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

Jyoti A. Mohite[1,2], Shubha S. Manvi[1,2], Shrinidhi Deshpande[4], Sanjana Patange[4], Rahul A. Bahulikar[3], Kajal Pardhi[1,2] and Monali C. Rahalkar[*1,2]

[1]C2-83,84, MACS Agharkar Research Institute, G.G. Agarkar Road, Pune 411004, Maharashtra, India

[2]Savitribai Phule Pune University, Ganeshkhind Road, Pune 411007, Maharashtra, India

[3]BAIF Development Research Foundation, Central Research Station, Urulikanchan, Pune 412202

[4]Modern College, Shivajinagar, Pune

Tel: +91-20-25325119

Fax: +91-20-2651542

*Corresponding author: monalirahalkar@aripune.org

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Abstract

Freshwater wetlands are a rich habitat for aerobic or micro-aerophilic methanotrophs. In the present study, we sampled a wetland ecosystem from Western India, a stone quarry with water situated amidst a hill. The wetland is surrounded by the macrophyte *Typha* sp. (cat-tail). To study the methanotroph diversity in this ecosystem, we collected mud samples from the mini-lakes and rhizospheric soil of *Typha* by random sampling. A serial dilution enrichment in liquid followed by isolation on agarose plates in a methane-air atmosphere was used. The high abundance of methanotrophs was indicated by the fact that growth was obtained till dilutions: 10^{-9} or 10^{-10}. In *Typha* root-associated soil, Type I methanotrophs: *Methylomonas koyamae* and uncultured *Methylococcus* were isolated from the lower dilutions: 10^{-3}-10^{-6}. Type II methanotrophs *Methylocystis sp.* and *Methylosinus* spp were isolated from the higher dilutions. The *Methylococcus* strain isolated was about 97.4% (16S rRNA gene similarity) related to *Methylococcus capsulatus* and *Methylococcus geothermalis* and was predicted to represent a putative novel species. Three more methanotrophs, non-axenic, related to *Methylomagnum-Methylocaldum spp*, *Methylocucumis oryzae*, and *Methylocystis* spp, were cultured. Their affiliation was predicted based on the *pmoA* gene sequence. The mud samples showed relatively less diversity and mainly yielded two *Methylomonas* strains related to *M. koyamae*.

Keywords: Wetlands; methanotroph; *Methylocystis* sp., *Methylomonas* sp.*; Methylococcus*; *Methylomagnum* sp., *Methylocucumis* sp.,; *pmoA* gene, *Typhus spp*

While various natural and anthropogenic activities emit methane, wetlands account for massive emitters and absorbers of methane gas [1]. 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 [1], thus simultaneously establishing an equilibrium between methanogenesis and methanotrophy. [1]. Methanotrophs are aerobic to micro-aerophilic bacteria that use methane as the sole carbon source for growth and energy [2]. 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 [3]. Methane-oxidizing bacteria in the oxic layer or the soil's oxic-anoxic interphase carry out methane oxidation. Arctic, Antarctic, temperate wetlands, and lakes have been studied in detail [4-7], but tropical wetlands have been less studied for their methanotroph ecology.

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 (known as ARAI hills), one of the only natural green habitats in the city. Due to the proximity of the institute ARAI (Automotive research association of India), the hill is popularly known as ARAI hill and is renowned as a potential niche for diverse flora and fauna [8] . The water-filled quarry contains 5-6 mini-lakes (Figure 1) and is rich in vegetation with aquatic weeds (*Typha* spp) and small water plants. One such aquatic weed is *Typha* spp, dominant in the west to the southwestern region of India. 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 [9]. Two earlier reports indicated that *Typha* species inhabit methanotrophs in their roots and rhizospheres [1,

10]. Almost no data is available on the methanotrophs dwelling in Indian freshwater wetlands apart from our study where a novel genus and species from Type Ib methanotrophs was cultured from a brackish water wetland near a beach, *Methylolobus aquaticus* [11] and *Ca.* 'Methylobacter oryzae' from a rice field near Allepey wetland in Kerala [12]. The present study is one of the first attempts to document the methanotroph diversity in another freshwater ecosystem using a cultivation method proven to culture a large diverse population [13].

Mud samples and soil adhered to *Typha* root samples were collected from the wetland patch in Pune, India 18°32´ N, 73° 48´E (Figure 1) on 30 January 2022 (one root soil sample) and 16 February 2022 (three rhizosphere samples and three mud samples), Figure 1. All the samples were collected using gloves in sterile plastic vials or sterile plastic bags and proceeded for enrichment and oxidation of methane. Roots of *Typha* with the attached soil were collected by uprooting the plant. The roots were briefly washed in distilled water, and only the adhered soil with cut roots was used as a sample. Mud samples were collected from three different mini-lakes present at this site using sampling vials (50 ml capacity) from the sides of the lakes with about 10-15 cm water layer on the top.

Initially, we saw growth of methanotrophs in a serial dilution series set up with a single root sample of a *Typha* collected on 30 January 2022, and growth was seen up to 10-6 dilutions within two weeks of incubation. Thus, this site was found to be promising for the enrichment and isolation of methanotrophs. Therefore, the second sampling was conducted on 16 February 2022 to study the overall methanotroph diversity in this area. Serial dilutions were set up using modified Nitrate Mineral Salts referred to as dilute NMS medium was prepared [14] 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 [14]. A headspace volume of 20% was removed with a sterile syringe and filled with 20% methane. All the further procedures were done as described in our method article [13], except we directly proceeded for isolation after the first serial dilution enrichment step, as

relatively high number of methanotroph like cells were seen. In all the samples collected from the quarry, growth was obtained till 10^{-9} or 10^{-10} dilutions in all the samples (Figure 2), indicating the high abundance of methanotrophs (~10^{-9}-10^{-10} cells/ g fresh weight of methanotrophs). The presence of abundant methanotrophs in our study site indicates that an active methane cycle is present here. Microscopically, methanotrophs were either in the form of large cells, oval or slightly elongated with dark interiors were seen in higher dilutions (indicative of Type I methanotrophs) or small coccoid cells (indicative of Type II methanotrophs) were observed in higher enrichment dilutions of 10^{-9} and 10^{-10} (Figure 2). Five pure cultures of methanotrophs named as strains AQ1-AQ5 were obtained in all from various dilutions (Figure 2, Table 1). DNA extracted from these pure cultures was subjected to 16S rRNA gene and *pmoA* gene sequencing. Three more cultures were obtained, showing a minor amount of small-sized bacteria which could be heterotrophs associated with the methanotrophs. A single morphotype with large cells of > 5µm length and oval shape indicative of Type I methanotrophs was seen in these cultures, indicative of *Methylomagnum* and *Methylocucumis*. We characterized the methanotroph present in these cultures (culture AQ6 and AQ7) using DNA extraction followed by *pmoA* PCR and sequencing and confirmed the predicted results obtained by morphological examination (Table 2). Both *Methylomagnum* [15]and *Methylocucumis* [16, 17] are known to be methanotrophs with large cells (~5µm) with dark inclusions, and hence characteristic in morphology. The third culture (AQ8) showed small coccoid cells was indicative of Type II methanotrophs, identified as *Methylocystis*.

Thus, from *Typha* root-associated soil, Type I methanotrophs from the genera: *Methylomonas*, *Methylococcus*, *Methylomagnum* and *Methylocucumis* were isolated/ cultured from the lower dilutions: 10^{-3}-10^{-6}. Type II methanotrophs *Methylocystis*, *Methylosinus* spp were isolated/cultured from the higher dilutions (10^{-9}), Table 1. The mud samples yielded two *Methylomonas* strains related to *M. koyamae*, Table 1. The *Methylococcus* strain isolated from

10^{-6} dilution was 97.4% related to *Methylococcus capsulatus and Methylococcus geothermalis*, on the basis of 16S rRNA gene and the partial *pmoA* gene was 95% related to that of *Methylococcus geothermalis* and the culture could represent a putative novel species. We found that small coccoid cells representing Type II methanotrophs of the Methylocystis-genus producing even milky, white turbidity were seen in all of the higher dilutions higher dilutions-10^{-8},10^{-9}-10^{-10} in the root-associated methanotroph community. This was confirmed by further isolating *Methylocystis* from 10^{-9} dilution. On the contrary, Type I methanotrophs representing *Methylomonas, Methylocaldum-Methylomagnum and Methylocucumis* were present in lower dilutions, strengthening the observations made earlier in a Chinese wetland study [1]. 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 [1]. *Typha* is a popular aquatic weed primarily occurs in tropical parts of the world. Strong associations of methanotrophs to the roots of emergent weeds have been published previously [10]. Further studies focusing on the methanotrophs present associated with *Typha* species in wetlands of various kinds would be more useful. The isolation of *Methylococcus* spp related to known species on a 97.4% similarity level indicates that this habitat may have yet un-cultured methanotrophs and could be a rich source for further isolations. Though *Methylococcus capsulatus* is the most studied methanotroph, *Methylococcus* genus has only two valid species, the classical methanotroph *Methylococcus capsulatus* [18] and the recently described *Methylococcus geothermalis* [19], and hence this niche could be an exciting habitat for the isolation of new species.

In Pune, temperatures can rise up to 42-43°C in summer, and the temperature of the water can increase in summer to a considerable extent. In summers, the wetland part also gets dried up, and hence we found that the roots were dominated by the presence of heat and desiccation-resistant methanotroph species: *Methylocystis*, *Methylosinus*, and *Methylococcus* [18, 19].

Annual monitoring of this habitat would give us a better picture of temporal diversity of methanotrophs.

The environmental relevance of methanotrophs as well as their potential applications in methane mitigation, are both equally important, and hence cultivation of methanotrophs becomes important to get deeper insight [20]. In the present study, we were successful in culturing important members of methanotrophs from a crucial ecological niche, a freshwater wetland, and methanotrophs associated with *Typha* spp. This study would lay a foundation for further studies focusing on methane mitigation from such sites.

AUTHOR CONTRIBUTIONS: MR designed the experiments, collected the samples, guided all the experiments, and wrote the manuscript. RB, JM, SM, SD, and SP did the sampling and performed the enrichment and isolation of the methanotrophs. JM performed the phylogenetic analysis and prepared the trees. KP assisted in the isolation and maintenance of cultures. RB and MR found this unique sampling site, did the initial sampling, and co-designed the experiments. RB, JM, and SM assisted in writing the manuscript. All the authors reviewed the manuscript and approved the final version.

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FIGURES:

Figure no. 1: Site of sample collection: 18°32´ N, 73° 48´ ARAI quarry, Pune a, b and c. *Typha* plants

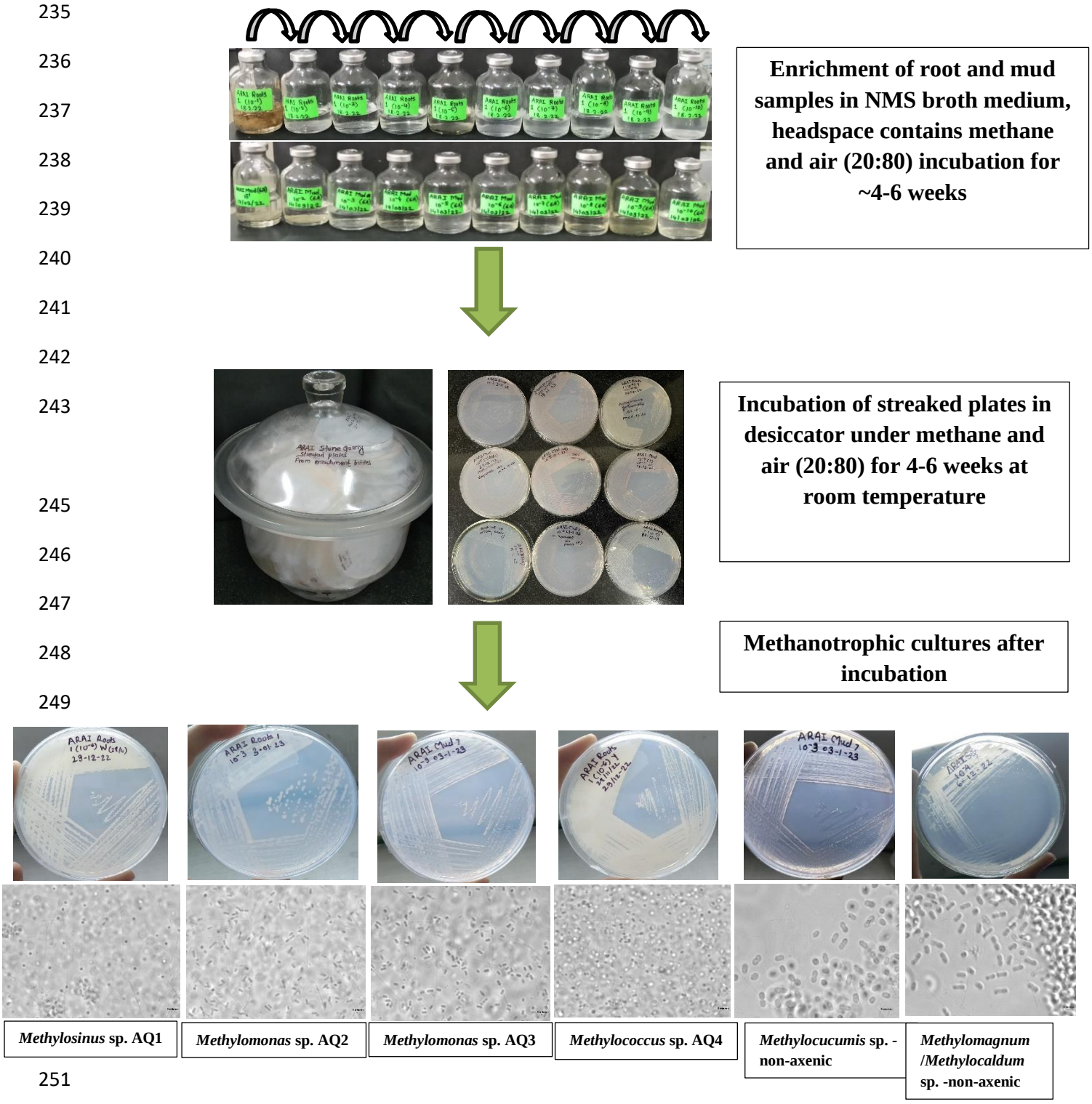

Figure No. 2: Enrichment, isolation, and identification of methanotrophs from ARAI stone quarry *Typha* plant root and mud samples

TABLE:

Table no. 1: Summary of type I and type II methanotrophs obtained in this study.

Sr No.	**Name of the methanotroph at genus level & NCBI accession number**	**Sample name & positive dilutions Sampling date: 30.01.2022 (1) Sampling date: 16.02.2022 (2)**	**Universal 16s rRNA gene sequence BLAST hit name, percent similarity and NCBI accession number**	***pmoA* gene sequence BLAST hit name, percent similarity and NCBI accession number**
1.	*Methylosinus* sp. AQ1 16s (OQ373008)	ARAI root (1) sample dilution 10^{-6} (2)	*Methylosinus sporium* (NR026512.1) 98.99%	*Methylosinus trichosporium* (CP023737.1) 94.55%
2.	*Methylmonas* sp. AQ2 16s (OQ373314)	ARAI root (1) sample dilution 10^{-3} (2)	*Methylomonas koyamae* (AP019777.1) 99.27%	*Methylomonas koyamae* (AP019777.1) 96.06%
3.	*Methylomonas* sp. AQ3 16s (OQ373316)	ARAI mud (7) sample dilution10^{-5} (2)	*Methylomonas koyamae* (AP019777.1) 99.27%	*Methylomonas koyamae* (AP019777.1) 96.29%
4.	*Methylococcus* sp. AQ4 16s (OQ373317)	ARAI root (1) sample dilution 10^{-6} (2)	*Methylococcus capsulatus* (NR042183.1) 97.47%	*Methylococcus geothermalis* (CP046565.1) 94.58%
5.	*Methylomonas* sp. AQ5 16s (OQ373316)	ARAI mud (7) sample dilution 10^{-9} (2)	*Methylomonas koyamae* (AP019777.1) 99.27%	*Methylomonas koyamae* (AP019777.1) 96.29%
6.	*Methylocucumis* sp. AQ6 *pmoA* (OQ354217)	ARAI root sample dilution 10^{-4} (1)	Non-axenic culture	*Methylocucumis oryzae* Sn10-6 (KT180167.1) 97.93%
7.	*Methylomagnum* sp. AQ7 *pmoA* (OQ354221)	ARAI water sample dilution 10^{-3} (1)	Non-axenic culture	*Methylomagnum ishizawai* (AP019783.1) 89.45%
8.	*Methylocystis* sp. AQ8 *pmoA* (OQ354219)	ARAI root sample dilution 10^{-9} (1)	Non-axenic culture	*Methylocystis hirsuta* CSC1 (DQ364434.1) 97.07%