Detection of planktonic coenzyme factor 430 in a freshwater lake: small-scale analysis for probing archaeal methanogenesis

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Abstract

We present the first data describing molecular evidence of planktonic coenzyme factor 430 (hereafter referred to as F430), a key signature of methanogenic archaea, obtained from a water column sample in a freshwater lake in Japan. We report concentrations of native F430 ranging from $6.8 \times 10^2$ to $35 \times 10^2$ femtomol g-wet$^{-1}$ and $8.5 \times 10^2$ to $86 \times 10^2$ femtomol g-wet$^{-1}$ in the water column and core-top sediment, respectively. Among these profiles, the epimer F430 of the deactivated chemical formula was very low overall in both planktonic and benthic environments. Small subunit (SSU) rRNA gene sequencing revealed that the planktonic microbial community was clearly different from that of the benthic sediment. Planktonic cyanobacteria (e.g., *Microcystis*) were predominant in the water column, whereas a wide variety of bacteria and archaea, including methanogens (e.g., *Methanobacterium*, *Methanoregula*, *Methanothrix*, *Methanosaarcina*, and *Methanocella*), were observed in the core-top sediment. These results suggest that the planktonic cyanobacterial community may be rapidly degraded at the water–sediment interface driven by activated benthic microbial heterotrophic processes.

Keywords: Planktonic methanogen, Coenzyme F430, mcrA, Lake Suwa, Surface hydrosphere

1 Introduction

Freshwater lakes are considered one of the main natural sources of methane (CH$_4$) emissions in terrestrial environments, accounting for 6–16% of CH$_4$ originating from natural sources (e.g., Bastviken et al. 2004; Cole et al. 2007; Holgerson and Raymond 2016; Günthel et al. 2019). The major CH$_4$ fraction released from freshwater lakes is produced by methanogenic archaea in anoxic sediments, and this is the terminal step of organic matter decomposition (e.g., Zepp Falz et al. 1999; Chan et al. 2005; Thauer et al. 2008; Conrad 2009; Borrel et al. 2011; Bukin et al. 2018). Among these biogeochemical processes, heterotrophic microbes degrade organic matter into H$_2$, CO$_2$, acetate, and methylated compounds, which are then adopted as the principal substrates for hydrogenotrophic, acetoclastic, or methylotrophic methanogenic pathways by methanogenic archaea. The CH$_4$ occurring in anoxic sediments then diffuses into the atmosphere or is oxidized via anaerobic or aerobic microbial processes in the lacustrine water column (e.g., Bastviken et al. 2002; Thauer et al. 2008; Kojima et al. 2014; Deutzmann et al. 2014; Donis et al. 2017; Iwata et al. 2018).

To date, biogeochemical evidence of CH$_4$ accumulation in oxygen-saturated freshwater and marine environments, commonly known as the methane paradox, has emerged (e.g., Repeta et al. 2016; Fernández et al. 2016; Sasakawa et al. 2008; Günthel et al. 2020). Three main hypotheses have been proposed to explain this enigmatic phenomenon: (1) methanogenesis by methanogenic archaea in anoxic microenvironments,
such as detritus or animal guts (e.g., Oremland 1979; Burke et al. 1983; de Angelis and Lee 1994; Schmale et al. 2018; Wäge et al. 2020); (2) release of CH₄ as a byproduct of the bacterial decomposition of potential precursors (e.g., methylphosphonate and dimethylsulfoniopropionate) under aerobic conditions (e.g., Damm et al. 2010; Wang et al. 2017; Khatun et al. 2019); and (3) methanogenesis by methanogenic archaea associated with photoautotrophic consortia in lacustrine environments (e.g., Grossart et al. 2011; McGinnis et al. 2015; Li et al. 2021).

In addition to the abovementioned sources, we note the other origins of seep methane derived from deep terrestrial and/ or oceanic sources along with some geological factors (e.g., Milkov and Sassen 2002; Naudts et al. 2006; Ritt et al. 2010; Valentine 2011 and the literature therein).

Within the context of planktonic methane production, Grossart et al. (2011) reported the coexistence of planktonic methanogen and cyanobacteria in the water column of an oligotrophic lake (Lake Stechlim, Germany) based on molecular analyses targeting the small subunit (SSU) rRNA gene and the methyl coenzyme M reductase A gene (mcrA) (cf. methyl coenzyme M reductase, McGinnis et al. 2015). It has been suggested that these cyanobacterial blooms may stimulate benthic CH₄ production in sediment by increasing the supply of organic matter (Wang et al. 2006; Yan et al. 2017). In addition, Bižić et al. (2020) demonstrated CH₄ production via pure cultures of cyanobacteria living in marine, freshwater, and terrestrial environments. Although it has been suggested that certain versatile methanogenic archaea thrive in anoxic microenvironments within planktonic cyanobacterial consortia (Batista et al. 2019) and/or in the interior of floating fecal pellets (Wäge et al. 2020), the entire CH₄ production process in water column environments remains unclear.

To elucidate enigmatic planktonic methane biogeochemistry, we applied a new tool, namely, archaeal methanogen-specific coenzyme factor 430 (F430), with ultrasmall-scale approaches (e.g., Takano et al. 2013; Kaneko et al. 2014, 2016). F430 is a hydrocorphinoid nickel complex and is the prosthetic group of methyl coenzyme M reductase, which catalyzes the final step of all methanogenesis pathways (e.g., Diekert et al. 1981; Thauer et al. 2008). F430 quantification indicates the ongoing activities of methanogenic archaea via rapid decomposition into its relic forms after the death of these archaea (e.g., Inagaki et al. 2015; Urai et al. 2021). To our knowledge, this is the first study to report the planktonic F430 concentration in a freshwater lake environment. Molecular SSU rRNA and mcrA gene analyses were also performed to assess the microbial community structure in both planktonic and benthic prokaryotic habitats.

### 2 Materials and methods

#### 2.1 Geological setting of Lake Suwa

Lake Suwa is located in Nagano Prefecture in the central part of Honshu Island, Japan, on the Itoigawa-Shizuoka tectonic line (Fig. 1). The lake exhibits a surface area of 13.3 km². The mean and maximum depths are 4.3 and 6.4 m, respectively, with a water catchment area of 513 km². The lake is a typical Japanese eutrophic lake affected by anthropogenic impacts originating from nearby cities,
despite being surrounded by mountains. The lake attains a high sedimentation rate (~1 cm year⁻¹) of organic-rich sediments (e.g., Nakazato et al. 1998; Suwa Construction Office 2019). In addition, cyanobacterial blooms, particularly those caused by *Microcystis* spp., have regularly occurred during the summer since the 1970s (Park et al. 1993, 1998; Watanabe et al. 2012). In an earlier study, we confirmed active CH₄ emissions derived from the surface sediments of Lake Suwa (Iwata et al. 2020; Additional file 1: Movie S1).

### 2.2 Sampling procedure for water, planktonic microbes, and sediment

Samples were collected along the southeast shore of the lake (36°2′36.92″N, 138°6′30.94″E; Fig. 2a) at a water depth of 1.8 m on September 28, 2018, and June 29, 2019. The water temperature and dissolved oxygen (DO) concentration were measured on-site at each depth with a DO meter (HQ-30D; Hach, Loveland, CO, USA). Lake water was collected in serum vials to measure the dissolved CH₄ concentration, while cyanobacteria floating on the surface (Fig. 2b, c) were collected with a plankton net (10-μm mesh) and placed in polypropylene bottles (Fig. 2d). Benthic sediment was collected at depths of 0–10 cm in September 2018 and at depths of 0–3 cm in June 2019 with a gravity core sampler (50-cm long, 4-cm diameter) (Fig. 2e). Planktonic cyanobacteria and benthic sediment samples were preserved in an ice box and transported to the laboratory, where they were frozen and stored at ~30 °C. The cyanobacterial samples were microscopically observed (PX-51; Olympus, Tokyo, Japan).

### 2.3 Analytical procedures

#### 2.3.1 Measurement of dissolved CH₄

The concentration of dissolved CH₄ in the sampled lake water was analyzed on June 27 and September 28, 2018, and on June 28, August 29, and October 10, 2019, according to previously described methods (Itoh et al. 2015). Lake water was collected in 30-mL vials from several depths (0, 10, 50, 100, 130, 150, and 160 cm) with syringes and tubes attached to a pier. CH₄ was quantified using a gas chromatograph with a flame ionization detector (GC-14B; Shimadzu, Kyoto, Japan) and a packed column (Porapack-Q, 80/100 mesh, 1.5 m × 3.0 mm inner diameter (i.d.); GL Sciences, Tokyo, Japan). The concentration of dissolved CH₄ was calculated with the ideal gas law and Bunsen solubility coefficient (Magen et al. 2014).

#### 2.3.2 Extraction of F430 by wet chemistry

F430 analysis was performed according to a previously described method (Takano et al. 2013; Kaneko et al. 2014). In summary, F430 was extracted from planktonic cyanobacteria and benthic core-top sediments by using 1% formic acid under ultrasonication for 30 min on ice and centrifugation (10,000g; 30 min; 4 °C). The supernatant was then recovered, and this step was repeated three times. The combined supernatants were introduced into an anion-exchange column (Q Sepharose; GE Healthcare, IL, USA) equilibrated with 50 mM Tris–HCl (pH 7.5) and washed with deionized water before use. The recovered eluent was introduced into a C₁₈ SPE column (Sep-Pack; Waters, MA, USA) equilibrated with methanol and conditioned with 1% formic acid. F430 was eluted with methanol and converted into F430 methyl ester (F430M) via derivatization with BF₃–methanol (40 °C; 3.5 h) before extraction with dichloromethane. To remove the organic matrix, the F430M fraction extracted from the samples was purified via silica gel chromatography (Kaneko et al. 2016). This pretreatment eliminates any potential analytical artifacts that may affect ultrasmall-scale analyses (~femto mol; e.g., Kaneko et al. 2014; Inagaki et al. 2015; Isaji et al. 2020) of tetrapyrrole compounds through chromatographic separation, which may induce ion suppression and enhancement effects in liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS).

#### 2.3.3 Qualification and quantification of F430 using LC/ESI-MS

The concentration of extracted F430M was determined via high-performance liquid chromatography/electrospray ionization-mass spectrometry (HPLC/ESI-MS; 1260 Infinity II LC System coupled to a 6490 Triple Quadrupole LC–MS system with an Agilent jet stream; Agilent Technologies, CA, USA) in positive ion mode. Chromatographic separation was performed with a ZORBAX Eclipse XDB-C18 column (0.5 × 150 mm; 5 μm, Agilent Technologies). Multiple reaction monitoring (MRM) analysis was performed at a fragmentor voltage of 380 V and a collision energy of 70 V. The F430M product ion was set to m/z 844.3. The mobile phases included 10 mM ammonium acetate with 1% acetonitrile (A) and acetonitrile (B) at a flow rate of 16 μL min⁻¹ and the following gradient: 0% B, 30% B after 3 min, and 90% B after 90 min (Mayr et al. 2008). The F430M concentration was determined with an external F430M standard (Kaneko et al. 2014, 2016).

#### 2.3.4 SSU rRNA gene tag sequencing

Total DNA was extracted from the cyanobacteria and benthic sediments collected in September 2018 using a Plant Genomic DNA Extraction Miniprep System (Viogene, Taipei, Taiwan) and a DNeasy Power Soil Kit (Qiagen, CA, USA), respectively, which were selected to achieve the most efficient DNA extraction from these samples. The concentration of the extracted DNA was...
Fig. 2. Photographs of the sampling site and samples in June 2019. a Sampling site in the present study. The pier extends ~ 40 m from the lakeshore and attains a depth of ~ 1.8 m. b, c Floating cyanobacteria near the sampling site. d Planktonic cyanobacterial sample collected in a polypropylene bottle. e Sediment core collection with a gravity core sampler.
measured with a Quant-iT™ dsDNA HS Assay Kit and a Qubit Fluorometer (Thermo Fisher Scientific, MA, USA). The SSU rRNA genes in the extracted DNA were amplified via polymerase chain reaction (PCR) using TaKaRa LA Taq (TaKaRa Bio, Shiga, Japan) according to the manufacturer’s protocol. The 530F/907R primer set (V4–V5 regions) covering most bacterial and archaeal SSU rRNA genes (Nunoura et al. 2012) was used. This primer set can also detect some eukaryotic SSU rRNA gene sequences, but it does not cover all eukaryotes. Therefore, eukaryote-derived sequences were excluded, and archaea- and bacteria-derived sequences, approximately 380 bp long of merged sequences that are the two-ended reads, were used to analyze the microbial community structure. The PCR amplification conditions were as described previously (Hirai et al. 2017). The amplified SSU rRNA gene sequences were analyzed using MiSeq (Illumina, CA, USA). Paired-end reads were merged using PEAR (Zhang et al. 2014). Low-quality (a Q score < 30 in over 3% of all sequences), short reads (<150 bp) and long reads (>500 bp) were filtered out with a customized Perl script. After chimeric sequences had been removed with Usearch61 (Edgar 2010) in QIME (Caporaso et al. 2010), operational taxonomic units (OTUs) at a 97% similarity level were selected using UCLUST (Edgar 2010) and assigned taxonomically (at the phylum, class, order, family, and genus levels) based on the SILVA 132 database (Quast et al. 2012).

2.3.5 Nucleotide sequence accession numbers
The SSU rRNA gene tag sequence data reported in this manuscript have been deposited in BioProject under accession number DRA009781.

2.3.6 PCR amplification of the archaeal 16S rRNA and mcrA genes
To confirm the presence of methanogenic archaea in the samples, the archaeal 16S rRNA and mcrA genes were amplified considering the extracted DNA by PCR with TaKaRa LA Taq (TaKaRa Bio) according to the manufacturer’s protocol. The archaeal 16S rRNA gene was amplified with the 340F/1000R primer set (Ganttner et al. 2011), while the mcrA genes were amplified using the Luton-mcrA F/R, MCR F/MCR R, ME 3F/ME 2R, and mls/mcrA-rev primer sets (Springer et al. 1995; Luton et al. 2002; Steinberg and Regan 2008; Sørensen et al. 2009). The PCR conditions involved denaturation at 95 °C for 9 min, followed by 45 cycles of denaturation at 94 °C for 40 s, annealing at 50 °C, 52 °C, or 55 °C for 30 s, extension at 72 °C for 40 s, and a final extension step at 72 °C for 7 min. The size of the PCR products was assessed via electrophoresis through a 1.5% agarose gel stained with RedSafe™ Nucleic Acid Stain Solution (FroggaBio, Toronto, Canada).

2.3.7 A culture experiment using the cyanobacteria
We performed a short-term culture experiment using the cyanobacterial bloom sample collected from Lake Suwa (date: 29-June-2019). The laboratory-based incubation was previously described in Han et al. (2012). Briefly, the cyanobacterial bloom sample was cultured in 10L plastic bottle with MA growth medium. The light/dark cycles was 12:12 (light: 9:00 to 21:00) and incubation temperature was 23 °C. CH₄ in the head space was analyzed by Ultraportable Greenhouse Gas Analyzer (Los Gatos Research, USA) under bright light conditions.

2.3.8 Carbon and nitrogen measurement
The total organic carbon (TOC) and total nitrogen (TN) contents and their stable isotopic ratios (¹³C/¹²C and ¹⁵N/¹⁴N, respectively) were measured in the planktonic cyanobacteria and core-top sediment collected in September 2018. All samples were freeze-dried, crushed, and homogenized prior to analysis. The benthic sediment was treated with 1 M HCl to remove carbonates and centrifuged (~2000g) for 5 min, and the precipitate was thoroughly washed with deionized water. This procedure was repeated three to five times before the sample was dried and pulverized. Measurements were conducted with an ultrasensitive elemental analyzer connected to an isotope ratio mass spectrometer (Flash EA1112 coupled to a Thermo Finnigan Delta plus XP via ConFlo III; Thermo Finnigan, CA, USA), as described previously (Ogawa et al. 2010). The isotopic composition was expressed in conventional δ notation:

\[ \delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000(\%o), \]

where R denotes the ¹³C/¹²C or ¹⁵N/¹⁴N ratio. The carbon and nitrogen isotope standards were Vienna Pee Dee Belemnite (VPDB) and atmospheric N₂ (AIR), respectively. The carbon and nitrogen contents and their isotopic compositions were calibrated against three reference materials: L-tyrosine (BG-T: C, 59.7%; N, 7.74%; δ¹³C, −20.83 ± 0.10 ‰; δ¹⁵N, +8.74 ± 0.09 ‰), dl-alanine (CERKU-01: C, 40.4%; N, 15.7%; δ¹³C, −25.36 ± 0.08 ‰; δ¹⁵N, −2.89 ± 0.04 ‰), and glycine (CERKU-03: C, 32.0%; N: 18.7%; δ¹³C, −34.92 ± 0.07 ‰; δ¹⁵N, +2.18 ± 0.04 ‰) (Tayasu et al. 2011). The analytical errors in the isotopic composition estimated via repeated BG-T analyses (5.8–22.7 µg C; 0.75–2.9 µg N) were ±0.07 ‰ (1σ; n = 9) for δ¹³C and ±0.18 ‰ (1σ; n = 9) for δ¹⁵N.
### 3 Results

#### 3.1 Water column chemical profiles with the carbon and nitrogen contents

Figure 3a shows the water column profiles based on the measurements conducted in September 2018. Both the water temperature and the DO decreased with depth and ranged from 20.8 to 17.2 °C and 10.97 to 6.76 mg L⁻¹, respectively. The DO saturation ranged from 134 to 77%, suggesting that the water column was highly oxygenated from the surface to the bottom. The maximum CH₄ concentration was observed between depths of 100 and 160 cm (3.5–3.6 μM) and decreased from 3.5 to 1.5 μM in water shallower than 100 cm. Similar trends were observed on the other measurement days (Fig. 3b).

We also analyzed the carbon and nitrogen contents and stable isotopic ratios of the samples collected in September 2018 (Table 1). Among the cyanobacterial samples, the TOC and TN contents were 41.1 ± 1.8 wt.% and 7.7 ± 1.08 wt.%, respectively, with δ¹³C and δ¹⁵N values of −26.5 ± 3.0 ‰ and +8.8 ± 0.5 ‰, respectively. Conversely, the sediment samples exhibited lower TOC and TN contents of 5.5 ± 0.9 wt.% and 0.3 ± 0.04 wt.%, respectively, with δ¹³C and δ¹⁵N values of −27.6 ± 0.4 ‰ and +3.0 ± 0.9 ‰, respectively. The observed carbon and nitrogen isotopic compositions were typical of the lacustrine range in Lake Suwa (cf. Yoshioka et al. 1988, 1994), and the cyanobacterial and sediment samples exhibited TOC/TN ratios of 5.4 ± 0.6 and 17.2 ± 0.6, respectively. The carbon and nitrogen isotope ratios presented in this study are in the typical cyanobacterial profiles (i.e., native feature of phototrophic N₂-fixation) and are consistent with the findings presented in previous reports (Yoshioka et al. 1988; Ohkouchi and Takano 2014).

#### 3.2 Concentration of F430

We detected F430 in the planktonic and benthic samples by using LC/ESI-MS/MS in MRM mode (Fig. 4). The planktonic samples collected in September 2018 and June 2019 exhibited F430 concentrations of 6.8 × 10² and 3.5 × 10³ femto mol g⁻¹, respectively (Table 2), or 9.2 × 10³ and 5.6 × 10⁴ femto mol g⁻¹, respectively, considering the water contents in the cyanobacteria, i.e., the water contents in both cyanobacterial samples were 92.5 and 93.8%, respectively. Conversely, the F430 concentrations in the core-top sediment obtained in September 2018 and June 2019 were 8.5 × 10² and 8.6 × 10³ femto mol g⁻¹, respectively. The peak ratios of native F430 to its epimers were 3.3 and 3.8 in the cyanobacteria and 9.0 and 11.1 in the benthic sediment, respectively (Table 2). The small amounts of F430M epimers detected in the samples collected in this study (Fig. 4b, c) indicated that most methanogenic archaea were activated.

#### 3.3 Microbial community structure in the planktonic and surface sediment samples

First, Fig. 5 shows a photomicrograph of the planktonic cyanobacteria collected in June 2019, in which large numbers of cells were confluent and formed colonies with a morphology similar to that of cyanobacteria of the genus *Microcystis* (Xu et al. 2016). The detailed microbial communities in the cyanobacteria and core-top sediment collected in September 2018 were investigated via SSU rRNA gene tag sequencing analysis. A total of 65,884 reads were obtained from the planktonic cyanobacteria, of which 99.5% were assigned to bacteria (Fig. 6a, b). More than 50% of the bacterial reads were assigned to a single OTU and were closely related to the cyanobacterium genus *Microcystis* in the order *Nostocales* (Fig. 6c).

We detected another cyanobacterium genus, *Pseudanabaena*, in the order *Pseudanabaenales*. In addition to cyanobacteria, bacteria closely related to the orders *Acetobacterales* (mainly *Acetobacteraceae*), *Betaproteobacteriales* (mainly uncultured *Nitrosonomonadaceae*, *Azohydromonas*, *Ramlibacter*, and *Novihersbaspirillum*), *Caulobacteriales* (mainly uncultured *Caulobacteraceae*), and *Cytophagales* (mainly uncultured *Microscillaceae*) were detected, which are generally regarded as aerobic heterotrophic or aerobic ammonia-oxidizing bacteria. A small proportion (0.3%) of the total sequence reads was assigned to eukaryotic 18S rRNA genes, mainly stemming from *Cnidaria*, *Conthreep*, *Bilateria*, and *Heterotricha*.

Second, a total of 46,182 reads were obtained from the benthic sediment, of which 92.8 and 6.9% were assigned to bacteria and archaea, respectively (Fig. 6d). Methanogenic archaea belonging to the classes *Methanobacteria* (*Methanobacterium*) and *Methanomicrobia* (largely *Methanoregula*, *Methanothrix*, *Methanosarcina*, and *Methanocella*) were confirmed in the archaeal community (Fig. 6e). The majority of the archaeal reads were assigned to the candidate phyla *Bathyarchaeota* and *Woesearchaeota* and the class *Thermoplasmata* (largely *Marine Benthic Group-D*). Although these predominant archaeal groups are also frequently encountered in marine and freshwater ecosystems, groundwater, and thermal springs, little is known about their physiology or metabolism due to the lack of cultured representatives (e.g., Evans et al. 2015; Tarnovetskii et al. 2018; Zhou et al. 2018, 2019). Compared to the planktonic cyanobacteria, the bacterial community in the sediment samples was extremely diverse (Fig. 6f), with relatively abundant bacterial reads closely related to the orders *Betaproteobacteriales* (mostly *Sulfuritalea*, *Quatritoncoccus*, uncultured *Nitrosonomonadaceae*, and uncultured *Burkholderiaceae*), *Gaiellales* (mostly uncultured), *Anaerolineales* (mostly uncultured *Anaerolineaceae*), and *Clostridiales* (mostly uncultured *Clostridialaceae*).
Romboutsia, Clostridium, and Sarcina). These bacteria include aerobic heterotrophic (Burkholderiaceae), anaerobic autotrophic (Sulfuritalea), anaerobic heterotrophic (Anaerolineales, Clostridiales), and ammonia-oxidizing (Nitrosomonadaceae) species, which have often been reported in the sediments of freshwater lakes (Buckles et al. 2013; Watanabe et al. 2017; Chen et al. 2017).

3.4 Genomic indicators of the methanogenic archaea
To detect methanogenic archaea in both the planktonic and the benthic samples, we amplified the archaeal 16S rRNA and mcrA genes in the total extracted DNA using PCR with the above one and four primer sets, respectively. The archaeal 16S rRNA and mcrA genes were successfully amplified from the benthic sediment (Table 3), while the PCR amplicons from the cyanobacteria were not significant after repeated attempts. A wet-chemical improvement to eliminate the sample matrix effect (e.g., extracellular polysaccharides stemming from cyanobacteria) is required for further confirmation of the current preliminary results.

3.5 The head-space methane during the culture experiment
Figure 7 showed the concentration CH$_4$ profile in the head-space with the incubation time. The CH$_4$ concentration was normalized by the difference of initial concentration ($t=0$, hour) to each corresponding time ($<t=9$). We confirmed a rapid increase in methane concentration within the incubation time. Therefore, the preliminary results indicated that the cyanobacterial bloom sample produced CH$_4$ under aerobic condition (cf. Bižić et al. 2020), and its production rate was determined about 1.39 nM h$^{-1}$. The data presented in this preliminary culture experiment supports the evidence for in-situ methane production (Fig. 7). In our future survey, it is necessary to conduct culture studies with different conditions (i.e., light and dark conditions, water temperature, medium) with a microbial ecological link to methane producing archaea.
Table 1 Carbon and nitrogen contents and their stable isotopic compositions in the planktonic and benthic samples

| Sample                               | TOC (wt%) | TN (wt%)  | δ¹³C (‰, vs VPDB) | δ¹⁵N (‰, vs AIR) | TOC/TN (wt%/wt%) |
|--------------------------------------|-----------|-----------|-------------------|------------------|------------------|
| Planktonic cyanobacteria (September 2018) | 41.1±1.8  | 7.7±1.1   | −26.5±0.3         | +8.8±0.5         | 5.4±0.6          |
| Sediment 0–10 cm depth (September 2018) | 5.5±0.9   | 0.3±0.0   | −27.6±0.4         | +3.0±0.9         | 17.2±0.6         |

Fig. 4  
(a) Structure of coenzyme factor 430 (F430), which has a chemical formula of C₄₂H₅₁N₆NiO₁₃. F430 is a hydrocorphinoid nickel complex that functions as an active site in MCR. The MCR–F430 complex catalyzes the last step of methanogenesis involving the reduction of methyl coenzyme M into CH₄.  
(b, c) Chromatograms of the planktonic cyanobacterial samples analyzed via LC/ESI–MS/MS in MRM mode (Kaneko et al. 2014). The F430M product ion was set to m/z 844.3. The concentration of native F430 with assessment of the F430 epimer ratio enabled us to estimate the biomass of the in situ living methanogenic archaea (Kaneko et al. 2021)
Discussion

4.1 Planktonic and benthic concentrations of F430 for potential methane production

F430 is unstable and rapidly epimerizes to its relic forms (13-epi-F430 and 12,13-diepi-F430) via a process occurring on a daily basis, even at room temperature (Diekert et al. 1981; Mayr et al. 2008; Inagaki et al. 2015); therefore, the native form of F430 is unlikely to accumulate in the environment after the death of methanogenic archaeal cells. The concentration of native F430 with assessment of the F430 epimer ratio enabled us to estimate the biomass of the in situ living methanogenic archaea (Kaneko et al. 2014, 2021). In this study, we detected native F430 in the planktonic cyanobacterial sample (i.e., $6.8 \times 10^2$ to $3.5 \times 10^3$ femto mol g-wet$^{-1}$), indicating the presence of methanogenic archaea in the water column environment (Fig. 4, Table 2). Although active photosynthesis simultaneously generates oxygen, certain methanogenic archaea are considered to tolerate oxygen exposure for long periods (Angel et al. 2011). Assemblages of heterotrophic bacteria are contained within the cyanobacterial phycosphere, and their respiration

### Table 2

Concentrations of coenzyme F430 and its epimer ratios in the planktonic and sediment samples collected from Lake Suwa

| Sample type            | Sample name                  | Concentration | F430/F430 epimer | References                        |
|------------------------|-------------------------------|---------------|------------------|-----------------------------------|
| Planktonic microbes    | Cyanobacteria (September 2018)| $6.8 \times 10^2$ femto mol g-wet$^{-1}$ | 3.3                 | This study                        |
|                        | Cyanobacteria (June 2019)     | $3.5 \times 10^3$ femto mol g-wet$^{-1}$ | 3.8                 | This study                        |
| Sediment               | Sediment 0–10 cm depth        | $8.5 \times 10^2$ femto mol g-wet$^{-1}$ | 9.0                 | This study                        |
|                        | Sediment 0–3 cm depth         | $8.6 \times 10^3$ femto mol g-wet$^{-1}$ | 11.1                | This study                        |
|                        | Kawatabi (Paddy soil)         | $8.7 \times 10^2$ femto mol g-wet$^{-1}$ | –                   | Kaneko et al. (2014)              |
|                        | Shimokita 11–4 (Marine sediment) | $6.3 \times 10^3$ femto mol g-wet$^{-1}$ | –                   | Kaneko et al. (2014)              |

We include a compilation of previous reference data (Kaneko et al. 2014)

Fig. 5 Photomicrograph of the planktonic cyanobacterial sample collected in June 2019. The cyanobacteria in Lake Suwa mainly include *Microcystis* (a) in addition to other cyanobacteria, such as *Aphanizomenon* (b). We note previous studies regarding the average cellular dry weight and average cellular volume of *Microcystis aeruginosa* ($2.0 \times 10^{-11}$ g cell$^{-1}$, Li et al. 2014; 65 µm$^3$ cell$^{-1}$, Olenina et al. 2006)
leads to an oxygen gradient (e.g., Ploug 2008; Dzialis and Grossart 2012; Batista et al. 2019). Very small amounts of F430 epimers were also detected from the planktonic cyanobacterial samples based on the ratio of native F430 to the epimer in these samples ranging from 3.3 to 3.8 (Table 2), suggesting that native F430 occurs in an active
state in the water column (cf. Urai et al. 2021). Therefore, the planktonic methanogen coexisting in the water column may produce CH₄ when oxygen production via photosynthesis ceases at night, as proposed by Bižić et al. (2020). We determined the gene extraction method for cyanobacterial bloom after comparing several extraction kits. Previous study reported that cyanobacterial cells are rich in polysaccharides, which make it difficult to obtain highly purified nucleic acid (e.g., Yoshida et al. 2003). However, we detected archaeal gene reads in cyanobacterial bloom, indicating that the method can detect archaeal gene from cyanobacteria. To evaluate the actual CH₄ production rate in the water column of Lake Suwa, preliminary short-term incubation experiments were performed by using the same planktonic cyanobacterial samples (Fig. 7).

SSU rRNA gene tag sequencing analysis detected a large number of 16S rRNA genes originating from aerobic heterotrophic bacteria, including Acetobacterales and Betaproteobacteriales, in the cyanobacterial sample (Fig. 6). Some Acetobacterales species produce acetate as a metabolite (Vu et al. 2019); therefore, these heterotrophic bacteria may supply methanogenic archaea with methanogenic substrates via decomposition of the organic matter produced during cyanobacterial photosynthesis. Berg et al. (2014) proposed that hydrogenotrophic methanogenic archaea utilize the hydrogen generated by cyanobacteria during nitrogen fixation; however, since Microcystis, which was dominant in our samples (Fig. 6c), lacks nitrogenase required for nitrogen fixation (Kim et al. 2019), its contribution to CH₄ production appears to be quite small in surface water. Although we detected F430 in the planktonic cyanobacterial samples (Fig. 4), the samples contained extremely small numbers of SSU rRNA gene sequences from known methanogenic archaea, and no PCR amplicons from archaeal 16S rRNA and mcrA genes were obtained (Fig. 4, Table 3). Previous studies have estimated the in situ cellular abundance of methanogenic archaea based on the F430 concentration (Takano et al. 2013; Kaneko et al. 2014); however, a technical discrepancy between the F430 concentration and molecular analyses has been reported (e.g., Inagaki et al. 2015; Kaneko et al. 2016; Urai et al. 2021). Although an equation has been proposed to convert the F430 concentration into the number of methanogenic archaea, deep insights are currently lacking to widely apply the equation. Therefore, method development is required in this preliminary study to refine the

### Table 3  
PCR amplification of the archaeal 16S rRNA and mcrA genes. The PCR products were loaded onto a 1.2% agarose gel with negative and positive controls and a DNA size standard.

| Target                  | PCR primer set | Target Archaeal 16S rRNA gene | mcrA gene     | Luton-mcrA F/R | MCR F/MCR R | ME 3F/ME 2R | mlas/mcrA-rev |
|-------------------------|----------------|-------------------------------|---------------|----------------|-------------|-------------|---------------|
| Planktonic cyanobacteria (September 2018) | 340F/1000R | – | – | – | – | – | – |
| Sediment 0–10 cm depth (September 2018) | + | + | – | + | + | + | + |

The presence or absence of the PCR product is indicated as (+) and (−), respectively. The annealing temperature for each primer set is listed in Table 4.

### Table 4  
PCR primers for archaeal 16S rRNA and mcrA genes for the present initial report.

| Target                  | Primer name     | Direction | Sequences (5’ → 3’) | Hybridization temperature (°C) | References          |
|-------------------------|-----------------|-----------|---------------------|-------------------------------|---------------------|
| Archaeal 16S rRNA gene   | 340F            | Forward   | CCCCAYGGGGGYYGASCAG | 52                            | Gantner et al. (2011) |
|                          | 1000R           | Reverse   | GGCCATGCACYWCTCT    |                               |                     |
| mcrA gene                | Luton-mcrA F    | Forward   | GGTTGTTGMGGATTCACACATAYGCWACAGC | 50                            | Luton et al. (2002)  |
|                          | Luton-mcrA R    | Reverse   | TTTATGCRTAGTTWGGRTAGT |                               |                     |
| mcrA gene                | MCR F           | Forward   | TAYGAYCARATHTGYT    | 50                            | Springer et al. (1995) |
|                          | MCR R           | Reverse   | ACRTTACNGCRTARTT    |                               |                     |
| mcrA gene                | ME 3F           | Forward   | ATGTCNGGTGHHGMGSSSTYAC | 55                            | Sørensen et al. (2009) |
|                          | ME 2R           | Reverse   | TCATBCRTAGTTDDIGRTAGT |                               |                     |
| mcrA gene                | mlas            | Forward   | GGTGTTGTMGGDTTACMCRTA | 55                            | Steinberg and Regan (2008) |
|                          | mcrA-rev        | Reverse   | CGTTCATBGCCTAGTTWGGRTAGT |                               |                     |
comparison to rRNA gene sequencing and quantitative examinations.

Regarding the benthic methane production process, we quantified F430 in the benthic sediment samples collected in September 2018 ($8.5 \times 10^2$ femto mol g-wet$^{-1}$) and June 2019 ($8.6 \times 10^3$ femto mol g-wet$^{-1}$), revealing levels similar to or higher than those previously reported in marine sediment ($0.026–1.9 \times 10^3$ femto mol g-wet$^{-1}$) and paddy soil ($0.3–2.0 \times 10^3$ femto mol g-wet$^{-1}$) (Kaneko et al. 2014, 2016), suggesting ongoing archaeal methanogenesis in the benthic sediment. The CH$_4$ production rate in the benthic sediment (5–30 cm depth) of Lake Suwa has been reported to range from

Table 6 Water column profiles at the sampling site between June 2018 and October 2019

| Date          | Depth | Temp (°C) | DO (mg/L) | CH$_4$ (mmol/L) |
|---------------|-------|-----------|-----------|-----------------|
| 2018.6.27     | 0     | –         | –         | 1.80            |
|               | 5     | 24.5      | 8.61      | –               |
|               | 10    | 24.5      | 8.61      | 2.09            |
|               | 20    | 24.5      | 8.61      | –               |
|               | 50    | 24.5      | 8.66      | 2.05            |
|               | 75    | 24.5      | 8.58      | –               |
|               | 100   | 24.5      | 8.64      | 2.26            |
|               | 130   | 24.5      | 8.43      | 1.83            |
|               | 150   | 24.5      | 7.83      | 2.05            |
|               | 160   | 24.5      | 7.16      | 2.36            |
|               | 180   | 24.5      | 6.48      | –               |
| 2018.9.28     | 0     | –         | –         | 0.92            |
|               | 5     | 20.8      | 10.97     | 1.52            |
|               | 10    | 20.7      | 10.11     | 1.73            |
|               | 20    | 20.2      | 9.34      | –               |
|               | 50    | 19.1      | 8.74      | 2.13            |
|               | 75    | 17.9      | 7.50      | –               |
|               | 100   | 17.5      | 7.68      | 3.50            |
|               | 130   | 17.3      | 7.67      | 3.60            |
|               | 150   | 17.2      | 7.24      | 3.53            |
|               | 160   | 17.2      | 6.76      | 3.45            |
|               | 180   | 17.2      | 6.80      | –               |
| 2019.6.28     | 0     | –         | –         | 0.28            |
|               | 5     | 22.9      | 7.16      | –               |
|               | 10    | 22.9      | 7.13      | 1.07            |
|               | 20    | 23.0      | 7.12      | –               |
|               | 50    | 23.0      | 7.11      | 1.17            |
|               | 75    | 23.0      | 6.94      | –               |
|               | 100   | 23.0      | 6.85      | 1.43            |
|               | 130   | 22.9      | 6.31      | 2.25            |
|               | 150   | 22.8      | 5.75      | 2.76            |
|               | 160   | 22.7      | 4.92      | 3.03            |
|               | 180   | 22.7      | 4.84      | –               |
| 2019.8.29     | 0     | –         | –         | 2.78            |
|               | 5     | 25.7      | 7.49      | –               |
|               | 10    | 25.6      | 7.51      | 0.61            |
|               | 20    | 25.5      | 7.54      | –               |
|               | 50    | 25.4      | 7.40      | 0.37            |
|               | 75    | 24.4      | 3.22      | –               |
|               | 100   | 23.9      | 1.32      | 0.23            |
|               | 130   | 23.4      | 2.77      | 0.94            |
|               | 150   | 22.6      | 1.86      | 3.38            |
|               | 160   | 22.4      | 1.22      | 13.59           |
|               | 180   | 22.3      | 0.83      | –               |

Principal component statistics and raw data profiles are shown in the Fig. 3b and Table 6, respectively.
7.8–19.8 nmol g-dry−1 h−1 at the typical summer maximum surface sediment temperature (Iwata et al. 2020). Since Lake Suwa is a eutrophic lake, its benthic sediment contains high concentrations of organic carbon (Table 1); therefore, this nutrient-rich substrate could support benthic CH₄ production (e.g., Zepp Fal et al. 1999). The correlation matrix of the water depth (cm), water temperature (°C), DO (mg/L), and CH₄ (mmol/L) in the early summer (n = 12) and autumn (n = 13) seasons during 2018–2019 is shown in Table 5.

### 4.2 Drastic change between the planktonic and benthic microbial communities

Archaeal 16S rRNA and mcrA gene analysis detected the presence of methanogenic archaea closely related to *Methanobacteriales* and *Methanomicrobiales* in the benthic sediment (Fig. 6, Table 3), including hydrogenotrophic, aceticlastic, and methylotrophic methanogenic archaea (Sakai et al. 2012; Schirmack et al. 2014). The 16S rRNA genes of anaerobic heterotrophic bacteria were also detected in the benthic sediment (Fig. 6). Members of *Anaerolineales* and *Clostridiales* anaerobically decompose organic matter via fermentation and supply methanogenic archaea with their metabolites, such as H₂, acetate, formate, and methanol (Yamada et al. 2006; Nishiyama et al. 2009). Although the bottom lake water contained DO (Fig. 3a), this DO may be rapidly consumed by aerobic heterotrophic bacteria in the benthic sediment, suggesting that CH₄ is produced in the benthic sediment via the anaerobic decomposition of organic matter by fermentative bacteria and methanogenic archaea. The benthic sediment was dominated by the 16S rRNA genes of archaea closely related to *Bathyarchaeota*, whose genomes reportedly contain divergent homologs of the genes required for methane metabolism (Evans et al. 2015). Thus, uncultured archaea belonging to *Bathyarchaeota* may also be involved in CH₄ production in the surface sediment.

The microbial community structure differed between the water column and benthic sediment. The planktonic observations are consistent with a previous study (*Microcystis* spp., Park et al. 1993). In contrast, a wide variety of archaeal and bacterial taxa occurred in the benthic habitat. Although dead cyanobacterial cells originating from the water column rapidly sank to the water–sediment interface, SSU rRNA genes from cyanobacteria constituted an extremely minor fraction (~2% of all sequences) in the benthic sediment (Fig. 6). Yan et al. (2017) reported that the decomposition of cyanobacterial cells produces nutrients (nitrate, phosphate, and organic substances) that may stimulate benthic microbial CH₄ production. In addition, a positive relationship was clearly observed between the CH₄ flux rate and productivity normalized to the chlorophyll a concentration in freshwater environments (Beaulieu et al. 2019). Furthermore, Iwata et al. (2020) reported that the observed seasonal variation in meteorological and limnological environments from 2016 to 2017 indicated stable stratification (i.e., a difference in temperature between the surface and bottom layers) from May to September. These results suggested that the migration of microorganisms derived from the benthic sediment to planktonic cyanobacteria was not notable in the water column.

### 5 Summary

1. We reported the first detection and concentration data of planktonic F430 in the water column by an ultrasmall-scale analysis (~femto mol g-wet⁻¹), suggesting the potential occurrence of planktonic methanogen archaea. To support this feasibility study, we should consider a method optimization of DNA/RNA-based molecular analysis, such as the elimination of the sample matrix effect (e.g., extracellular polysaccharides stemming from massive cyanobacteria) with other precise analytical protocols (e.g., Wäge et al. 2020).

2. SSU rRNA gene analysis revealed that the microbial communities differed dramatically between the water column and benthic sediment. After eventual detritus sinking, therefore, the planktonic primary producers contributed as a nutrient source to the benthic microbial habitat mostly at the water–sediment interface.

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**Table 6 (continued)**

| Date       | Depth cm | Temp °C | DO mg/L | CH₄ mmol/L |
|------------|----------|---------|---------|------------|
| 2019.10.10 | 0        | –       | –       | 0.32       |
|            | 5        | 20.9    | 10.06   | –          |
|            | 10       | 20.9    | 10.06   | 0.29       |
|            | 20       | 20.9    | 10.07   | –          |
|            | 50       | 20.8    | 10.06   | 0.99       |
|            | 75       | 18.9    | 8.98    | –          |
|            | 100      | 17.8    | 7.01    | 0.90       |
|            | 130      | 16.9    | 5.50    | 1.83       |
|            | 150      | 16.9    | 5.45    | 2.36       |
|            | 160      | 16.8    | 5.23    | 2.37       |
|            | 180      | 16.8    | 5.07    | –          |
Along with the short summary above, multiple novel chemical and microbial approaches are required to elucidate the issues associated with the methane paradox in water column environments.

Abbreviations
F430: Coenzyme factor 430; HPLC: High-performance liquid chromatography; ESI-MS: Electrospray ionization-mass spectrometry; EIC: Extracted ion chromatography; MRM: Multiple reaction monitoring; DO: Dissolved oxygen; mcrA: Methyl coenzyme M reductase A gene; OTU: Operational taxonomic unit; PCR: Polymerase chain reaction; SSU rRNA: Small subunit ribosome RNA; TN: Total nitrogen; TOC: Total organic carbon.

Supplementary Information
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Additional file 1: Movie S1 Observation of lacustrine surface methane emission near the sampling site at Lake Suwa. The surface sediment has a large amount of CH4-rich gas because of microbial activity, including that of methanogenic archaea. This movie was taken at the present study site. Stimulation with a rod confirms that abundant bubbles are released from the surface sediment.

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Authors’ contributions
AU, MM, NO, and YT proposed the topic and designed the study framework. MK, Y1 and NO outlined the small-scale analytical development of F430 molecules in a decade. AU, MM, HDP, YT, and HI collected the samples. AU, MM, HI, HDP, and MO performed the experiments. AU, MM, HI, MK, NOO, NO, and YT analyzed the samples and interpreted the results. AU and YT wrote the present report with contributions from NO and MM. All authors edited and approved the final manuscript.

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Availability of data and materials
Declaration of nucleotide sequence accession numbers: The SSU rRNA gene tag sequence data have been deposited in BioProject PRJNA 9444 under accession number DRA009781. The Global Lake and River Ice Phenology Database are compiled by the National Snow and Ice Data Center (NSIDC), Data Set https://doe.org/10.7265/NSW66HP. The database of fixed point observations (temperature, dissolved oxygen, turbidity, live camera capture) in Lake Suwa is released by the project of Suwa Smart Society 5.0 at https://ssss50.harmonia-cloud.com. The other data sets supporting the conclusions drawn in this article are included within the article.

Declarations
Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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