For the degradation products, moieties in red have an archaeal origin and blue moieties have a bacterial origin.

Methods

During the drilling campaign, sediments were sampled from core catchers using sterile tools, and kept in the freezer until further processing. The main characteristics of samples used for lipid biomarkers are given in Table S1. Samples were freeze-dried, ground and extracted through sonication cycles (methanol x2, methanol/dichloromethane (1:1, x2) and dichloromethane x3). Elemental sulfur was removed with activated copper. Lipid extracts were filtered out and separated over an inactivated column of silica gel (5% H_2O) into five fractions of increasing polarity. Fraction F1 was eluted with hexane/dichloromethane (9:1), fraction F2 with hexane/dichloromethane (1:1), fraction F3 with dichloromethane, fraction F4 with ethyl acetate and fraction F5 with methanol. Fractions F3 and F4 were silylated with pyridine/BSTFA 2:1 (v/v). Fraction F5 was trans-esterified by incubating at 60°C overnight with toluene (0.5 ml) and 2% H_2SO_4 in methanol (2 ml). NaCl 5% and hexane:dichloromethane were then added and the organic upper phase was extracted three times, washed with $NaHCO_3$ (2%) and dried with sulfate. The dry extract was then silylated with pyridine/BSTFA 2:1 (v/v) before analysis by gas chromatography-mass spectrometry (GC-MS). GC-MS analyses were performed on a HP 6890 Series Plus gas chromatograph equipped with a cool on-column injector and coupled to an Agilent 5975C (VL MSD) mass spectrometer. The samples were injected at 60°C and the oven temperature was programmed as follow: 60 °C isotherm for 30 sec, 20 °C /min to 130 °C, 5 °C /min to 250 °C and 3 °C/min to 300 °C. Identification of compounds was based on interpretation of their mass spectral characteristics and comparison of their mass spectra and retention times with those of authentic standards or literature data. Quantification was realized using stepwise dilution of external standards of alcohol, ester and alkanes. Peak areas were integrated manually using the ChemStation software. Isoprenoid fatty acids are less polar than linear acids and partly eluted in the alcohol fraction.

Analyses of C organic content were determined using an Elementar Vario Micro Cube coupled to a Thermal Conductivity Detector. Between 1 and 8 mg of bulk samples were weighted into tin capsules (Elemental Microanalyses, 11.5*7mm) with a CPA26P Sartorius microbalance (2.10^{-6}g). The capsules were individually introduced into a combustion furnace (950°C) with an excess of oxygen. Copper oxide was used as oxidation catalyst and He as carrier gas. Reduction of N_xO_y to N_2 and removal of excess O_2 was achieved with reduced copper at 550°C. Water was removed with a phosphorous pentoxide chemical trap. N_2 and CO_2 were separated with a purge and trap desorption column. The working standard, IVA sediment, was measured every ten samples. Normalization was made using a working standard of IVA sediment containing 9.15% of C.

Calculation of the branched C_{17} fatty acids ratio (Fig.1) was realized using the ratio of 10-methyl C_{17} fatty acid over the sum of 10-methyl, iso and anteiso C_{17} fatty acids. To enhance the visualization of the alcohol and acid fragments, selected ions of m/z 103+133+159+205+218 were added on a single chromatogram (Fig. 2b).