Environmental Chemistry Letters
https://doi.org/10.1007/s10311-023-01653-8

ORIGINAL ARTICLE

Floating macrophyte phyllosphere as a habitat for methanogens

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Received: 27 February 2023 / Accepted: 7 September 2023

Abstract

Methane generation has a significant impact on climate change. Aquatic plants have been widely studied for their role in methane emissions. Most research has focused on the contribution of the plant itself whereas the potential involvement of the phyllosphere microbiota associated with aquatic plants in methanogenesis remains largely unexplored. This study investigated the phyllosphere microbiota on both the top and bottom sides of lotus (*Nelumbo nucifera*), a commonly found aquatic plant in Asia, sampled from two urban ponds, using sequencing for both the 16S rRNA gene and the methanogenesis functional marker gene. Methanogenesis potential was assessed through laboratory-based ^{13}C labeling experiments. Results show that methanogenic archaea were successfully detected on the floating lotus leaves but not on the standing leaves. Among the methanogens detected, *Methanobacterium* was identified as the predominant group, with significantly higher relative abundance on the bottom sides of floating leaves compared to the surrounding pond water. This study demonstrates the metabolic activity of the phyllosphere methanogens of floating lotus. The microbiota associated with the bottom sides of floating leaves exhibited the ability to produce methane and incorporate ^{13}C carbon into methane during incubation. Overall, this study provides the first compelling genetic and functional evidence for the existence of epiphytic methanogens on the phyllosphere of aquatic plants. Hence, the phyllosphere of floating-leaved macrophytes represents a previously unrecognized habitat for methanogens and a potential microbial source of methane at the water–atmosphere interface.

Keywords Methanogenesis · Lotus · Epiphytes · Water–atmosphere interface · Methane cycling

Introduction

Methane is a potent greenhouse gas with a global warming potential 28 times higher than carbon dioxide (IPCC 2013). It is estimated that half of global methane emissions originate from aquatic ecosystems (Rosentreter et al. 2021). The methane flux in aquatic systems is largely influenced by aquatic plants (Emilson et al. 2018). Aquatic plants can enhance the production of methane via releasing compounds that function as methane precursors (Ernst et al. 2022; Hilt et al. 2022). They also enable methane emissions to the atmosphere through plant-mediated transport and affect its diffusive and ebullitive pathways (Kosten et al. 2016; Villa et al. 2020). However, most relevant studies have focused on the effects of the plant itself, such as its inherent gas transport capacity and the methylated compounds of plant materials (Bhullar et al. 2013; Hilt et al. 2022). Yet, very few studies have considered the role of plant-associated microbes in aquatic methane fluxes.

Methane consumption by methanotrophs is the main biological methane sink and contributes substantially to methane

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Published online: 26 September 2023

mitigation under both oxic and anoxic conditions (Cai et al. 2016; Guerrero-Cruz et al. 2021). The surface of aquatic plants has been generally considered as a niche for methanotrophs (Yoshida et al. 2014). In contrast, the methane production has primarily been attributed to the activity of methanogenic archaea in anoxic environments (Costa and Whitman 2023; Evans et al. 2019). For inland water ecosystems, methanogens have been intensively studied in anoxic sediments, wetlands, and paddy soils (Hernández et al. 2019; Peng et al. 2018; Xia et al. 2019). However, their presence on the phyllosphere of plants has received limited attention. While anoxic microsites are potentially present on the bottom sides of floating leaves that are in contact with surface water, the methanogenic potential of the phyllosphere microbiota of floating-leaved macrophytes remains unexplored.

Floating-leaved macrophytes often dominate in ponds and are commonly found in various water bodies, such as shallow lakes, ditches, canals, lowland brooks, and small rivers (Lind et al. 2022). It is estimated that ponds and small lakes smaller than 0.01 km^2 comprise 99% of all lakes in terms of quantity and cover 31% of the total area (Hornbach et al. 2020). As such, the collective leaf area of floating-leaved macrophytes could be extensive, although precise estimations are currently lacking. Therefore, understanding the role played by the phyllosphere of floating-leaved macrophytes in methane turnover is vital, given the potential of resident microbiota for methanogenesis. Such investigations will pave the way for accurate assessments of the phyllosphere microbiota's contribution to methane dynamics in aquatic ecosystems, particularly in the context of global warming.

Here, we aimed to address two fundamental research questions: Are methanogens present in the phyllosphere of aquatic plants? Do these methanogens possess the capacity for methanogenesis? To investigate the methanogenic potential of the phyllosphere microbiota of floating-leaved macrophytes, we selected the lotus plant (*Nelumbo nucifera*) as a representative model, given its widespread occurrence as a common aquatic plant in Asia. Our objective is to unravel the composition of the phyllosphere microbiome involved in methanogenesis and subsequently validate their methanogenic activity. These results are expected to shed light on the hitherto neglected roles of phyllosphere microbes associated with floating-leaved macrophytes in methane production and turnover.

Experimental

Sampling

Lotus leaves were collected from two ponds in Ningbo, China, in early August 2021. The ponds are located in two urban parks, namely, Reader Park (29°54′2599″ N and 121°50′1356″ E) and Central Park (29°53′5272″ N and 121°50′3325″ E). We chose urban ponds due to their frequent association with eutrophication, which can increase methane emissions and influence the abundance of floating-leaved macrophytes in aquatic systems (Beaulieu et al. 2019; Bornette and Puijalon 2011). The leaves were designated as floating or standing depending on whether they were floating on the water or perched above the water. Six intact healthy floating and standing leaves of lotus were collected from each pond, respectively. The surrounding pond water was sampled using sterile glass bottles, with independent triplicates from each pond. In total, we got 12 floating and 12 standing lotus leaves and six pond water samples. The stems of lotus were cut with a long sterile scissor. All lotus leaves were put into sterile bags and transferred to the laboratory on ice immediately. The analytical procedure for the basic physicochemical properties of the leaf and water samples and the data are shown in Text S1 and Table S1.

DNA extraction

The leaf surfaces were rinsed with 500 mL of 0.01 M autoclaved phosphate-buffered saline solution using a dental scaler (BAIR, M3, China). The top and bottom sides were rinsed separately and treated carefully to avoid mutual contamination. Afterwards, the two sides of the leaves were scraped using sterile cotton swabs, respectively. The pre-rinsing procedure enabled the ultimate removal of microbes from leaf surfaces by scrapping, particularly for the relatively dry sides, including the standing leaves and the top side of floating leaves. The resulted solution was filtered through a 0.22-μm sterile glass fiber membrane (Jinjing, China). The membranes and cotton swabs from each side of each leaf were ground into powder with liquid nitrogen and were mixed as one sample for DNA extraction. The pond water samples were filtered as described above, and the resulted membranes were ground using liquid nitrogen. The DNA was extracted using the FastDNA Spin Kit for Soil (Fisher Scientific, USA) following the manufacturer's instructions.

Library preparation and sequencing

The 16S rRNA gene of archaea was amplified using a nested polymerase chain reaction (PCR) approach with primer sets Arch340F/Arch1000F and Uni519F/Arch806R, respectively (Zhang et al. 2015; Zhang and Lu 2016). The diagnostic indicator of methanogens is *mcrA* gene that encodes methyl coenzyme M reductase. The amplification of *mcrA* gene was conducted using the primer set mlas/mcrA-rev as described in the literature (Steinberg and Regan 2008). In addition, the primer pair 799F and 1193R was used for the bacterial 16S rRNA gene amplification (Chelius and Triplett 2001). The detailed

PCR conditions are described in Text S2. The amplicon libraries were sequenced on Illumina NovaSeq platform (Illumina, USA). Bioinformatics and statistics details are provided in Text S3.

Isotopic labeling experiments

To evaluate the metabolic activity of the phyllosphere methanogens of floating lotus, we conducted a ^{13}C labeling experiment to verify the production of ^{13}C-labeled methane. In late July 2022, we sampled floating lotus leaves and the surrounding water in proximity in triplicates, from the same two ponds as described above. The analyses and results of physicochemical properties for the leaf and water samples are shown in Text S1 and Table S1. To exclude the possibility of methane production originated from the lotus plant (Matthews and Seymour 2014; Riya et al. 2020), and possibly endophytes, we separately washed microbes off both sides of the leaves for incubation. Leaf epiphyte collection procedures are shown in Text S4.

Five mL of the obtained microbial culture was transferred into a 120-mL sterile serum bottle with 50 mL corresponding filter-sterilized pond water as the natural medium. The cell-free pond water was set as control and performed in triplicates. All bottles were sealed with sterile butyl rubber stoppers and then subjected to five cycles of evacuation and flushing with sterile helium gas of 99.999% purity, and finally vented to atmospheric pressure. Thereafter, 2 mL of a 50 mM sterile anoxic (helium gas) stock $NaH^{13}CO_3$ (98 atom % ^{13}C, Merck, Germany) solution was injected into each serum bottle, conducted in an anoxic glove box (N_2 atmosphere, MBRAUN UNIlab, Germany). This resulted in a final ^{13}C content in carbon dioxide of 32.97 ± 2.31 atom % for leaf bottom side samples from Reader Park and 36.63 ± 6.66 atom % for leaf bottom side samples from Central Park, as indicated by GC-C-IRMS (Text S5). All anoxic bottles were incubated at 28 °C in the dark for 9 weeks. The cell density was monitored by measuring the optical density at the wavelength of 600 nm (OD_{600}) at the beginning and at 2, 4, 6, and 9 weeks of incubation. After 9-week incubation, 12 mL of headspace gas was sampled and injected into 12-mL pre-evacuated glass vials for subsequent methane and carbon dioxide content and isotope analyses, as described in Text S5. All sampling procedures were performed in the anoxic glove box. At the end of incubation, the culture solution of the epiphyte-containing microcosms was filtered through the 0.22-μm sterile mixed cellulose ester membrane (Bandao, China). The resulted membrane was ground using liquid nitrogen, and the DNA was extracted and amplified using the archaeal 16S rRNA gene, and then sequenced, as described earlier.

Results and discussion

Methanogenic and methanotrophic groups on floating lotus phyllosphere

We evaluated the presence of methanogens on lotus leaves by sequencing the archaeal 16S rRNA gene (Fig. 1a). The libraries were recovered from all bottom sides of floating leaves and eight out of twelve floating top side samples. Only three libraries were obtained from the standing leaves, including two from bottom sides and one from top sides. In the phyllosphere of the floating leaves, known methanogens, such as *Methanobacterium*, were detected as dominant groups. However, the archaeal community on the standing leaves was mainly composed of *Nitrososphaera* and *Nitrosopumilus*, known primarily as ammonia-oxidizing archaea (Fig. 1a). The methanogenic community was further investigated by sequencing the methanogenesis functional marker gene *mcrA* (Fig. 1a). The *mcrA* libraries were obtained from all bottom sides of floating leaves and half of the floating top side samples. No *mcrA* libraries were recovered from the standing leaves. The methanogens on floating leave surfaces were mainly assigned to *Methanobacterium*, followed by *Methanothrix* (Fig. 1a). In general, *mcrA* sequencing and 16S rRNA sequencing exhibited a similar profile of methanogens, but with slight differences in the relative abundance. The previous research has established *Methanobacterium* as a hydrogenotrophic methanogen widely distributed in various environments, including rice paddy soil and many other habitats (Evans et al. 2019; Li et al. 2021). Intriguingly, *Methanobacterium* has also been found to dominate and even be isolated from the heartwood of trees, as demonstrated in the previous studies (Yip et al. 2019; Zeikus and Ward 1974). This highlights the versatile nature of *Methanobacterium* and its adaptability to different ecological niches. We also examined the bacterial taxonomic profile. The most abundant bacterial genus in the phyllosphere of the floating ($n = 12$) and standing ($n = 12$) lotus leaves was *Aquabacterium* and *Exiguobacterium*, respectively (Fig. 1a). Methanotrophic groups were detected on both sides of both leaf types ($n = 12$ for each), among which *Methylobacterium* appeared with the highest relative abundance (Fig. S1). The detection of bacterial aerobic methanotrophs on floating and standing macrophyte leaves aligns with the previous research, as such leaves occupy oxic environments (Yoshida et al. 2014).

Permutational multivariate analysis indicates that both the archaeal and bacterial communities on the bottom sides of floating leaves differed from the surrounding pond water ($p < 0.05$) (Fig. S2). Pairwise comparison results show that *Methanobacterium* in the archaeal group was enriched on

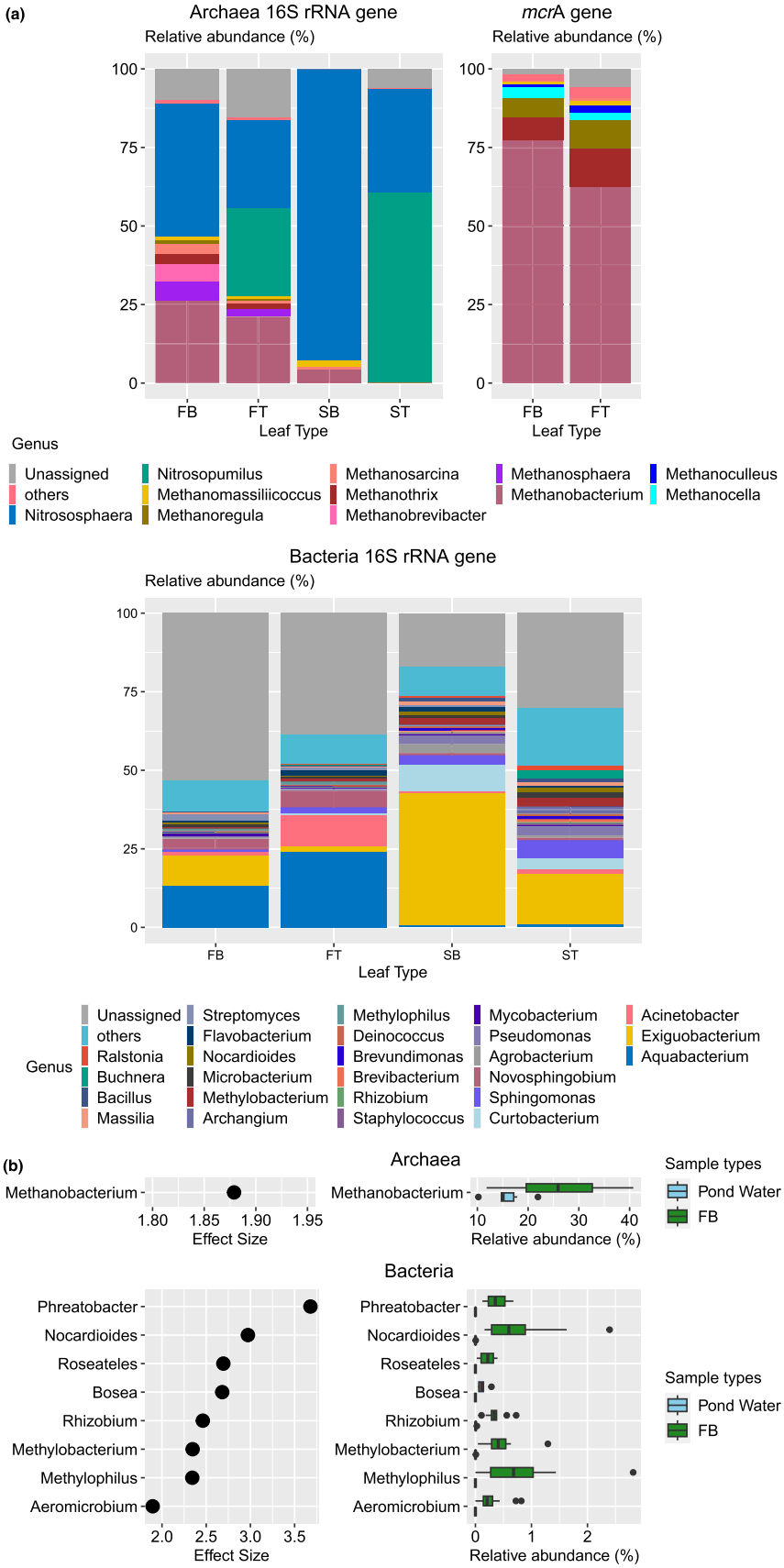

Fig. 1 **a** Taxonomic profile of lotus phyllosphere microbiota at the genus level. Results are based on sequencing of the archaeal 16S rRNA gene, methanogenesis functional gene *mcrA*, and bacterial 16S rRNA gene. The mean relative abundances are reported solely for the samples that were detected. Results demonstrated the presence of methanogens on floating lotus leaves. *FB* floating leaves' bottom sides, *FT* floating leaves' top sides, *SB* standing leaves' bottom sides, and *ST* standing leaves' top sides. **b** Enriched microbiota on the bottom sides of floating leaves in comparison with the surrounding pond water. The relative abundance of archaeal and bacterial groups on the bottom sides of the floating lotus leaves ($n=12$) was significantly higher than that of the surrounding water ($n=6$). The left panels illustrate the effect size of the comparison, and the right panels present the relative abundance of the enriched taxa in each sample type

the bottom sides of floating leaves (Fig. 1b). For the bacterial community, eight genera were enriched on the bottom sides of floating leaves compared to the pond water, including known methanotrophs such as *Methylophilus* and *Methylobacterium* (Fig. 1b). It suggests that the floating lotus phyllosphere appears to be a preferential habitat for methane-cycling microbes at the water–atmosphere interface.

Metabolic activity of phyllosphere methanogens on floating lotus leaves

We evaluated the methanogenesis capacity of the phyllosphere microbiota of floating lotus leaves through an isotopic tracer experiment. Epiphytes of floating lotus leaves were incubated in sterile pond water with ^{13}C-labeled $NaHCO_3$ as an external carbon source (Fig. 2a), as most observed methanogens in our study are hydrogenotrophic. In samples from Reader Park, two replicates showed high-methane production (37.17 and 47.92 µmol/bottle), while one replicate emitted lower methane (0.03 µmol/bottle) (Table 1). This observation was in line with their biomass status (Table 1). The OD_{600} of high-methane-producing replicates increased from 0.089 at the start of incubation to 0.16 after 4 weeks and then kept stable. The low-methane-producing replicate showed minimal change in OD_{600} over time. The ^{13}C enrichment in methane was 27.48 ± 2.77 atom % (Fig. 2a), showing incorporation of ^{13}C carbon from $NaHCO_3$ into methane. Other biological treatments, including epiphytes on the bottom sides of floating lotus from Central Park (Table 1) and the top sides of floating lotus from the two parks (not shown), did not produce measurable methane, and the OD_{600} values remained unchanged during incubation. Moreover, methane was not detected in the sterile control treatments. Together, these results provide evidence for methanogenesis by microbes on the bottom sides of floating lotus leaves.

We then specifically examined the archaeal taxonomic profile of incubated microcosms with bottom side samples of floating leaves. The two high-methane-producing microcosms, i.e., Replicate 1 and Replicate 3 from the Reader Park, were dominated by methanogens, accounting for over 83% of the archaeal community, mainly

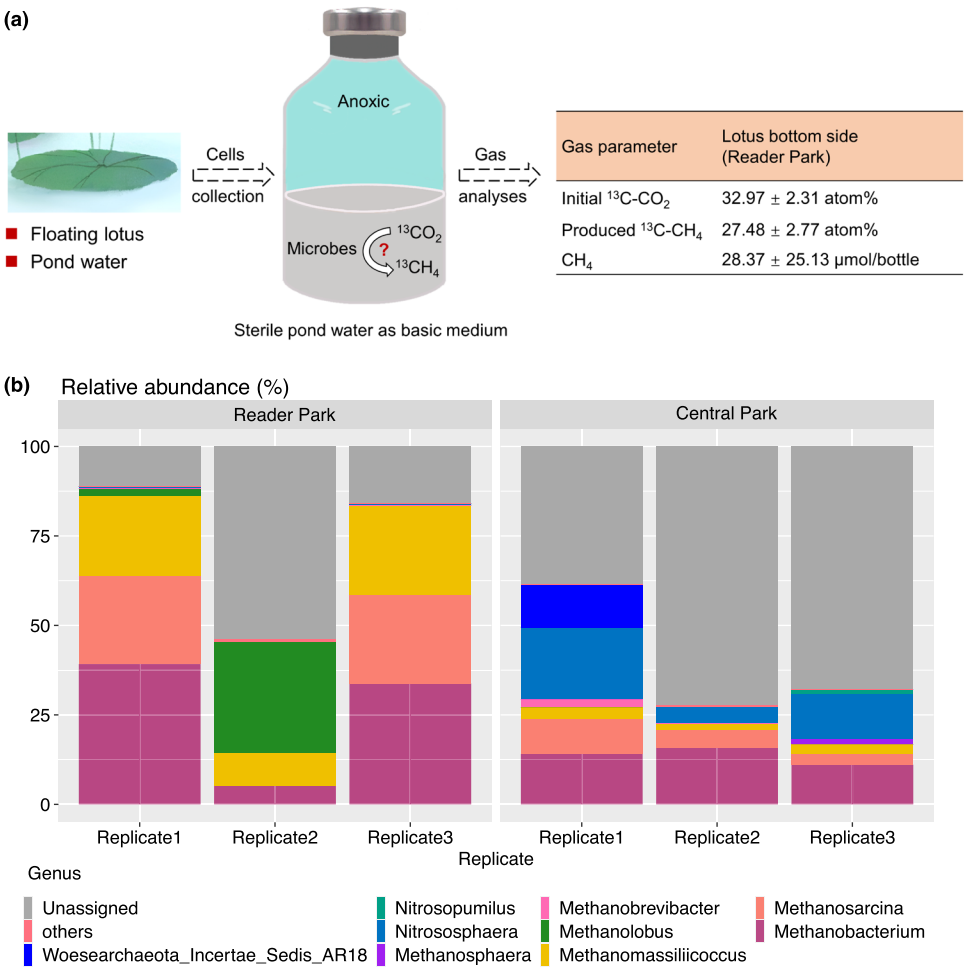

Fig. 2 **a** ^{13}C labeling experiment procedures and methanogenesis activity of epiphytes from floating lotus leaves at Reader Park. Total methane production was quantified by summing up both headspace and aqueous methane (Text S5). Data are means ± standard deviations ($n = 3$). The phyllosphere microbiota on the bottom sides of floating lotus leaves exhibited methanogenic activity. **b** Archaeal taxonomic profile of incubated microcosms at the genus level after 9 weeks of anoxic incubation of epiphytes from the bottom sides of floating lotus leaves. Incubated microcosms of leaf epiphytes from Reader Park generally had a higher relative abundance of methanogens compared to Central Park

Table 1 Methane production volume and biomass after 9 weeks of anoxic incubation of epiphytes from the bottom sides of floating lotus leaves

	Reader Park			Central Park		
	Replicate 1	Replicate 2	Replicate 3	Replicate 1	Replicate 2	Replicate 3
CH_4 production (μmol/bottle)	37.17	0.03	47.92	ND	ND	ND
OD_{600} (sampling)	0.163	0.070	0.165	0.053	0.064	0.066
OD_{600} (initial)	0.090	0.057	0.089	0.053	0.063	0.064

OD_{600} Optical density measured at the wavelength of 600 nm, *ND* not detected

Methanobacterium, *Methanosarcina*, and *Methanomassiliicoccus* (Fig. 2b). The low-methane-producing microcosm, i.e., Replicate 2 from the Reader Park, had less abundant methanogens, accounting for around 45%, with *Methanolobus* being the most abundant group, followed by *Methanomassiliicoccus* and *Methanobacterium* (Fig. 2b). We observed a positive correlation between the overall relative abundance of methanogens and both methane production and cell biomass, implying the occurrence of methanogenesis coupled with microbial growth (Fig. 2b; Table 1). In the microcosms without detected methane, i.e., the three replicates from the Central Park, the relative abundance of methanogens was much lower, ranging from 18 to 29% (Fig. 2b). This disparity suggests that methanogens may require additional growth substrates to sustain viability under our simplified incubation conditions. Nonetheless, we acknowledge the necessity for further investigations, specifically using DNA-/RNA-based stable isotope probing, to unequivocally identify the specific microbial communities associated with methanogenic activity. Altogether, our findings indicate that the floating-leaved macrophyte phyllosphere can serve as a niche for methanogens and a potential microbial source of methane at the water–atmosphere interface.

Environmental implications

Our main findings are summarized and conceptually visualized in Fig. 3. To our knowledge, this provides the first genetic and functional evidence of epiphytic methanogens on aquatic plants, particularly floating-leaved macrophytes. Conventionally, the origin of methane from aquatic plants has mainly been attributed to plant-mediated transport from the sediment and direct emission from the plants themselves. Aquatic macrophytes are known to employ pressurized ventilation to transport methane from the rhizome through stomata on the leaves, as an adaptive strategy to transport oxygen from shoots to roots for thriving in the anoxic environment of sediments (Riya et al. 2020; Villa et al. 2020). Additionally, studies have shown that plants can generate methane by producing methane precursor compounds (Keppler et al. 2006; Lenhart et al. 2015). Here, we introduce another potential source of plant-derived methane, i.e., the microbial methane formation by phyllosphere methanogens.

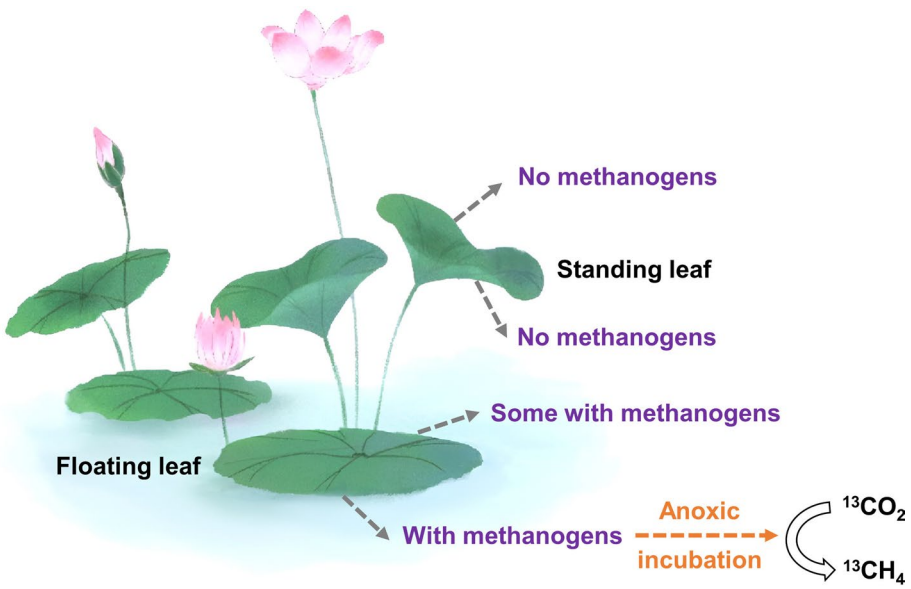

Fig. 3 Spatial distribution of methanogens on lotus leaf surfaces. The methanogenic potential can be detected in microbes present on the bottom sides of floating lotus leaves

The presence of methanogens on the phyllosphere of floating macrophyte leaves, located at the water–atmosphere interface, may contribute to the methane production observed in oxic waters (Hilt et al. 2022) and represent an overlooked microbial component related to the "methane paradox" for oxic environments (Tang et al. 2016). Further laboratory incubations can be conducted to enrich methanogenic archaea or even isolate them for biochemical studies. In the context of global climate change, additional research is warranted to examine the presence of methanogens in other floating-leaved aquatic plants and their ecophysiology at larger spatial and temporal scales. Note that bacterial aerobic methanotrophs were also detected in our study. The estimation of the net flux of methane from the floating macrophyte epiphytes in natural settings still needs to consider the entire microbial community.

Conclusion

We demonstrated the presence of methanogens on the surface of floating lotus leaves through a combined analysis of both phylogenetic and functional gene sequencing data. Additionally, we validated the methanogenesis metabolic activity of these methanogens using ^{13}C labeling incubation experiments. These results highlight the phyllosphere of floating-leaved macrophytes as a previously overlooked habitat for methanogens and a potential microbial source of methane. Our findings thus open new perspectives on the complex interactions between aquatic plants and methane emissions. The microbial origin of methane needs to be considered in the future studies when assessing the implications of aquatic plants for global methane dynamics and climate change.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10311-023-01653-8.

Authors' contribution SL and LY conceived the research and designed the experiments. SL, LY, and YZ conducted the experiments. LY performed bioinformatics analysis. YZ and SL performed routine analyses and isotope measurements. LY and SL prepared the figures and tables and wrote the manuscript with input from all co-authors.

Funding This work was financially supported by the National Natural Science Foundation of China (Grant Nos. 42007294 and 42021005). JP acknowledges National Natural Science Foundation of China (Grant No. 41977038). AK acknowledges infrastructural support by the German Research Foundation (DFG) under Germany's Excellence Strategy, cluster of Excellence EXC2124, Project ID 390838134.

Availability of data and materials The sequence data of bacterial 16S rRNA gene, archaeal 16S rRNA gene, and *mcrA* gene were deposited in the GSA database (https://bigd.big.ac.cn/gsa) in BioProject PRJCA009184 under the accession number CRA006684, CRA007369, and CRA007370, respectively.

Declarations

Conflict of interest The authors declare no conflicts of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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