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Community composition and methane oxidation activity of methanotrophs associated with duckweeds in a fresh water lake

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Methanotrophs are the only biological sink of the greenhouse gas methane. To understand the ecological features of methanotrophs in association with plants in the methane emitting environments, we investigated the community composition and methane oxidation of methanotrophs associated with duckweeds in a fresh water lake. Duckweeds collected from Lake Biwa, Japan over three summers showed methane consumption activity between 0.0067 and 0.89 μmol h^{-1} g^{-1} (wet weight), with the highest values occurring from the end of July to August. The methanotrophic community on duckweeds consisted primarily of γ-proteobacterial groups including the genera *Methylomonas* and *Methylocaldum*. Further analysis of co-cultures of a methanotroph isolate with sterilized duckweed revealed that the duckweed plant as well as the duckweed spent culture supernatant exerted an enhancing effect on methane oxidation. These results indicate that duckweeds not only provide a habitat for methanotrophs but also stimulate methanotrophic growth.

[**Key words:** Methanotroph; Duckweed; Methane oxidation; *Methylomonas*; *Spirodela*]

Methane is a greenhouse gas that is 21 times more heat-trapping than carbon dioxide, and increases in its atmospheric concentration contribute to global warming. In natural ecosystems, methane is biologically produced by methanogenic archaea that transform carbon dioxide and hydrogen, formate, or acetate to methane in anoxic environments (1). Plants emit methane generated by methanogens via their aerenchyma (2), and some plants also produce methane originating from methyl ester groups of pectin coupled with photosynthesis (3). A major portion (more than 80%) of the methane emitted into the atmosphere is oxidized by hydroxyl radicals in the troposphere, and a portion of the remaining methane is biologically oxidized by methane-oxidizing microorganisms, methanotrophs (4). The balance between methane sources and sinks is important to maintain the atmospheric methane concentration and the global climate. However, human activities have raised methane emissions from paddy fields, livestock, landfills, oil factories, and waste-treatment equipment. As a result, the atmospheric methane concentration increased by a factor of 2.5 in the past 200 years (4). Therefore, social demands are not only to decrease methane emission sources, but also to develop technologies for the removal of methane at production sites.

Methanotrophs are the only biological sink of methane and they play an important role in the global carbon cycle between two major greenhouse gases, methane and carbon dioxide, which is called the methane cycle (4). Methanotrophs utilize methane as sole carbon and energy sources and represent a subset of methylotrophs, which utilize one-carbon compounds (5). Methanotrophs consist of both bacteria and archaea, and the bacterial groups are classified in three phyla: α-Proteobacteria, γ-Proteobacteria and Verrucomicrobia (6). While methanotrophic archaea are anaerobes, methanotrophic bacteria (MOB) are aerobic oxidizers of methane via the enzyme methane monooxygenase. Methanotrophs (hereafter referred to as MOB) are distributed in a wide range of environments such as soils, wetlands, lakes, and oceans.

Aquatic environments such as wetlands and lakes are major methane emission sites. Global methane emission from lakes has been estimated to represent as much as 6–16% of the total natural methane emission (7). Methanotrophs have been detected in lake water and sediments, and the surface layers of sediments show high methane oxidation activity (8–12). Previous studies also revealed that methanotrophs inhabit submerged hydrophytes (13–16). It is noteworthy that hydrophytes have high methane oxidation activity (17–21). Thus, hydrophytes are considered to be a niche for methanotrophs in aquatic environments, and the ecosystem of methanotrophs and hydrophytes may contribute significantly to the global carbon cycle.

Duckweeds are floating hydrophytes that are often found in calm waters or fresh water lakes. The duckweed plant is recognized as an ideal biomass for biofuel production and animal feed based on its characteristics of global distribution, high starch content, and rapid growth (22). Techniques for cultivation under aseptic conditions and genetic engineering have also been developed for

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duckweeds (23,24). Recent studies reported that bacteria-duckweed interactions are important for enhanced biomass production as well as bioremediation; duckweed-growth promoting ability has been demonstrated for several bacterial isolates from duckweeds, including *Acinetobacter*, *Aquitalea*, *Pseudomonas*, and *Exiguobacterium* (25–27). In addition, *Acinetobacter* and *Exiguobacterium* were shown to degrade phenolic compounds and remediate chromium toxicity, respectively (27,28). Also, inoculation of the isolated bacteria on the duckweed plant resulted in the formation of biofilms in the rhizosphere of host plants (28). Furthermore, duckweeds inhabit the interface between water and air; in other words, in oxic environments where methane oxidation occurs and anoxic environments where methanogenesis occurs. They have air spaces that provide buoyancy and can be a temporal reservoir for methane generated in anoxic water environments. These characteristics suggest that duckweeds may be favorable sites for methane oxidation by methanotrophs. However, whether methanotrophs inhabit and exhibit methane oxidation activity on duckweeds is currently unknown.

In this study, we collected duckweed samples from Lake Biwa, a fresh water lake in Japan over three summers, and analyzed the community composition and methane oxidation activity of methanotrophs associated with duckweeds. Furthermore, we describe the ecology of methanotrophs associated with duckweeds, and demonstrate that positive interactions between methanotrophs and duckweeds enhance methane oxidation activity.

MATERIALS AND METHODS

Sampling Duckweeds and lake water were sampled at the shore of Lake Biwa (Shiga Prefecture, Japan) in the summers (July, August, and September) of 2011, 2012, and 2013. The latitudes of the sampling sites were as follows: site A (35°7′52″N 135°93′91″E), site B (35°6′90″N 135°93′54″E) and, site C (35°3′15″N 135°91′29″E). In each sampling site, duckweeds and the ambient lake water were collected from approximately 1 m^2 area and put together into a plastic container, and the samples were conveyed to the laboratory. Methane consumption analysis of these samples was started within several hours after sampling. For DNA extraction, the duckweed samples were stored at −80°C until use.

Analysis of methane consumption by environmental samples Environmental samples, 3 g (wet weight) of duckweed or 3 mL of lake water, were placed in 25 mL glass vials. After sealing the vials with butyl rubber caps and aluminum seals, 1 mL of methane was added. Vial were incubated statically at 28°C in the dark. A duplicate or triplicate experimental setup was prepared from the sample collected at each sampling site. Methane concentrations in the headspace of the vials were determined using a Shimadzu GC-14B gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and Porapak Q column (Shinwa Chemical Industries, Kyoto, Japan). Nitrogen gas was used as the carrier. Analytical temperatures of the oven, injector and detector were 100°C, 120°C and 225°C, respectively. Methane consumption activity was calculated from the linear portion of a graph of methane concentration against incubation time (approximately 15–40 h after the start of incubation).

Clone library analysis of the *pmoA* and *mxaF* gene DNA was extracted from 0.5 g (wet weight) of duckweeds using ISOIL for Beads Beating (Nippon Gene, Tokyo, Japan). The *pmoA* and *mxaF* genes were amplified from the extracted DNA with Ex Taq DNA polymerase (Takara Bio, Shiga, Japan) using primer sets A189-mb661 and mxa1003-1561, respectively (29). PCR products were cloned into pMD20 (Takara Bio) using the TA cloning strategy, and the plasmid DNA was sequenced.

Methane oxidation analysis by *Methylomonas* sp. BLU1 The methanotrophic strain *Methylomonas* sp. BLU1 was isolated from a duckweed sample from Lake Biwa (site A, July 12, 2011). The isolation procedure was as follows: After enrichment with methane in nitrate mineral salts (NMS) liquid medium (ATCC 1306 medium), the serially diluted cultures were spread on NMS agar plates, which were incubated under a methane/air atmosphere. A single colony formed was transferred to NMS liquid medium. *Methylomonas* sp. BLU1 was grown in NMS medium with methane at 28°C.

The duckweed *Spirodela polyrhiza* was sterilized by treatment with 5% bleach for 10 min, and washed with sterilized water. The sterilized duckweed was aseptically cultivated in half-strength Hunter's medium (1/2H medium) under light/dark conditions at 25°C in a plant growth chamber (Nippon Medical & Chemical Instruments, Osaka, Japan). Hunter's medium includes only inorganic nutrients and has been used for duckweed culture (30). Several colonies were inoculated in 30 mL of 1/2H medium in a 100-mL flask with a culture plug. After 1-month cultivation, duckweed colonies and the culture medium (supernatant after centrifugation) were used for the following assays.

One mL of the methanotroph culture ($OD_{600} = 0.1$, adjusted with NMS medium by dilution), 1 mL of methane, and one of the following additives were placed into a 25 mL glass vial; the additives were five individual samples of duckweed, 100 μL of filter-sterilized duckweed spent culture supernatant, or 100 μL of 1/2H medium. The methane concentration in the headspace of the vial, which was incubated statically at 28°C in the dark, was analyzed. Dead duckweeds were prepared by treating duckweeds at 60°C for 30 min. Boiled duckweed culture supernatant was prepared by incubating the duckweed spent culture supernatant in boiling water for 30 min. Either 1 mL of air, carbon dioxide, or oxygen was added to the vial to test the effect of additional gases.

Nucleotide sequence accession numbers Partial sequence of the 16S rRNA gene of *Methylomonas* sp. BLU1 has been deposited in DDBJ under accession number LC440492.

RESULTS

Methane consumption by duckweed samples from Lake Biwa To investigate the methane oxidation potential of methanotrophs associated with duckweeds, we sampled duckweeds growing on Lake Biwa over three summers. Duckweeds began to vigorously proliferate in July and disappeared in October at all sampling sites. Judging from the morphology of duckweeds, main portions of collected duckweeds were *S. polyrhiza* and *Lemna aequinoctialis*. The methane consumption activity of duckweeds and the ambient lake water was measured under *in vitro* conditions as described in Materials and methods. Fig. 1A shows the typical methane consumption of duckweeds and the lake water. The methane concentration in the vial with duckweeds collected from site A on July 19, 2012 significantly decreased with incubation time, while that with the lake water slightly decreased. All the tested duckweeds showed the methane consumption activity, and the values were varied between 0.0067 and 0.89 μmol h^{-1} g^{-1} (wet weight) by sampling site and period (Fig. 1B). The rate with duckweeds was more than one-order of magnitude higher than that with the lake water, and thus the methane consumption activity of the lake water attached to duckweeds could be considered as background level. These results indicate that duckweeds growing in natural environments have the potential for methane oxidation. At every site, the peak of the methane consumption activity was observed from the end of July to August.

Analysis of the methanotrophic community on duckweeds Next, in order to analyze the methanotrophic community composition on duckweeds, the *pmoA* gene, which encodes a subunit of particulate methane monooxygenase (pMMO), was amplified by PCR from the DNA extracted from duckweed samples from site A, and ligated with a TA cloning vector to generate a clone library. Analysis of the *pmoA* sequences revealed that the duckweeds predominantly harbored γ-proteobacterial methanotrophs, whereas *Methylocystis* was the only detected α-proteobacterial methanotroph (Fig. 2A). The composition of methanotrophs changed substantially during the first month (from July 12 to July 29 in 2011 and from July 19 to Aug 29 in 2012) (Fig. 2A). The methanotrophic community was diverse, harboring three to seven genera, and members of the genera *Methylomonas* and *Methylocaldum* represented the core components throughout the sampling period. Additionally, the *mxaF* gene, which encodes the large subunit of methanol dehydrogenase, was sequenced to assess the methylotrophic community including methanotrophs and methanol utilizers (Fig. 2B). The ratio of methanotrophic members was relatively low among the methylotrophic community on duckweeds. *Hyphomicrobium* was present on all the tested duckweeds, and *Methyloversatilis* and *Methylobacterium* were also frequently found.

Analysis of methane oxidation in methanotroph-duckweed co-cultures To investigate whether duckweeds only provide

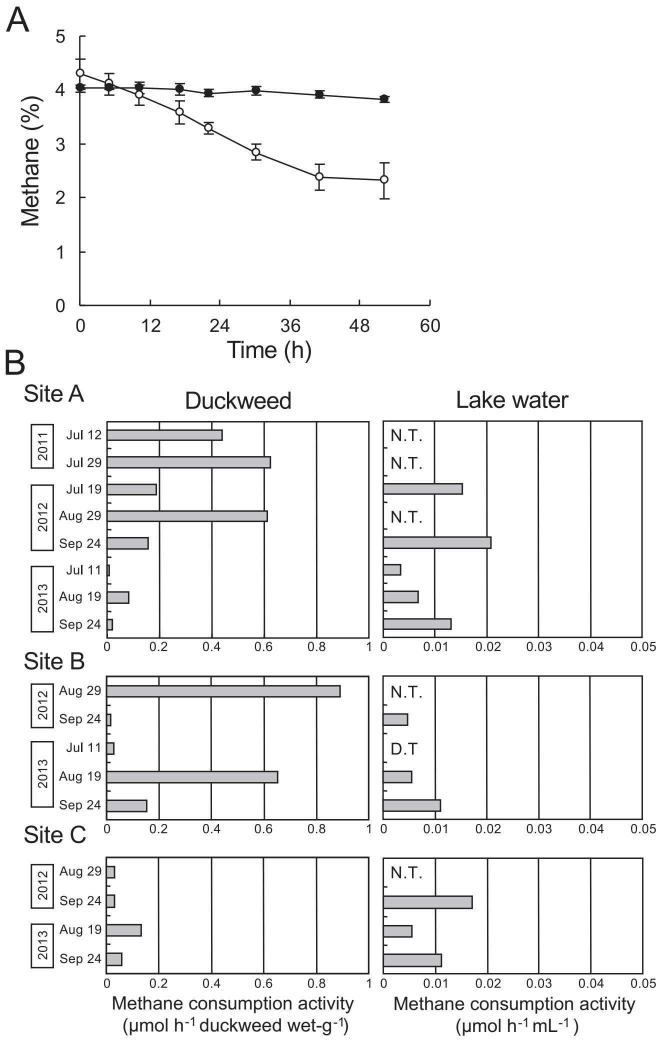

FIG. 1. Methane consumption activity of duckweeds and ambient lake water. (A) Methane consumption of the duckweed sample (open circles) and lake water (filled circles) collected at site A on July 19, 2012. Duckweeds or lake water samples were enclosed in vials with methane, and the methane concentration in the headspace of the vials was analyzed over time. Data are provided as the means ± standard deviations (N = 3). (B) Methane consumption activity of duckweeds and lake water. Duckweeds or lake water samples from Lake Biwa collected at the indicated sites on the indicated date were enclosed in vials with methane, and the methane concentration in the headspace of the vials was analyzed over time. Methane consumption activity was determined as described in Materials and methods. Data are provided as the means (N = 1–3). N.T., not tested. D.T., under the detection limit.

methanotrophs with a habitat in aquatic environments or have symbiotic interactions with methanotrophs, we examined the methane oxidation activity of methanotrophs in the presence and absence of duckweed. The methanotroph *Methylomonas* sp. BLU1, which was isolated from duckweeds in Lake Biwa, was subjected to cultivation tests with sterilized duckweed, *S. polyrhiza*. A pure culture of *Methylomonas* sp. BLU1 grew well on NMS medium containing methane as the sole carbon source without the addition of any growth factors such as vitamins (data not shown), and methane was consumed along with cultivation time (Fig. 3). When *Methylomonas* sp. BLU1 was cultured with duckweeds, methane oxidation by *Methylomonas* sp. BLU1 was enhanced (Fig. 3). Addition of dead duckweeds that was prepared by treatment at 60°C also resulted in enhanced methane oxidation at the same rate as co-cultures with living duckweeds (Fig. 3). These results suggest that methane oxidation activity or growth of *Methylomonas* sp. BLU1 is stimulated in the presence of duckweeds regardless of whether they were living or dead.

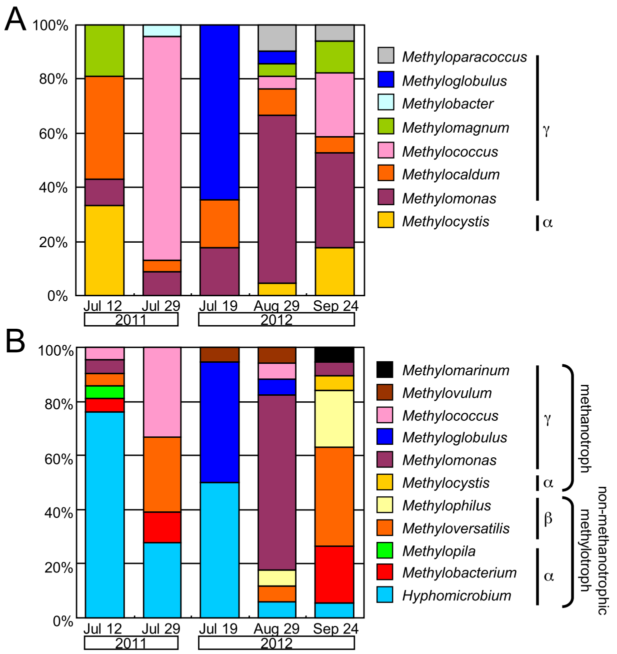

FIG. 2. Community composition of methanotrophs and methanol utilizers on duckweeds. The *pmoA* and *mxaF* genes were amplified by PCR with the DNA extracted from duckweed samples collected at site A on the indicated date, and plasmid clone libraries were constructed for sequencing. The methanotrophic community composition (A) was assessed by the analysis of *pmoA* gene sequences (N = 17–23) and the methylotrophic community composition (B) was assessed by the analysis of *mxaF* gene sequences (N = 17–21). α, β, and γ indicate subgroups of the Proteobacteria.

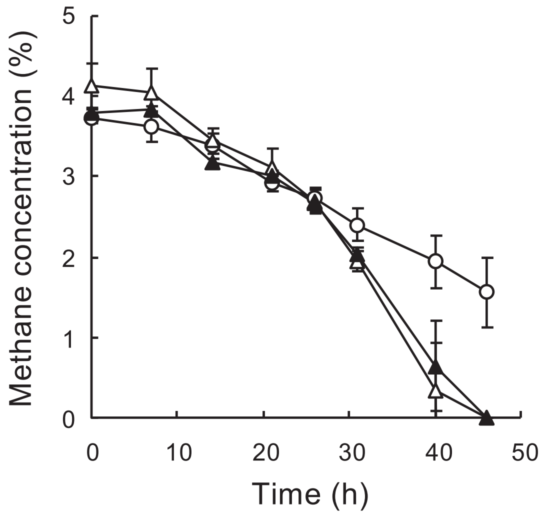

FIG. 3. Effect of duckweeds on methane oxidation by *Methylomonas* sp. BLU1. Cells of *Methylomonas* sp. BLU1 were incubated with or without duckweeds in sealed vials filled with methane, and the methane concentration in the headspace of each vial was analyzed over time. Data are provided as means (N = 3) and error bars represent standard deviations. Open circles, *Methylomonas* sp. BLU1; open triangles, *Methylomonas* sp. BLU1 with living duckweeds; filled triangles, *Methylomonas* sp. BLU1 with dead duckweeds.

Effect of duckweed culture supernatant on methane oxidation by *Methylomonas* sp. BLU1 Because dead duckweeds enhanced the methane oxidation activity of *Methylomonas* sp. BLU1 (Fig. 3), some metabolites of duckweeds seem to have stimulatory effects. Next we tested whether duckweed culture supernatant enhances methane oxidation by *Methylomonas* sp. BLU1. As shown in Fig. 4A, spent duckweed culture supernatant

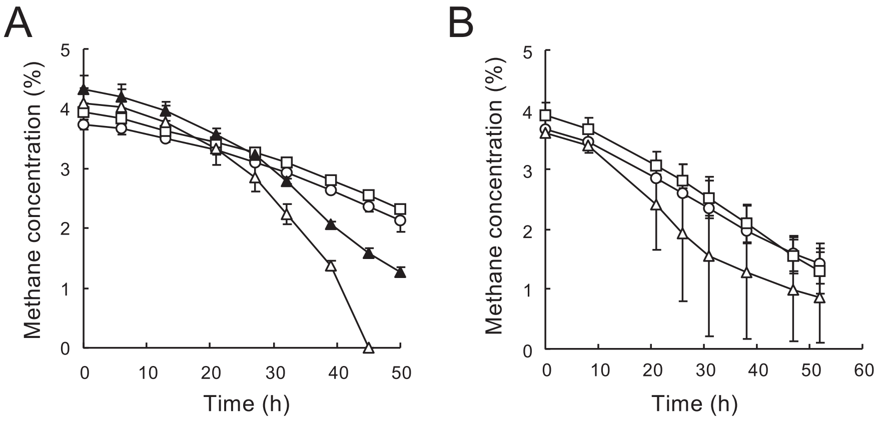

FIG. 4. Effect of duckweed culture or additional gases on methane oxidation by *Methylomonas* sp. BLU1. Cells of *Methylomonas* sp. BLU1 were incubated with or without duckweed culture supernatant (A) or additional gases (B) in sealed vials filled with methane, and the methane concentration in the headspace of each vial was analyzed over time. Data are provided as means ($N = 3$) and error bars represent standard deviations. (A) Open circles, *Methylomonas* sp. BLU1; open squares, *Methylomonas* sp. BLU1 with 1/2H medium; open triangles, *Methylomonas* sp. BLU1 with duckweed spent culture supernatant; filled triangles, *Methylomonas* sp. BLU1 with boiled duckweed culture supernatant. (B) Open circles, *Methylomonas* sp. BLU1 with air; open triangles, *Methylomonas* sp. BLU1 with carbon dioxide; open squares, *Methylomonas* sp. BLU1 with oxygen.

also enhanced methane oxidation, whereas the 1/2H medium did not. These results indicate that the stimulatory effect is mainly achieved by plant-derived compounds. When boiled duckweed culture supernatant was added, the methane oxidation rate was slightly enhanced (Fig. 4A), indicating that the major compounds responsible for the stimulatory effect are heat-sensitive.

In general, methanotrophic growth and methane oxidation by methanotrophs are affected by the concentrations of two kinds of gases, carbon dioxide and oxygen. Since both gases can be generated by photosynthesis and respiration by duckweeds, we further examined the effect of carbon dioxide or oxygen on methane oxidation by *Methylomonas* sp. BLU1. As shown in Fig. 4B, addition of carbon dioxide but not oxygen had an enhancing effect. Although carbon dioxide emitted by duckweeds may affect the methane oxidation activity of methanotrophs, judging from the results with dead duckweeds and boiled culture supernatant, metabolites generated by duckweeds seem to be the major factors that enhance methane oxidation by methanotrophs.

DISCUSSION

To reduce the increasing concentration of methane in the atmosphere, management of both methane production and removal in natural and man-made environments is required. In this study, we revealed that the ecosystem of methanotrophs and floating hydrophytes, duckweeds, has high methane oxidation potential (Fig. 1B). Lake sediments that generate methane are known to also abundantly consume methane (31), and our results showed that methanotrophs associated with duckweeds have high methane oxidation activity. Thus, duckweeds that expand over the water surface can function as a biological filter of methane generated deep under water, and prevent methane emission into the atmosphere.

The duckweeds examined here harbored a diverse methanotrophic community that consisted of up to seven methanotrophic genera in addition to methanol utilizers (Fig. 2). γ-Proteobacteria were the dominant population among the methanotrophic community on duckweeds. This community composition is a common feature in lake water and sediments (9,10,32,33), indicating that duckweeds share a common methanotrophic population with the ambient environment. Since the detected methanotrophic genera are obligate methanotrophs with the exception of limited *Methylocystis* strain that have been reported to be facultative methanotrophs, they are assumed to predominantly utilize methane on duckweeds. It is known that terrestrial plants emit methanol (34); however, this metabolic process has been not reported for duckweeds. The methanol utilizers detected in this study represent facultative methylotrophs, which can utilize methanol as well as complex carbon compounds secreted from duckweeds or present in the lake water. There may be a trophic relationship in which methane metabolites such as methanol, which are produced by methanotrophs are provided to methanol utilizers (35).

Variations in the methane consumption activity are probably related to *in situ* methane concentrations from the sites where duckweeds were sampled. The highest methane oxidation potential by duckweeds was found from the end of July to August (Fig. 1B), during which the atmospheric temperature reached the highest of the year in this area. A possible explanation for this observation is that high temperature enhances biomass production including duckweed growth, biomass degradation, and methanogenic activity (36), which lead to an increase in methanotrophic growth. Based on our previous study, the high methane oxidation activity of duckweeds is likely to be due to the large population of associated methanotrophs (20). The methane oxidation potential of duckweeds reported here (0.0067–0.89 μmol h^{-1} wet-g^{-1}) was lower than those in other studies with *Elodea canadensis* (9.7 μmol h^{-1} dry-g^{-1}) and *Egeria densa* (22 μmol h^{-1} dry-g^{-1}) (17,20). This may be due to the structure of the duckweed plant, which has limited area for methanotrophs to inhabit, whereas duckweed plants have the advantage of growing to high density. Two reports showed that bacterial biofilm formed on duckweed roots (28) and higher methanotrophic activity occurred in submerged parts of plants (21), suggesting that methanotrophs constitute root biofilms on duckweeds with other bacteria rather than living on the frond body. Further analysis by fluorescent *in situ* hybridization with a methanotroph-specific probe could reveal the localization of methanotrophs on duckweeds.

We revealed that the duckweed plant has an enhanced effect on methane oxidation by methanotrophs. In microbe-plant interactions in soil, plant roots are considered to release nutritional compounds and oxygen (37). But in this study, oxygen addition did not affect methane oxidation by methanotrophs (Fig. 4B), and therefore oxygen does not appear to be the limiting factor in the surface water where duckweeds live. Since dead duckweeds as well as the spent culture supernatant of duckweeds enhanced methane oxidation (Figs. 3 and 4A), metabolites produced by duckweeds appear to have a stimulatory effect on methanotrophs and it seems that living duckweeds are not necessary for the effect. The addition of carbon dioxide, which is a respiration product of duckweeds, also enhanced methane oxidation (Fig. 4B). However, carbon dioxide is probably not the key compounds that enhanced methane oxidation, since both the dead duckweeds and the boiled culture supernatant, which are not expected to generate carbon dioxide, enhanced methane oxidation (Figs. 3 and 4A). It is well known that carbon dioxide supports the carbon metabolism of γ-proteobacterial methanotrophs via the Calvin cycle (38,39).

The stimulatory compounds produced by duckweeds were characterized as heat sensitive (at 100°C) (Fig. 4A), but their chemical identification remains for future study. In general, photosynthetic products of plants, such as sugars and organic acids, can be used as nutrients by plant-associated bacteria in natural environment where nutritious substances are limited. By contrast, most of methanotrophs including *Methylomonas* sp. BLU1 are obligate C1-utilizers that are only capable of growth on methane or methanol as the sole carbon source. However, several additional organic compounds such as organic acids, yeast extract, and peptone have been reported to stimulate the growth of some methanotrophs (40–42). Bacterial interactions can also stimulate methanotrophic growth. Our previous study showed that vitamin B_{12} produced by *Rhizobium* sp. stimulated the growth of some methanotrophs (43). Stock et al. (44) proposed that bacterial metabolites such as quinones and vitamins B_6 and B_{12} also stimulate methanotrophic growth. Knowledge of the molecular mechanism of plant–methanotroph interactions is limited to studies with *Sphagnum* mosses in which the interaction is mediated by carbon dioxide, oxygen, and ammonium (45); no plant products that have stimulatory effects on methanotrophs have been identified.

Duckweeds are useful biological materials for removing nitrogen, phosphorus, and heavy metals, as well as organic pollutants from wastewater (28,46). These removal activities are attributed to utilization by duckweeds or degradation by root-associated bacteria. However, treatment ponds, which are intended for pollutant removal through activities of microorganisms or plants, are also a source of greenhouse gases including methane. A study showed that the removal of duckweeds from a stormwater treatment system increased methane flux and proposed a role for root-associated bacteria in methane flux (47), although methanotrophic activity was not examined. We expect that if methanotrophs can be stably colonized on duckweed roots together with other functional bacteria that degrade pollutants, the duckweed ecosystem would add the function of methane removal, mitigating methane emission into the atmosphere.

In conclusion, duckweeds living in fresh water lakes are inhabited by methanotrophs and have high methane oxidation activity. Duckweeds can stimulate methanotrophic growth, presumably by contributing some duckweed metabolites that are heat-sensitive. Future studies analyzing the molecular mechanism of the duckweed-methanotroph interaction will open the way to the application of methanotrophs for wastewater treatment with duckweeds.

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