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Sedimentary membrane lipids recycled by deep-sea benthic archaea

Yoshinori Takano[1*], Yoshito Chikaraishi[1], Nanako O. Ogawa[1], Hidetaka Nomaki[1], Yuki Morono[2], Fumio Inagaki[2], Hiroshi Kitazato[1], Kai-Uwe Hinrichs[3] & Naohiko Ohkouchi[1,4,5]

[1]Institute of Biogeosciences, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima, Yokosuka 237-0061, Japan.

[2]Kochi Institute for Core Sample Research, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), Monobe B200, Nangoku, Kochi 783-8502, Japan.

[3]Department of Geosciences and MARUM Center for Marine Environmental Sciences, University of Bremen, D-28359, Bremen, Germany

[4]Department of Environmental Science and Technology, Tokyo Institute of Technology, 4259 Nagatsuda, Midori-ku, Yokohama, Kanagawa 226-8502, Japan

[5]Department of Earth and Planetary Sciences, University of Tokyo, 7-3-1 Hongo, Tokyo 113-0033, Japan

E-mail: takano@jamstec.go.jp; nohkouchi@jamstec.go.jp

Fax: +81-46-867-9775

Since the work of Woese et al.[1], Archaea are recognized as one of three domains of life and cosmopolitan microbes in marine environments[2-5]. Recent molecular evidence suggests that uncultured heterotrophic archaea relying on relic organic carbon dominate biomass in marine sediment[6]. Archaeal membranes are well characterized, and are comprised of a glycerol backbone and a non-polar isoprenoid chain. However, the ecology of sedimentary archaea remains elusive, because it is difficult to grow them in the laboratory. Here, we trace the fate of ^{13}C-labelled glucose added to marine sediments in Sagami Bay, Japan, to determine the *in situ* mechanisms of membrane synthesis. We found that the ^{13}C was incorporated into the glycerol backbone of archaeal membranes; ^{13}C was apparent after 9 days of incubation, but most pronounced following 405 days. The isoprenoid chain of the membranes remained unlabelled, however. Based on the differential uptake of ^{13}C, we suggest that the glycerol unit is synthesized *de novo*, whereas the isoprenoid unit is synthesized from exogenous relic archaeal membranes and detritus, due to the prevalence of these compounds in marine sediments. We therefore suggest that some benthic archaea build their endogenous membranes by recycling sedimentary organic compounds for their growth and maintenance.

Deep-sea sediments harbor a novel and vast biosphere with yet unconstrained importance in the global carbon cycle[6-14]. Carbon isotopic signatures of archaeal polar lipids from sediments dominated by benthic archaeal communities indicate utilization of sedimentary organic carbon[6,15]. On-shore laboratory studies aimed at understanding

microbial processes in deep-sea sediments remain problematic because most indigenous microbes appear viable but non-culturable[16]. To further constrain the substrates utilized by benthic archaea, we conducted an *in situ* ^{13}C-tracer experiment to provide direct evidence of their metabolism *via* an investigation of membrane lipid biomarkers.

The experiment was performed on the seafloor at the central part of Sagami Bay, Japan (35°00.8'N, 139°21.6'E; 1453 m depth)[17] for 0-405 days (Supplementary Figure 1). Sedimentary processes relevant to carbon burial and benthic activity have been previously documented[18]. In our experiment, an aqueous solution of 16.5 mg of ^{13}C-labeled glucose ($^{13}C_6H_{12}O_6$) was injected into the headspace of the chamber using dual 5-mL syringes attached to the top (Figure 1). The incubation cores and a reference core were recovered at 0, 9 and 405 days after deployment (hereafter, the culture cores are referred to as G-*i*, with *i* indicating the length of incubation in days).

Already after 9 days, ^{13}C-tracer incorporation was unambiguously detected in caldarchaeol ($\delta^{13}C$ = +46‰) and crenarchaeol (+22‰) from the surface sediment (0–1 cm depth), reflecting the active metabolism and growth of the benthic archaeal community (Figure 2). ^{13}C uptake increased after 405 days. Depth profiles of carbon isotopic compositions of caldarchaeol and crenarchaeol are positively correlated (R^2 < 0.83; *p*-value > 0.07) with those of organic matter in the sediment. $\delta^{13}C_{caldarchaeol}$ (<-21‰) and $\delta^{13}C_{crenarchaeol}$ (<-22‰) in the experimental blank are consistent with values previously observed in benthic[15] and planktonic[19] archaeal communities (Figure 2). ^{13}C-tracer uptake process is either due to heterotrophic assimilation of glucose or autotrophic assimilation of $^{13}CO_2$ after glucose has been oxidized by undefined microbial

community members (cf. DIC, $\delta^{13}C_{DIC}$ > +4200‰ in G-9 experiment). In the latter case, the signal could result from assimilation of $^{13}CO_2$ by marine group I (MGI) crenarchaeota. 16S rRNA genes affiliated with the MGI group dominate the corresponding clone library in the G-9 experiment (Figure 3). However, given previous demonstration of rapid ^{13}C-DIC uptake into the biphytanyl moieties of caldarchaeol and crenarchaeol by closely related planktonic MGI crenarchaeota[4], this autotrophic scenario appears less likely (see Supplementary Information).

The carbon isotopic compositions of the acyclic, bicyclic and tricyclic archaeal biphytanes (hereafter referred to as BP[0], BP[2] and BP[3], respectively, see Methods) released from caldarchaeol and crenarchaeol were around –22‰ in experiment G-405, whereas the bulk GDGT core lipids caldarchaeol (+69‰) and crenarchaeol (+132‰) were significantly ^{13}C-enriched (Figure 2, Supplementary Tables 1 & 2). This disparity indicates that the ^{13}C tracer is unevenly distributed in the core lipid molecule, *i.e.*, ^{13}C-tracer is concentrated in the lipid's 2,3-*sn*-glycerol backbone. We can estimate the carbon isotopic composition of 2,3-*sn*-glycerol ($\delta^{13}C_{2,3\text{-}sn\text{-glycerol}}$) by mass balance (Supplementary Information).

$$86 \times \delta^{13}C_{caldarchaeol} = 80 \times \delta^{13}C_{BP[0]} + 6 \times \delta^{13}C_{2,3\text{-}sn\text{-glycerol}} \quad \text{Eq. [1]}$$

$$86 \times \delta^{13}C_{crenarchaeol} = 40 \times \delta^{13}C_{BP[2]} + 40 \times \delta^{13}C_{BP[3]} + 6 \times \delta^{13}C_{2,3\text{-}sn\text{-glycerol}} \quad \text{Eq. [2]}$$

Accordingly the 2,3-*sn*-glycerol moieties in caldarchaeol and crenarchaeol in the 0–1 cm interval are enriched in ^{13}C by 900‰ and 500‰, respectively, after 9 days, and by

1300‰ and 2200‰ after 405 days (Figure 2). For further confirmation of our observation, the validity of the estimates was confirmed by direct measurement of the carbon isotopic compositions of 2,3-*sn*-glycerol (Supplementary Information). Then, the carbon isotopic composition of glycerol moieties derived from the purified caldarchaeol and crenarchaeol in G-405 samples were up to +1900‰ and +2780‰, respectively. These values are consistent with ^{13}C-mass balance estimations. The large heterogeneity suggests a predominance of the benthic archaeal metabolic pathway toward 2,3-*sn*-glycerol rather than isoprenoid lipid biosynthesis. The variable differences of $\delta^{13}C_{2,3\text{-}sn\text{-glycerol}}$ values of caldarchaeol and crenarchaeol are consistent with changes in community composition and associated changes of the sources of these two compounds during the course of the experiment. Structural and biosynthetic studies of archaeal lipids[20] suggest the presence of a pathway leading to GDGTs *via* formation of 2,3-*sn*-glycerol and isoprenoid moieties (Supplementary Information). When synthesizing *sn*-glycerate, Archaea utilize either the Embden-Meyerhof (EM), the modified EM or the Entner-Doudoroff (ED) pathway[21]. In contrast, they produce isoprenoids through either the mevalonate (MVA) or the modified MVA pathway from acetyl-CoA[22].

16S rRNA and quantitative PCR (qPCR) analysis indicated a community shift in the composition of the archaeal community and its abundance (10^5-10^7 copies g-sed^{-1}) during the course of the experiment, whereas the relative abundances (%) of tetraether archaeal lipids were almost constant during 405 days (Figure 3). The initial community consisted predominantly of *Crenarchaeota* including MGI, the Soil Crenarchaeota Group (SCG), and the Miscellaneous Crenarchaeotal Group (MCG). After 9 days of incubation, the

relative proportion of individual archaeal groups, and especially MGI and MCG, changed substantially. After 405 days, the relative proportions of clones of these two groups resemble again those at initial G-*0*. Benthic MGI were previously identified in organic-rich (*e.g.*, Peru Margin, ODP Leg 201) and also organic-poor (*e.g.*, Equatorial Pacific, ODP Leg 201) marine sediment[13]. Furthermore, genes of the Methanosarcinales accounted for approximately 20% of the archaeal assemblages after 405 days.

Given the properties of the outer membrane of prokaryotic cells[23] in combination with the extremely energy-limited sedimentary archaeal communities[11], the following scenario is consistent with the intramolecular isotopic distribution. Benthic archaea could synthesize caldarchaeol and crenarchaeol via recycling of exogenous precursor compounds, such as biphytane diols resulting from decomposition of decayed archaeal cells, while only 2,3-*sn*-glycerol is synthesized *de novo* process as signaled by exclusive ^{13}C-label uptake in this molecular moiety. Price and Sowers[24] estimated that the metabolic energy required for growth of microbial cell is 6 orders of magnitude larger than that needed for survival. Therefore, the recycling of fossil molecules could be a crucial strategy for coping with conditions of energy starvation in sedimentary environments and aid in minimizing energy expenditures for growth and maintenance[12,15]. Transport of these exogenous building blocks may take place *via* permeable channels in the outer membrane[23]. Passage of solutes across the outer membrane channel is generally permeable to hydrophobic molecules with molecular weight in excess of 1000 Da[23]. Specifically, transport initiated by adsorption of hydrophobic functional groups (e.g.,

hydroxyl groups) onto porin proteins are proposed for long-chain fatty acids and other organic molecules across the outer membrane channel (Supplementary Information).

Relic archaeal tetraether core lipids are among the most abundant molecular types in marine sediments[19]; these compounds could potentially serve as substrate for supplying the building-blocks, although this would require biochemical scission of ether-bonds[25], a process associated with an appreciable investment of energy (<360 kJ mol^{-1}). This experiment strongly suggests that deep-sea benthic archaea utilize yet unknown mechanisms to minimize energy expenditures.

Methods

Bulk and compound-specific isotope analysis

We extracted archaeal core GDGTs (glycerol dialkyl glycerol tetraethers) from dried sediments using an improved method[26] and purified them by preparative high-performance liquid chromatography combined with atmospheric-pressure chemical ionization mass spectrometry (HPLC/APCI-MS) without ether-bond cleavage treatment (Supplementary Figure 2). To verify an analytical bias during HPLC, we carefully evaluated potential co-elution by fraction collection both before caldarchaeol peak and after crenarchaeol peak on the chromatogram (Supplementary Figure 3). This CSIA method was performed with an elemental analyzer coupled to an isotope ratio mass spectrometer (EA/IRMS). Bulk isotope analysis ($\delta^{13}C_{TOC}$) and total organic carbon (TOC) in the sediment were determined by conventional methods (see Supplementary Information).

To analyze the biphytanes of caldarchaeol and crenarchaeol, we performed an ether cleavage treatment[27], followed by reduction[28]. We determined the carbon isotopic compositions of resulting biphytane and glycerol derivatives using an online gas chromatograph/combustion/isotope ratio mass spectrometer (GC/C/IRMS), enabling the (i) calculation of 2,3-*sn*-glycerol ($\delta^{13}C_{2,3\text{-}sn\text{-glycerol}}$) using the mass balance equation between caldarchaeol, crenarchaeol and their isoprenoid moieties, (ii) direct measurement of $\delta^{13}C_{2,3\text{-}sn\text{-glycerol}}$. The validity of this method was verified using another marine sediment sample prior to application (Supplementary Information).

2. Phylogenic analysis of 16S rRNA and qPCR for benthic archaeal community

Bulk genomic DNA was extracted from 0.2–0.5 g of sediments using the ISOIL for Beads Beating Kit (Nippon Gene, Tokyo, Japan). Extracted DNA was further purified using the Montage PCR Kit (Millipore, Billerica, MA), and archaeal 16S rRNA gene fragments were amplified with Arch21F-1492R primers[2,29]. The PCR conditions were as follows: denaturation at 95°C for 60 sec, annealing at 50°C for 60 sec, and extension at 72°C for 120 sec, for 25–30 cycles. A total of 528 clones (more than 160 clones per sample) were selected from the libraries, and the inserted 16S rRNA gene sequences were determined using a 3130xl Genetic Analyser (Life Technologies, Carlsbad, CA). For analysing phylogenetic affiliation, the sequences were aligned and grouped using ARB software (AB561312-AB561824; 513 entries)[30]. To estimate copy numbers of archaeal 16S rRNA genes, quantitative PCR (qPCR) was performed with a SYBR® Premix DimerEraser® (Takara Bio Inc.) by StepOnePlus™ real-time PCR system[6] according to the

manufacturer's instructions (Applied Biosystems). Three replicate reactions per sample were performed with the primer set of ARC806F and ARC958R and the thermal condition of 95°C for 30 sec for initial denaturation, up to 50 cycles of 95°C for 5 sec, 55°C for 30 sec, 72°C for 30 sec. The standard curve for archaeal 16S rRNA genes were obtained from genome DNA of *Pyrococcus horikoshii* (R^2= 0.995).

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Author contributions

Y.T. performed present lipids analysis and wrote the paper; Y.C. & N.O.O. supported on carbon isotope standard reagents and IRMS analysis; H.N. & H.K. ^{13}C-substrate set up, *in-situ* deployment of the chamber, core processing during NT06-04, -05, -22 and NT08-02 cruise; Y.M. and F.I. phylogenic molecular analysis of 16S rRNA and qPCR; K-U.H. contributed technical aspects and was involved in study design; Y.T. and N.O. contributed to this study and all authors discussed the results and commented on the manuscript.

Additional information

Supplementary Information accompanies this paper on www.nature.com/naturegeoscience. Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions. Correspondence and requests for materials should be addressed to Y.T.

Figure captions

Figure 1. Distribution of benthic archaeal lipids and their carbon isotopic compositions during *in situ* ^{13}C-tracer experiment during 405 days. a, b, Total organic carbon (TOC, wt%) and carbon isotopic composition ($\delta^{13}C_{TOC}$, ‰ vs. PDB).

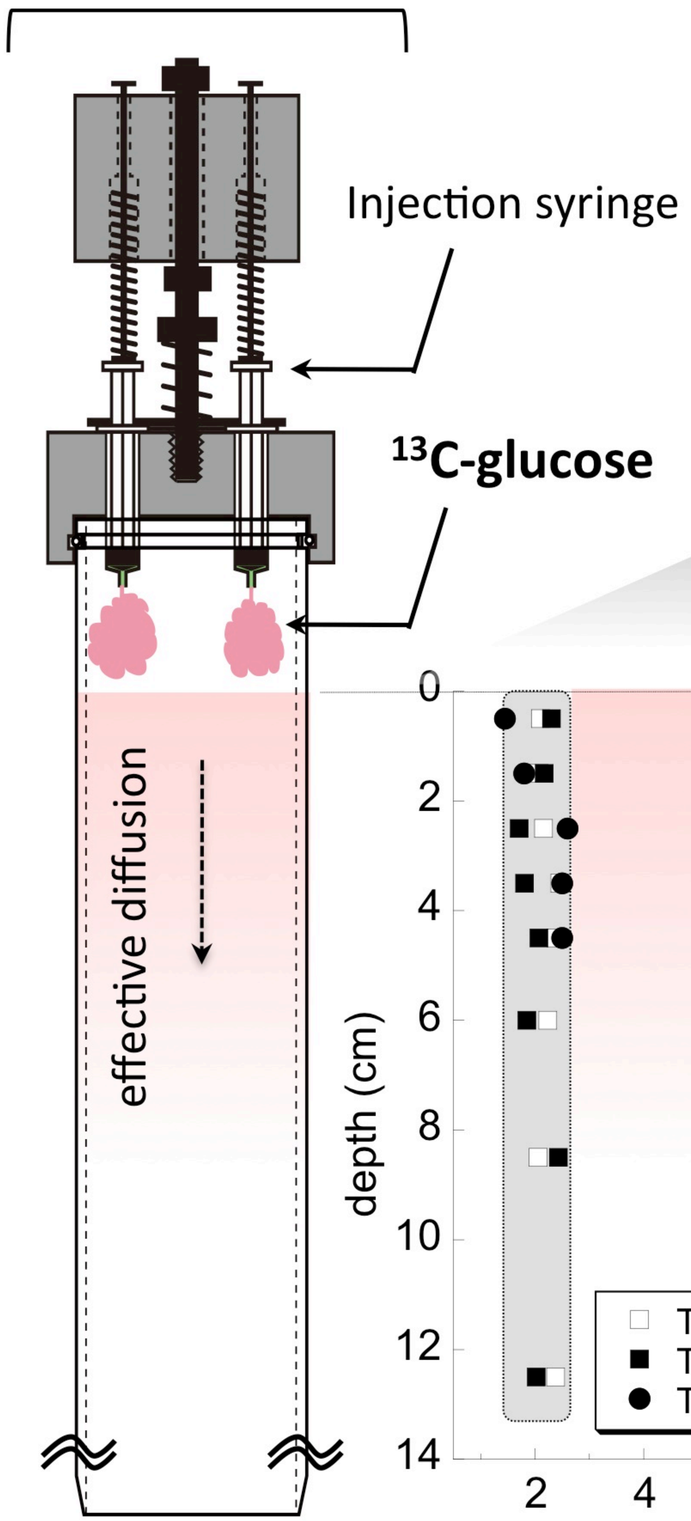
c, Carbon isotopic compositions of caldarchaeol and crenarchaeol ($\delta^{13}C_{caldarchaeol}$ and $\delta^{13}C_{crenarchaeol}$) for 0 day (G-0), 9 days (G-9), and 405 days (G-405) experiments. The experiments using I-K incubation chamber[17] were performed using the ROV *Hyper-Dolphin* and the R/V *Natsushima*. Bottom-water properties at the station are stable throughout the year, with temperatures of 2.3±0.1°C and salinity of 34.5±0.2 ‰ (see also Supplementary Figure 1).

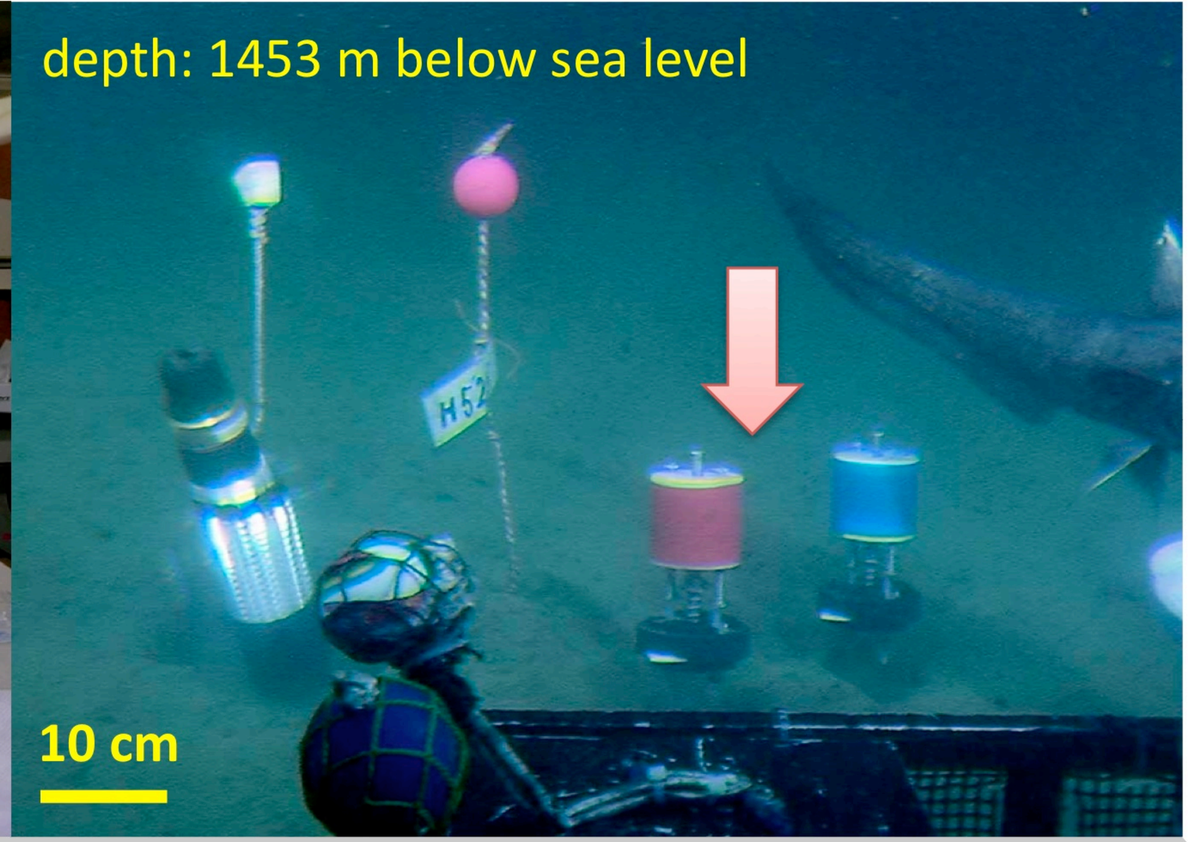
Figure 2. Results of carbon isotopic compositions of $\delta^{13}C_{caldarchaeol}$, $\delta^{13}C_{crenarchaeol}$, $\delta^{13}C_{biphytane}$ and $\delta^{13}C_{2,3\text{-}sn\text{-}glycerol}$ *in situ* ^{13}C-tracer experiments during 405 days. The sample analyzed for the time-course profiles was 0–1 cm depth below the seafloor each. Here, $\delta^{13}C_{2,3\text{-}sn\text{-}glycerol}$ of caldarchaeol and crenarchaeol (*see* molecular structure) were determined as follows: $\delta^{13}C_{2,3\text{-}sn\text{-}glycerol} = (86 \times \delta^{13}C_{caldarchaeol} - 80 \times \delta^{13}C_{BP[0]}) / 6$, where $\delta^{13}C_{caldarchaeol}$ and $\delta^{13}C_{BP[0]}$ were independently determined by GC/C/IRMS. Furthermore, $\delta^{13}C_{2,3\text{-}sn\text{-}glycerol} = (86 \times \delta^{13}C_{crenarchaeol} - 40 \times \delta^{13}C_{BP[2]} - 40 \times \delta^{13}C_{BP[3]}) / 6$, where $\delta^{13}C_{crenarchaeol}$, $\delta^{13}C_{BP[2]}$ and $\delta^{13}C_{BP[3]}$ were determined by CSIA. $\Delta\delta^{13}C_{glycerol\text{-}BP}$ stands for the difference in $\delta^{13}C$ values between 2,3-*sn*-glycerol and isoprenoid moieties.

Figure 3. Long-term monitoring for abundances of archaeal lipids and phylogenic analysis of the benthic archaeal community. a, Relative abundances (%, *n*=3) of GDGTs during 405 days. **b,** Natural assemblage of benthic archaea in the sampling site (mid-depth 1.5 cm), *in situ* experiment for 9 days and 405 days. Abbreviations; ANME2-d, Anaerobic oxidation of methane 2-d; MBG-D, Marine Benthic Group D; MEG/TMEG, Miscellaneous Euryarchaeotal Group/Terrestrial Miscellaneous

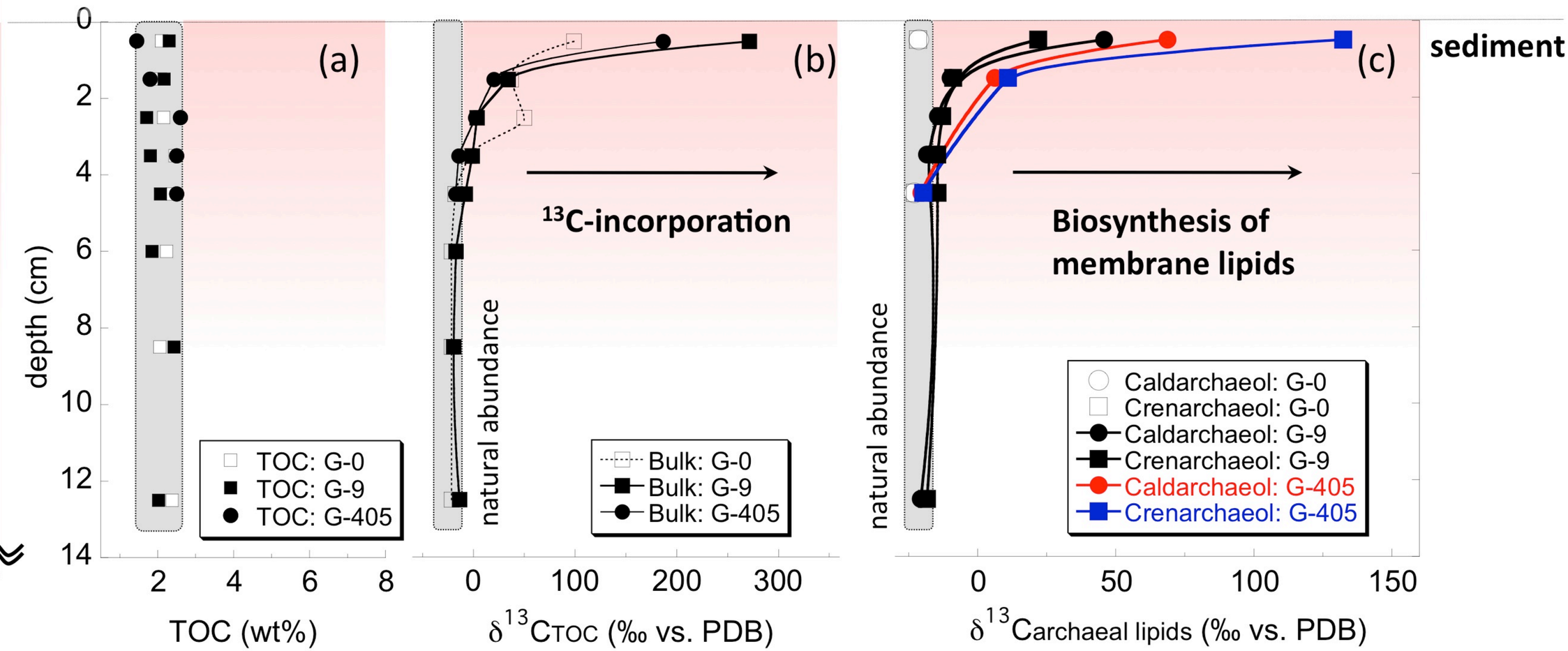
Euryarchaeotal Group; GSAG, Deep-Sea Archaeal Group; SAGMEG, South African Goldmine Euryarchaeotal Groups; MCG, Miscellaneous Crenarchaeotic Group. Species richness, community diversity, and abundance of the benthic archaea were also monitored during ^{13}C-incubation.

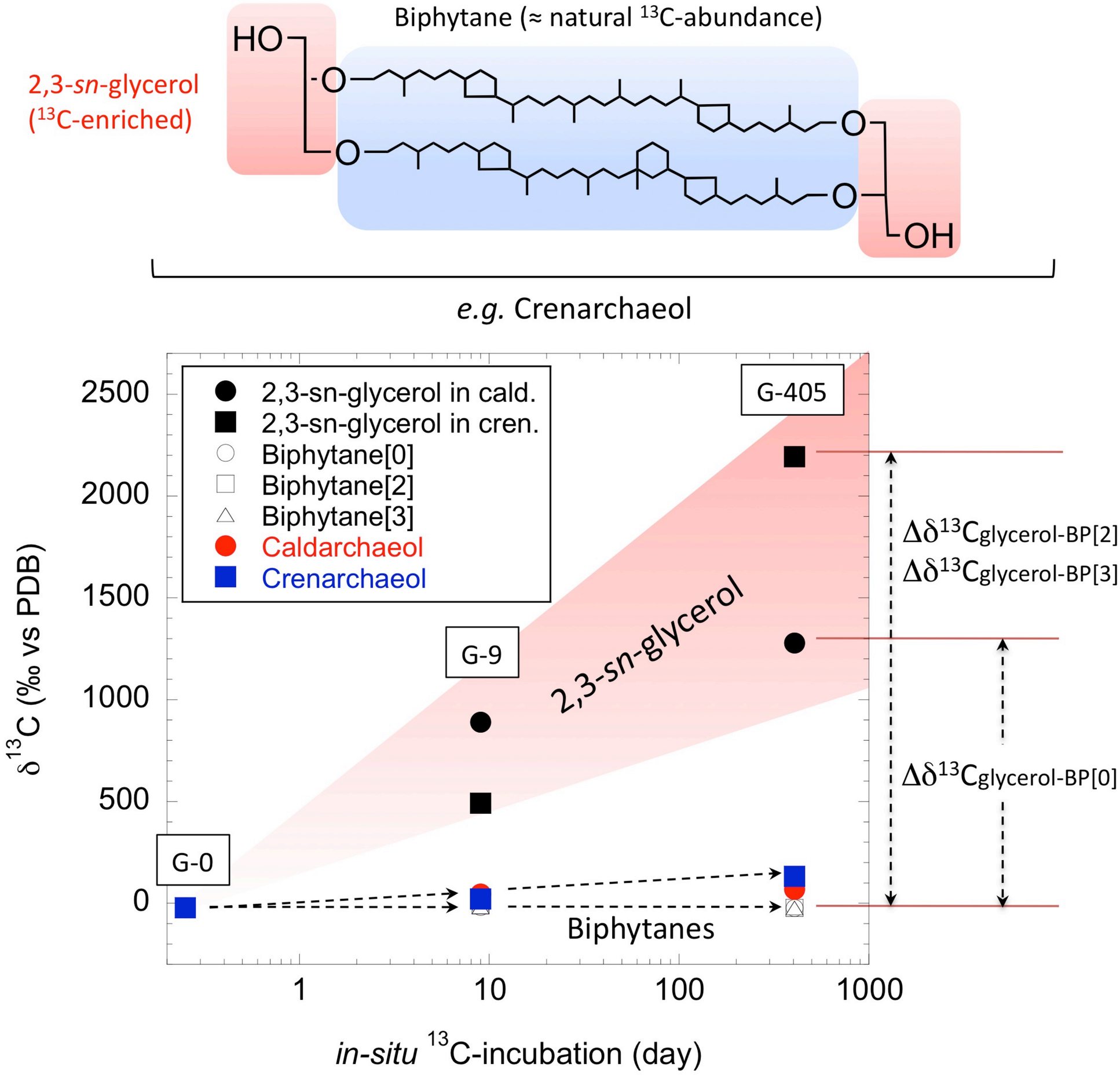
***In-situ* ^{13}C-incubation**



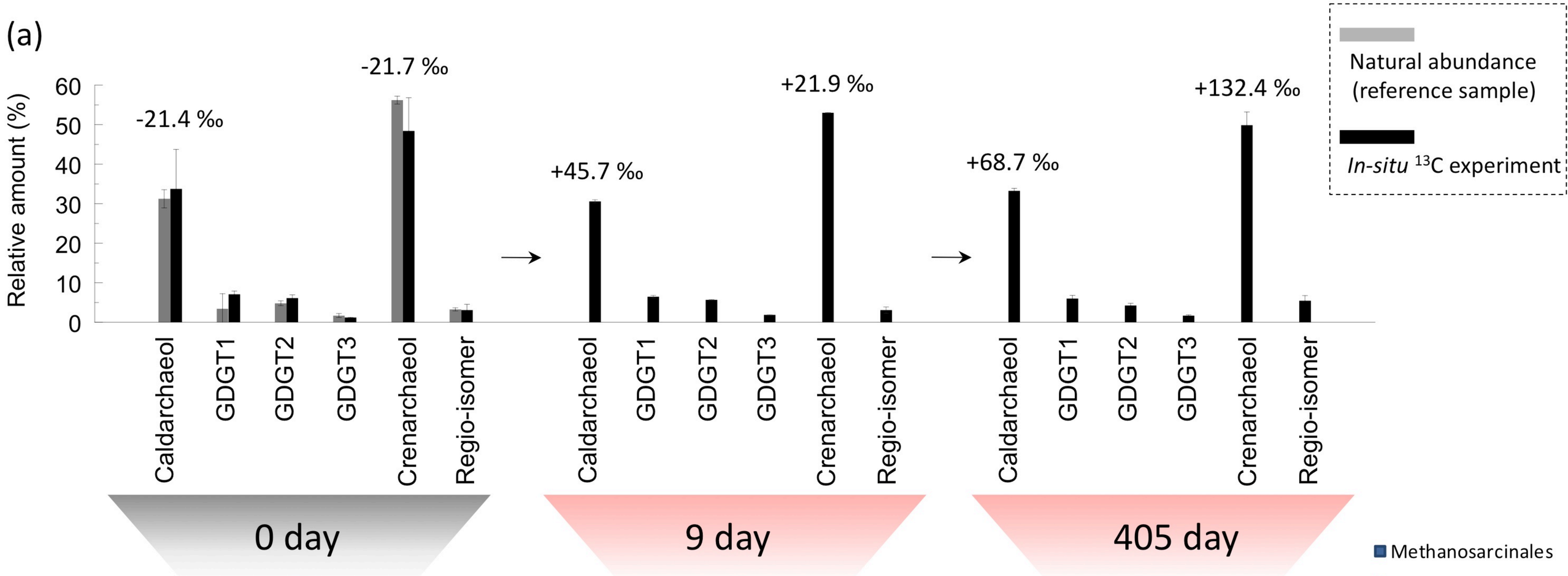


Sedimentary organic carbon **Benthic archaea**

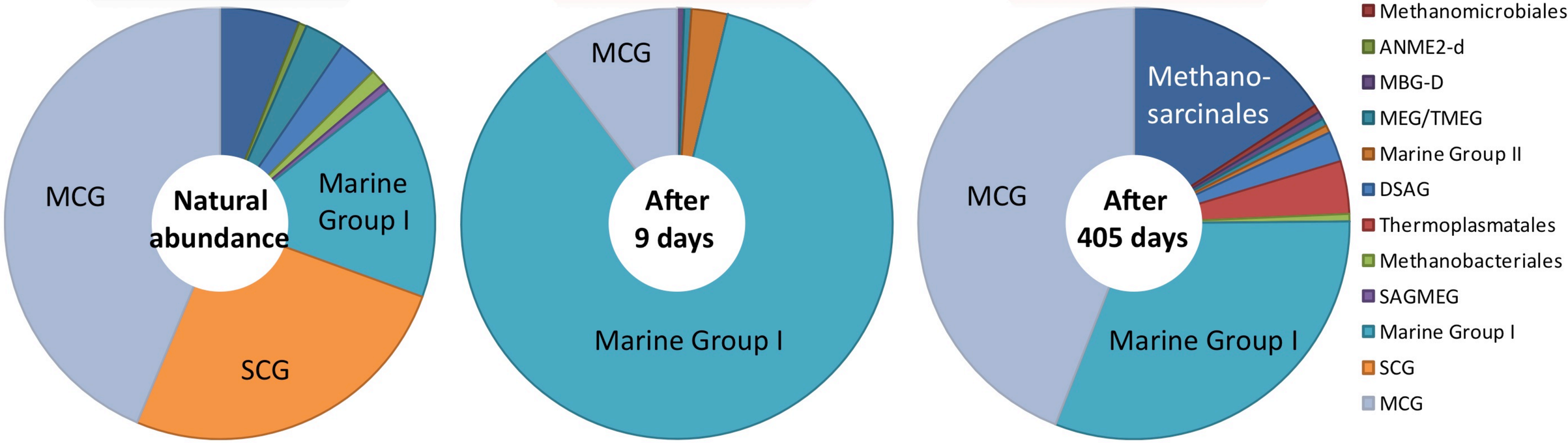



Biphytane (≈ natural 13C-abundance)
2,3-sn-glycerol
(13C-enriched)
HO
O
O
O
O
OH
e.g. Crenarchaeol
2,3-sn-glycerol in cald.
2,3-sn-glycerol in cren.
Biphytane[0]
Biphytane[2]
Biphytane[3]
Caldarchaeol
Crenarchaeol
G-405
G-9
G-0
2,3-sn-glycerol
Biphytanes
Δδ13Cglycerol-BP[2]
Δδ13Cglycerol-BP[3]
Δδ13Cglycerol-BP[0]
δ13C (‰ vs PDB)
2500
2000
1500
1000
500
0
1
10
100
1000
in-situ 13C-incubation (day)

(a)



(b)



Species richness	**2647**		**260**	**1684** : **Chao1 index**
Community diversity	**4.83**	→	**4.22**	→ **4.93** : **Shannon-Wiener index**
Archaeal abundance	**1.5 x 10^6** (± 5.7 x 10^4, *n*=3)		**8.6 x 10^7** (± 2.1 x 10^6, *n*=3)	**1.8 x 10^6** (±1.7 x 10^4, *n*=3) : **qPCR (copies g-sed^{-1})**