Diverse methanotrophs from alpha and gammaproteobacteria dwell in a stone quarry: a study from Western India wetland

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Abstract

Freshwater wetlands are global methane sources and have become a rich habitat for methanotrophs. In the present study, we sampled a wetland ecosystem in a stone quarry situated amidst a hill in Pune city, popularly known as ARAI hills. Sediment samples from the wetland lake, water samples, and soil associated with *Typhus angustifolia* plant roots were collected in the winter season of 2022 (31st Jan and 15th Feb 2022). Enrichments were set up in a serial dilution from 10^{-1} and 10^{-12} by incubating the diluted samples in an air: methane atmosphere (80:20). A high abundance of methane-oxidizing bacteria was indicated by the fact that growth was obtained till dilutions 10^{-10}. Methanotrophs isolated from the enrichments belonged to various known methanotrophs: *Methylomonas*, *Methylomagnum*, *Methylocucumis*, and *Methylocystis* genus, spanning the entire range of aerobic methanotrophs [Type Ia, Type Ib (from gamma proteobacteria), and Type II (from alpha-proteobacteria) methanotrophs]. This is one of the first studies on cultured methanotrophs from Indian freshwater wetland habitat from a stone quarry. The present study also indicated that the wetland site was a rich source of methanotrophs in high abundance. The isolates could be helpful as model organisms for mitigation applications and for studying various biotechnological applications.

Keywords: Wetland methanotrophs, *Methylomagnum* sp., *Methylocucumis* sp., *Methylocystis* sp., *Methylomonas* sp., *pmoA* gene, methane-oxidizing bacteria, *Typhus angustifolia*

Introduction

Methanotrophs are aerobic to micro-aerophilic bacteria that use methane as the sole carbon source for growth and energy [1]. Wetlands occupy 3.8% of the Earth's land surface, amounting to about 20–40% of global CH_4 emissions. However, the wetlands' net emission results from the delicate balance between methanogenesis and methanotrophy. Methanotrophs live in the uppermost oxic layer or at the oxic-anoxic interface in the rhizospheric regions [2] of wetland plants before reaching the atmosphere [3]. They also harbour a rich community of methanotrophs which, with their methane monooxygenase enzyme activity, utilize over 18 to 90% of the methane, thus simultaneously establishing an equilibrium between methanogenesis and methanotrophy. Methane-oxidizing bacteria in the supra-aerobic layer or the wetland soil's oxic-anoxic interphase influences methane oxidation. It ensures that the least methane is emitted into the air [4]. Amongst the two types of wetlands, methanotrophs from arctic wetlands have been studied in much detail [5; 6], but less information prevails on the diversity of methanotrophs from tropical wetlands. The inherent methanotrophs from tropical wetlands are adapted to high methane concentrations and temperatures Wetlands harbor a great variety of emergent macrophytes, which along with being water biofilters, are also mediators of methane emissions to the atmosphere. Most such investigations have been done on a few plants like rice and *Sphagnum* moss; however, various studies on the interaction between methanotrophs and plants will deepen our understanding of the carbon cycle and help regulate it [4].

Wetlands can produce and absorb greenhouse gases, a significant component of global climate change. About 90% is consumed by methanotrophic bacteria when passing through the aerobic soil layer. Between 18 and 90% of the produced CH_4 in the root zone of emergent macrophytes in wetlands is consumed by aerobic methanotrophs. Even though wetlands account for 38% of the

total methane production globally, a large portion of the methane is consumed and utilized by methane-oxidizing bacteria or methanotrophs dwelling in these ecosystems. Aerobic methanotrophs associated with the roots of emergent aquatic plants are the primary colonizers of aquatic environments by making carbon available via methane consumption. Methane consumption by methane-oxidizing bacteria decreases the amount of methane that could be emitted by vegetation. However, limited data is available on the diversity and methanotrophic bacterial community of the wetlands found across varied ecosystems, especially from tropical regions. Stone quarries are unique ecosystems, and the lakes created naturally in such quarries become a unique habitat for methanotrophs. In the present study, we sampled a wetland ecosystem associated with a stone quarry situated amidst hills in Pune city (popularly known as ARAI hills), one of the only natural green habitats in the city. The water-filled quarry is rich in vegetation from aquatic weeds and plants. One such aquatic weed named *Typhus angustifolia* is dominant in the west to the southwestern region of India and is found growing abundantly in a water-filled quarry on a hill in Pune, Maharashtra, India; the soil firmly adhered to the roots of this aquatic weed; was investigated for studying the methanotrophic community structure. Almost no data is available on the methanotrophs dwelling in Indian freshwater wetlands. The present study is one of the first attempts to document and study the cultivable methanotroph diversity in such an ecosystem by cultivating the dominant members.

Materials and Methods

Sampling sites:

Various sediment samples were collected from the wetland patch, including roots of *Typhus* associated sediment samples from the wetland patch, and water samples (Figure 1a and b) in January 2022 and February 2022, representing the winter sample. All the samples were collected using gloves in sterile plastic vials or sterile plastic bags and proceeded for enrichment and oxidation of methane. Roots and the surrounding sediments of *Typhus angustifolia* emergent species were collected by uprooting the plant. The roots with the adhered mud were collected in sterile, clean zip-lock bags and used for enrichment experiments.

Enrichment set up:

Serial dilutions were set up using modified Nitrate Mineral Salts referred to as dilute NMS medium was prepared [2]. The constituents were: $MgSO_4.7H_2O$, 1g; KNO_3, 0.25g; $CaCl_2$, 0.2g per litre. The samples were serially diluted from 10^{-1} to 10^{-10} by adding 1g of the sample to a 9 ml sterile (dilute NMS) medium in 35 ml serum bottles [2]. A headspace volume of 20% was removed with a sterile syringe and filled with 20% methane. The gas was added using a syringe and a needle through a sterile filter (0.2 microns). The enrichment bottles were incubated under static conditions and at 25°C in the dark. Chemito 8510 Gas Chromatography, India, equipped with a flame ionization detector (FID), was used to check the methane oxidation of enriched bottles. The bottles were incubated for ~4-6 weeks, and the gas phase was replaced every ten days with the same gas mixture described above. A simultaneous methane decline accompanied by visual turbidity, pellicle, or biofilm growth at the bottom indicated methanotrophic growth and a positive

enrichment. Later, the methanotrophic growth was confirmed by phase contrast microscopy also (Figure 2).

Microscopic identification of methanotrophs:

The morphology of all the positive enrichments was examined microscopically using wet mounts, and images were taken. The typical characteristics of the methanotrophs, such as their growth rate, appearance, and growth in liquid broth pellicle, turbidity, or biofilm, helped categorize them primarily as Type I and Type II methanotrophs.

Isolation of methanotrophs: All positive enrichments were streaked on dilute NMS agarose plates, and methanotrophs were isolated as described earlier [3].

DNA extraction, PCR amplification, and sequencing:

DNA extraction from colonies was done using the GenElute TM Bacterial Genomic DNA kit (Sigma). The partial methane monooxygenase gene, the *pmoA* gene, was amplified from the DNA samples using the universal *pmoA* primer pair A189f-mb661r (Costello et al., 2002). All amplifications were carried out in 25 µl total volume in ABI thermal cycler (Veriti) using EmeraldAmp® GT PCR Master Mix. Purified PCR products were sequenced in-house using both primers. All forward and reverse sequences of the *pmoA* gene were assembled using the Lasergene software. An NCBI BLAST search was performed using GenBank (http://www.ncbi.nlm.nih.gov/) (Altschul & Lipman, 1990). Partial *pmoA* sequences of methanotroph-type strains that showed the closest match to query sequences were included in the phylogenetic analysis. Online alignments were done using the MAFFT aligner (http://mafft .cbrc. jp/), and the phylogenetic tree was constructed using the maximum likelihood algorithms implemented in MEGA 11.0 with 1000 bootstraps (Kumar et al., 2018)

Results

Growth was seen in all the enrichments arising from roots, sediments and water collected from the quarry wetland. After incubation of 4-6 weeks, growth was seen in the form of turbidity, pellicle, and clumps accompanied by a decline in methane concentrations in the bottles. The growth pattern was similar in both duplicates. Microscopically, methanotrophs were either in the form of large cells of oval or slightly elongated with dark interiors were seen in higher dilutions (indicative of Type I methanotrophs) [7] or small coccoid cells (indicative of Type II methanotrophs) were observed in higher enrichment dilutions of 10^{-9} and 10^{-10}. After serial dilutions of each grown culture, we obtained almost pure cultures of methanotrophs (from 10^{-3}, 10^{-4}, 10^{-5}, $10^{-9,}$ and 10^{-10}) where a single morphotype observed in microscopic observations (Figure no. 4). It was seen that the sequences of the *pmoA* gene were of good quality, reflecting that each culture had a single methanotroph. On performing NCBI BLAST, it was indicated that the cultured members belong to the genus *Methylocystis* genus, *Methylocucumis* genus, *Methylomagnum* genus, and *Methylomonas* genus (1strain), representing four genera (Table no. 1, Figure 3). The higher positive dilutions (10^{-9}, 10^{-10}) showed the presence of *Methylocystis* whereas in the lower dilutions *Methylomonas*, *Methylomagnum* and *Methylocucumis* were observed (10^{-3}, 10^{-4}, 10^{-5}). Almost pure cultures of these methanotrophs were obtained (~80-90% of the cells observed under the microscope), Figure 4. As the main aim of this study was to document the methanotroph diversity dwelling in this habitat, the cultures were identified at the level by sequencing the *pmoA* gene without obtaining a pure culture. Each pure *pmoA* sequence indicated that there was a single methanotroph in each culture.

The percent homology of the *pmoA* sequence with the sequences from valid methanotrophic species was 89–98% indicating the presence of known and putative novel methanotrophs.

Methane oxidation rates of various samples: Methane was oxidized almost instantaneously within eight days from 20% to 1-2%. The root samples of the *Typha* plant showed the highest rates of methane oxidation.

Discussion

Methanotrophs are aerobic to micro-aerophilic bacteria that use methane as the sole carbon source for growth and energy (Conrad, 2009). While various natural and anthropogenic activities emit methane, wetlands account for massive emitters and absorbers of methane gas (Cui et al., 2020a). Wetlands occupy 3.8% of the Earth's land surface, amounting to about 20–40% of global CH_4 emissions. Wetlands have anoxic zones where methane is produced. Methanotrophs utilize over 18 to 90% of the methane in wetlands [3] thus simultaneously establishing an equilibrium between methanogenesis and methanotrophy. Methane-oxidizing bacteria in the oxic layer or the soil's oxic-anoxic interphase carry out methane oxidation. It ensures that the least amount of methane is emitted into the air [4]. Amongst the two types of wetlands, methanotrophs from arctic wetlands have been studied in much detail, but less information prevails on the diversity of methanotrophs from tropical wetlands. The inherent methanotrophs from tropical wetlands are adapted to high methane concentrations and temperatures [3]. Tropical wetlands are spots of biodiversity and important natural carbon and nitrogen reserves.

We aimed to culture the methanotroph community dwelling in the sediments of wetlands and in the rhizospheres of the plant *Typhus angustifolia.* We used an already successful enrichment and isolation strategy developed by us, which includes serial dilution enrichment, and prolonged incubation followed by isolation of methanotrophs [8]. In a Chinese wetland, it was observed that *Methylomonas* and *Methylocystis* dominated the root associated community of *Typha* species, and the active community was composed of *Methylocystis* mainly [3]. The emergent macrophytes have

been reported to consume methane far more than other emergent plants, such as leaves of woody plants, macro phytic marine algae, and seagrass [4]. The tropical wetlands showed the dominance of *Methylococcaceae*, in general (Type Ia and Type Ib together), whereas in the subtropical area, Type II methanotrophs were dominant.

This study reports the growth of Type I and Type II methanotrophs, isolated from the sediments surrounding the water and roots of *Typhus angustifolia* found growing in a small wetland patch of a hillock in Pune. Firm associations of methanotrophs to the roots of the emergent weeds have been published previously [9] and it is believed that the aerenchyma of *Typhus angustifolia* is favourable for the mitigation of methane, causing methane emissions of negligible concentrations in the vicinity; proven by Maltis-Landry et al., in 2009 when constructed wetlands planted with *T. angustifolia* emitted lesser methane as compared to the constructed wetlands planted with other emergent weeds like *Phragmites australis* and *P. arundinacea* [3]. *Typha angustifolia* L. is a perennial herbaceous plant of genus *Typha*. This cattail is an "obligate wetland" species that is commonly found in the northern hemisphere in brackish locations. The plant-root epidermis secretion of various organic and inorganic chemical compounds alters the methane oxidizing community around the roots (Chowdhury & Dick, 2013). The sediment's seasonal changes and redox potential significant impact the methanogenic growth and diversity. Similar to the phenomenon observed in rice plants, aquatic weeds have a specific life-cycle phase when the growth is exponential, and their surrounding is enriched well with methanotrophs due to the rich secretion of organic and inorganic compounds; thus, methane is efficiently transported and consumed by the methane-oxidizing bacterial community around the root tips (Chowdhury & Dick, 2013). In the wetlands, redox potential plays a crucial role in methane oxidation; methane-oxidizing bacteria grow in oxic zones of water-sediment juncture and the roots of the aquatic plants

(Chowdhury & Dick, 2013). As few reports talk about methanotrophs associated with roots of weeds in natural wetlands, the current study intended to investigate the methanotrophs associated with *T. angustifolia*. This widely popular aquatic weed mainly occurs in tropical parts of the world. It was observed that the roots of *Typhus* were typically inhabited by *Methylomonas* species in lower dilutions and by *Methylocystis* species in higher dilutions. Temperature sensitivity experiments suggested that all the methanotrophs were mostly mesophilic and grew best in the range of 25-30°C, and only *Methylocystis* grew at 37°C.

Conclusions

In this study, the methanotrophic diversity around the rhizosphere of *T. angustifolia* was investigated prevailing in a wetland patch of Pune though it is a small wetland patch, the diversity. The particular hillock is renowned as a potential niche for diverse flora and fauna, and as methanotrophs initiate ecological succession, it was interesting to observe the species abundance here. The cultivation of single methanotroph dominant cultures concluded that *Methylomagnum*, *Methylocucumis, Methylomonas,* and *Methylocystis* thrive well within the aerobic-anaerobic zone. This study contributes to understanding how sediments and plant species affect the methanotrophic community and their role in sequestering carbon and hence running the carbon-nitrogen cycle. Methanotrophs are natural methane mitigation agents. The methanotroph isolates isolated from native habitats such as rice fields [10; 11; 12; 13; 14; 15] and wetlands [11] enable us to use them as models for studying methane mitigation. The successful cultivation of methanotrophs from more and more natural habitats opens an avenue for numerous biotechnological applications [16].

Author contributions: Conceptualization: MCR and RAB; manuscript writing: MCR, SM, RAB and JAM; Experimental work: Strain isolation, characterization, maintenance, and growth experiments: JAM, SD, SP and SSM; Fund acquisition and supervision: MCR; Phylogenetic analysis and sample collection: MCR, JAM and RAB; Seen and approved: All

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Conflicts of Interest: The authors declare no conflict of interest.

TABLE No. 1: Details of enriched and isolated methanotrophs from ARAI quarry samples

Dilutions	**Visual Observation**	**Isolated methanotroph BLAST results related to the closest hit based on partial *pmoA* gene sequence with %identity**
10^{-3}	Turbidity (within 2 weeks)	*Methylomagnum ishizawai* 89.45%
10^{-4}	Turbidity (within 2 weeks)	*Methylocucumis oryzae* Sn10-6 97.93%
10^{-5}	Orange-colored flakes (within 2 weeks)	*Methylomonas koyamae Fw12-Y* 95.82%
10^{-9}	Turbidity in 4-6 weeks	*Methylocystis hirsuta* CSC1 97.06%
10^{-10}	Turbidity in 4-6 weeks	*Methylocystis hirsuta* CSC1 93.03%

Figure 11a: Stone quarry site 1b. Typha plants

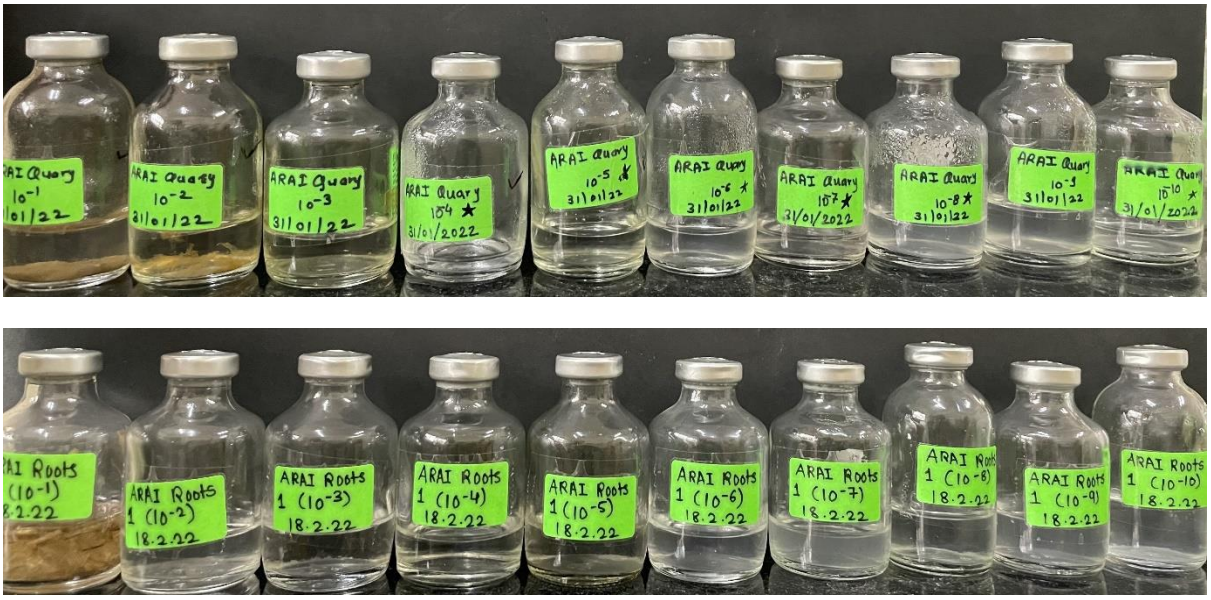


Figure No. 2: Enrichment set up of a sample in 35ml serum bottles in NMS medium collected from tropical wetland habitat

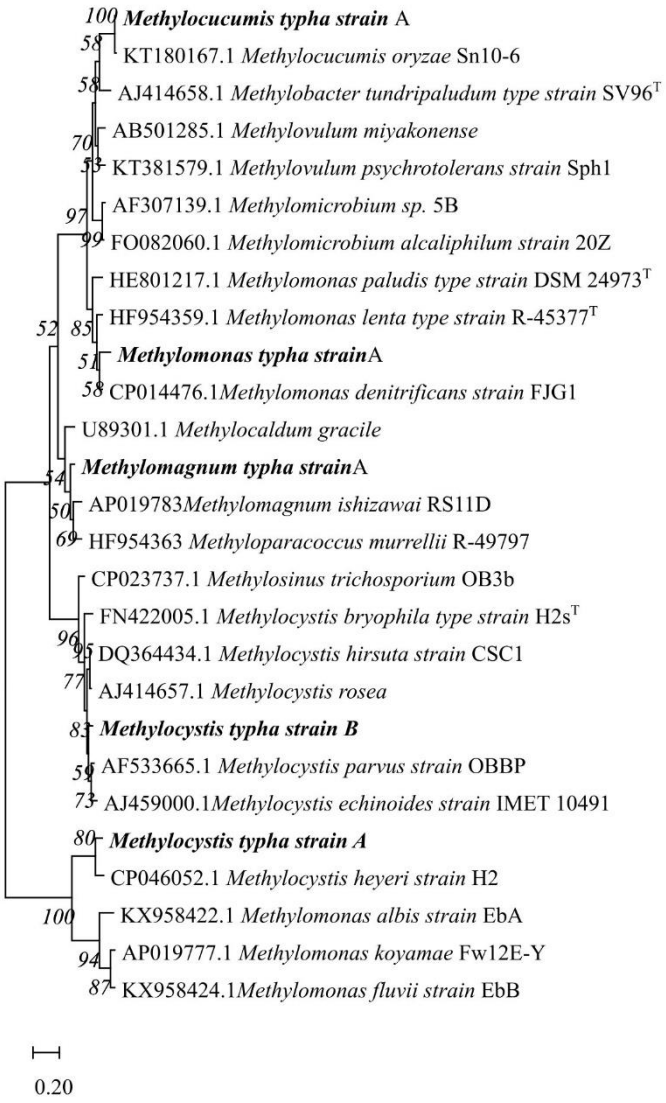
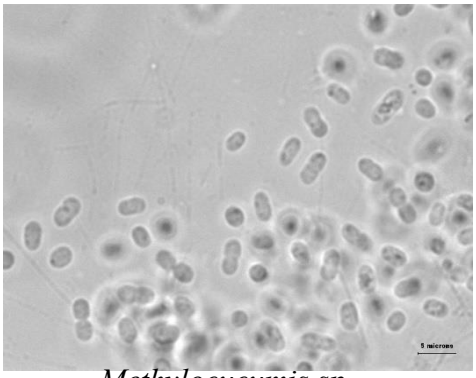
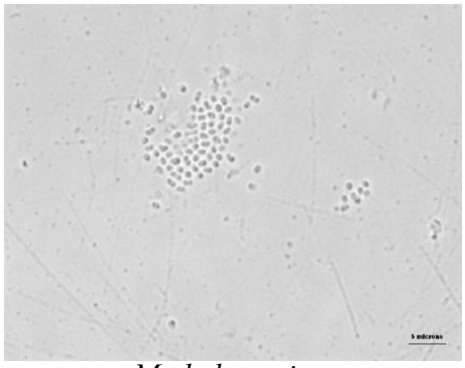


Figure no. 3: Maximum-likelihood tree of the *pmoA* gene sequence of strains associated with an aquatic *Typha* species in tropical wetland habitat compared with other methanotrophic cultured members of valid species using MEGA 11 software (Kumar *et al*., 2018). GenBank accession numbers are given in parentheses. The bar represents 20% sequence divergence.

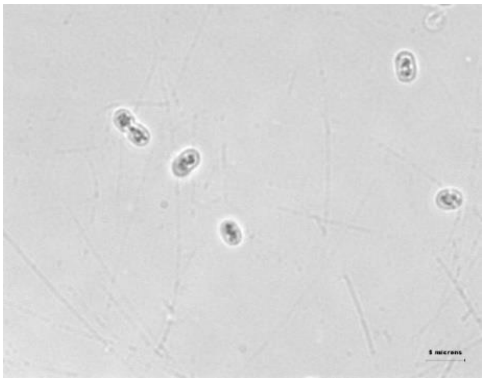
Figure no. 4: Phase Contrast micrographs of *Methylocucumis sp.*, *Methylocystis sp.*, *Methylomagnum*, and *Methylomonas sp.* using 100X Oil emersion lens



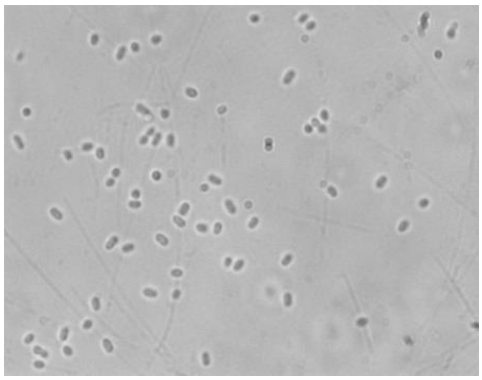
Methylocucumis sp.



Methylocystis sp.



Methylomagnum spp.



Methylomonas spp.

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