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Marine and estuarine methylotrophs: their abundance, activity and identity

Daphne Faria and P. A. Loka Bharathi*

Biological Oceanography Division, National Institute of Oceanography, Dona Paula, Goa 403 004, India

Methanotrophs were up to 1 and 0.65% of the total counts in estuarine waters and offshore sediments respectively. Experimental tests on methanol utilization showed that the estuarine isolates grew best at 4% methanol whereas offshore ones grew at 5% at an optimum pH of 6 or 7. Methanol, when used as an additional carbon source, in the presence of nutrient broth concentration ranging from 0.08 to 0.4%, enhanced growth by 129% and respiration by 177% in estuarine isolates. Biochemical and physiological characteristics showed that estuarine methylotrophs exhibited taxonomic affinities to *Pseudomonas* I or II sp. The offshore genera were more varied and belonged to *Flavobacterium* and *Pseudomonas* I or II sp. The abundance, activity and identity suggest that these physiological groups could be widespread and therefore could perhaps contribute significantly to the changes in C_1 compounds and even their derivatives in marine and estuarine environments.

Keywords: Adaptation, estuarine, methylotrophs, marine, methanol.

METHYLOTROPHIC bacteria (MTB) are obligate aerobic microorganisms recognized by their ability to grow on carbon compounds more reduced than CO_2, without any C–C bonds. They are even able to assimilate compounds such as HCHO or a mixture of HCHO and CO_2. MTB capable of oxidizing methane are methanotrophs (MOB). They play an important role in the geochemical cycling of methane and its derivatives. The oxidation of methane can have major implications on the structure of food webs and climate, especially in the current global scenario. Hence, a study on their ecology would be pertinent to understand the dynamics of methane and methane-derived compounds, especially in marine and estuarine systems.

Though much work has been carried out on the molecular[1–3] and taxonomic aspects[4] of methanotrophs, the study is either restricted to lacustrine environment[5,6] or terrestrial regions[7–9]. Work on the marine environment is limited[10]. Hence, the present study assesses the retrievable abundance and distribution of methylotrophs and methanotrophs. It also examines the activity of methylotrophs from estuarine beach and offshore regions.

Sampling was carried out during low tide in September, representing the end of the southwest monsoon season at Dona Paula beach (15°27′N, 73°48′E), a sheltered

*For correspondence. (e-mail: loka@nio.org)

beach in North Goa situated at the confluence of the Mandovi and Zuari estuaries. Three adjacent sediment cores were collected using hand-corers. The cores were sectioned into 0–5, 5–10 and 10–13 cm. Water and core samples were transported to the laboratory in iceboxes and analysed within 2–3 h of collection.

Water samples were collected into sterile polypropylene bottles from which 5 ml was transferred into vials and fixed with 250 µl buffered formalin. Total bacterial populations were estimated using acridine orange direct counts (AODC) method[11]. For sediment samples, approximately 1 g was transferred to a 15 ml centrifuge tube filled with 9 ml autoclaved filtered sea water, vortexed for 5 min and allowed to settle for 1 min. Five millilitre of supernatant was transferred into vials and fixed with 250 µl buffered formalin. One millilitre of the fixed sample was mixed with acridine orange (final concentration 0.01% w/v) for 5 min. The contents were then filtered onto 0.22 µm pore-size black-stained Nuclepore filter paper. Bacterial counts were made at 100X with an epifluoresence microscope (BH) using 515-barrier filter.

Isolation of methylotrophs and methanotrophs was carried out onto ATCC #1306 medium[12]. The medium contained [g l^{-1} sea water (50%) at pH 6.8]: $MgSO_4.7H_2O$, 1.0; $CaCl_2.6H_2O$, 0.2; $FeNH_4EDTA$, 0.004; KNO_3, 1.0; KH_2PO_4, 0.272; $Na_2HPO_4.12\ H_2O$, 0.717 and 0.5 ml trace element solution. Plates were solidified with 1.8% of purified bacto agar. Approximately 5 g of sediment sample was suspended in a conical flask containing 45 ml of sterile sea water, vigorously shaken for 1 min and diluted up to 10^6. Suitable aliquots (50–100 µl) from 10^2 to10^6 dilutions were surface-plated onto ATCC #1306 medium. The plates were incubated with methane in Gas Pak jars in the dark for 3–4 weeks and checked for bacterial growth against a control. For the isolation of methylotrophs the procedure was the same, except that methanol was supplied in the vapour phase[13] from a petri plate placed at the bottom of Gas Pak jar. Growth was recorded for 7 to 10 days period against a control. The counts were expressed as CFU g^{-1} dry sediment or CFU ml^{-1}. Plates were divided into sectors and a sector was randomly chosen and all CFU were isolated, checked for purity and used for experiments and characterization. Colonies of similar morphotypes were isolated (in replicates of 4 to 5). Thus six methylotrophic isolates each ES6, ES7, ES31, OF401, OF504 and OF507 from estuarine and offshore regions represent about 30 original isolates which were identified according to Gerhardt[14].

Adaptation to aqueous methanol was achieved by sequential transfer of cultures into media with progressively higher concentration of methanol (0.1, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0% v/v). Subsequently, experiments were carried out with 4% methanol for estuarine and 5% for offshore isolates. Experiments were carried out to measure growth and respiration under varying methanol, organic carbon concentrations, and pH. Growth was estimated at definite intervals by direct cell counts on a haemocytometer. Simultaneously, respiration was monitored in terms of formazan production from TTC[15,16]. Cultures were incubated at (30 ± 2°C) for a period of 8 days.

To test the effect of organic carbon on growth and respiration, isolates were grown in mineral media containing methanol supplemented with various concentrations (0.08, 0.16 and 0.4%) of nutrient broth.

To test the effect of pH on growth and respiration, isolates were grown in mineral media containing methanol adjusted to various pH values, viz. 6–9. Control tubes without methanol were included.

Microbial Adhesion to Hydrocarbons (MATH) Assay was carried out to assess the ability of the isolates to utilize hydrocarbons, as outlined by Rossenberg[17]. The assay was carried out as follows: To a thick suspension of a day old culture, 4 ml of phosphate buffer (pH 8) was added and the same was vortexed and its initial OD was adjusted to 0.2 at 550 nm. To this, 0.5 ml of *n*-undecane was added and vortexed for 2 min. This was allowed to stand at room temperature for 20 min for phase separation. Turbidity of the lower aqueous phase was measured again and fraction of adherence calculated.

Fraction of cell adherence is defined as the ratio of the difference between the initial and final turbidity over the initial value.

$$\text{Fraction of adherence} = (A - C)/A,$$

where A is the initial turbidity and C the final turbidity.

Both water and sediment from the estuarine and offshore regions were tested for the presence of methylotrophs and methanotrophs. Culturable methanotrophs ranged between 0.13 and 6.12×10^3 CFU g^{-1} estuarine sediment and between 0.17 and 0.85×10^3 CFU g^{-1} offshore sediment. However, methanotrophs from water were two orders more at 807.0×10^3 CFU ml^{-1} in the estuary than in the sea, where they ranged between 10^1 and 10^3 ml^{-1}. However, Faria *et al.*[18] reported that the MOB could range higher from 10^4 to 10^6 g^{-1} much later in the year (post-monsoon) in offshore sediments of the Arabian Sea and these were over three orders higher than the present values. Yet another comparison showed that abundance of methanotrophs in this study is one order less than the values reported by Takeuchi *et al.*[19] from sediment using MPN technique. The difference in abundance could be partly due to differences in ecosystems and also differences in the techniques employed. Like methanotrophs, methylotrophs retrieved on methanol-containing media varied from 0.63 to 66.9×10^3 CFU g^{-1} estuarine sediment, but were below detection limits offshore. Methylotrophic abundance was higher in the estuarine waters at 49.60×10^3 CFU ml^{-1} than offshore (Table 1). Ross *et al.*[20] reported higher values for methylotrophic bacterioplankton that varied between 0.6 and 1.2×10^6 ml^{-1} in the winter of 1994, and 0.8 and 5.5×10^6 ml^{-1} in the summer of 1994–95 in the floodplain lake in northeastern Victoria, Australia

Table 1. Comparison of abundance of methylotrophic and methanotrophic bacteria with other studies

Source	Number of MTB	Number of MOB	Method	Reference
Marine sediment (g^{-1})				
Arabian Sea sediment–January to February 2002	BDL	$0.17–0.85 \times 10^3$	Surface plating	Present study
Arabian Sea sediment–November 2002	ND	$10^4–10^6$		Faria *et al.*[18]
Water (ml^{-1})	$0.08–4.00 \times 10^3$	$0.08–7.3 \times 10^3$		Present study
Estuarine sediment	$0.63–66.9 \times 10^3$	$0.13–6.12 \times 10^3$		
Water	49.60×10^3	807.00×10^3		
Others				
Aquifer sediment	–	10^4	MPN	Takeuchi *et al.*[20]
Floodplain lake water				
1994 Winter	$0.6–1.2 \times 10^6$	–	16S rRNA probes	Ross *et al.*[21]
1994–95 summer	$0.8–5.5 \times 10^6$			

BDL, Below detection limit; ND, Not done; MTB, Methylotrophic bacteria; MOB, Methane-oxidizing bacteria; MPN, Most probable number technique.

Table 2. Total counts of bacteria from estuarine and offshore waters

	Estuarine		Offshore	
Total counts	Water	Sediment	Water	Sediment
(ml^{-1}/g^{-1})	$\times 10^7$	$\times 10^5$	$\times 10^7$	$\times 10^8$
	1.8–1.9	2.4–1.3	1.15–8.19	0.09–1.18

(Table 1). Thus, our study shows that methylotrophs are two orders less than the estimates made by Ross *et al.*[20] using 16S rRNA probes. Irrespective of the region, water recorded higher abundance than sediment in the present study. The present study shows that the methylotrophs and methanotrophs were generally more abundant in estuaries with water showing a higher concentration than sediment, suggesting that the substrates or substrate producers for these organisms could be more abundant in estuarine waters. Particles in estuarine waters could harbour higher number of fermentative and methanogenic bacteria which could provide these substrates.

The total bacterial counts in water were in the order of $10^7\ ml^{-1}$. However, in the estuarine beach and offshore sediments, they were in the order of 10^5 and $10^8\ g^{-1}$ respectively (Table 2). Takeuchi *et al.*[19] reported total counts in the order of $10^4\ g^{-1}$ dry sediment, which is lower than that observed in the present study. Methanotrophs formed up to 1% of total counts in estuarine water and 0.005% in sediment. The contribution of these forms amounted to 0.65 and 0.03% of the total counts in offshore sediments and water respectively. These values are congruent with those reported by other authors. Gilbert and Frenzel[21] reported that MOB estimated in a planted paddy soil accounted for 1% of total counts (direct counts of total bacteria vs MPN of MOB). MOB are also known to account for the same number as aerobic heterotrophic bacteria[22]. However, Vecherskaya *et al.*[23] reported that MOB estimated by immunofluorescence formed 1–23% of total counts in peat soils from Siberian tundra.

Takeuchi *et al.*[19] reported high percentages of 15.71, 4.78 and 5.08 for three aquifers from TCE-contaminated site in Chikura, Chiba, Japan. The higher percentages of MOB reported by these authors could perhaps be attributed not only to the different ecosystems, but also to the lower total bacterial counts obtained from that system. MPN methods are known to yield higher numbers. Dubey *et al.*[24] reported that most quantification of MOB population size relies on MPN methods. Frenzel[22] highlights some of the limitations of this method. These include microcolonies that could be counted instead of single cells, the medium could be selective for certain strains, and cells may be in an unculturable state.

Despite the widely reported toxicity of methanol to obligate methylotrophs[25–27], growth on methanol at high concentrations is clearly possible for *Methylocystis parvus* OBBP up to 4% w/v[28], *Methylococcus* NCIB 11083 up to 0.2%, v/v[28] and *Methylosinus trichosporium* OB3b up to 4% v/v[29]. In the present study, some of the estuarine isolates grew and respired at 4 or 5% v/v concentration of methanol. The mechanism by which these organisms adapt to growth on methanol at high concentrations is unknown, but may reflect physiological adaptations of the population to the substrate or the selection of a mutant to either methanol or formaldehyde[29]. Loss of this ability of methanol-grown organisms on methane or substrate analogues has been reported for bacterium B6 (ref. 30) and for three other methylotrophs[31]. In contrast, other workers have demonstrated the retention of methane-oxidizing activity in type I methylotrophs[25,28]. Results of Best and

Higgins[13] were in contrast with those of Hou *et al.*[31] for the same strain of *M. trichosporium*. In the present study the six isolates also displayed retention of such methanol-oxidizing activity, suggesting that the enzyme is either constitutive or induced by methanol.

Many methylotrophs are facultative or restricted facultative, that grow on sugars, fatty acids, amino acids, inorganic substrates, and complex media as well as one-carbon compounds. However, in the present study, methylotrophs could utilize either methane or methanol effectively for their growth in the presence of low concentration of nutrient broth (0.1%). It has been reported that some cultures utilizing methanol grow better on heterotrophic substrates[30,32], and it is suggested that methanotrophs with heterotrophic tendencies would be more widespread than strict autotrophic ones. According to Griffiths *et al.*[33], there is extensive documentation that methane-utilizing bacteria can utilize a large number of organic compounds that include not only simple hydrocarbons, but also more complex organic molecules[33–36].

Experiments with isolates showed that maximum growth and respiration were recorded after a period of 8 days for all methylotrophs with the exception of OF507, which showed maximum growth and respiration after 6 days. With increasing organic carbon from 0.08 to 0.4%, enhanced growth (129%) and respiration (177%) were recorded for estuarine isolates. However, there was a 17% decrease in growth and 396% increase in respiration for their marine counterparts.

The optimum pH recorded for the estuarine and offshore isolates was 6 or 7. On an average, growth of offshore isolates was 199% of the control at pH 6 or 7, and was better than its estuarine counterparts, which was only 166%. Respiration was stimulated by 80 and 70% of the control for offshore and estuarine methylotrophs respectively. At 4 and 5% methanol concentration for estuarine and offshore respectively, their ability to grow at different pH values, and organic carbon concentration (0.08–0.4%) showed that these isolates have an optimal pH at 6 or 7. Borne *et al.*[37] reported methane oxidation from pH 3.5 to 8.0. However, Dedysh *et al.*[38] reported isolation of methanotrophs from *Sphagnum* peat bogs incapable of growth at pH values below 5.0. Calhoun and King[39] report isolation of methanotrophs from freshwater macrophytes at pH values between 6 and 7. Irrespective of the region, isolates could grow in a broad range of pH values with an optimum at 6 or 7 in this study.

Molar growth yield on methanol was 2×10^{10} cells ($\pm$ 3.4) and 4×10^{10} cells ($\pm$ 1.2) for offshore and estuarine methylotrophs respectively. The molar growth yield constant on methanol for offshore methylotrophs was 0.02 and for estuarine methylotrophs 0.04. Offshore forms are lower in yield, but higher in activity (Tables 3 and 4). Faria *et al.*[18] also reported that offshore MOB are significantly higher in abundance than near-shore ones ($P > 0.001$). Thus it is suggested that offshore MOB are not only more abundant in certain seasons, but also could be higher in activity.

MATH assay, a cell-surface hydrophobicity test indicates that growth of four of the six methylotrophs under the conditions described displayed a hydrophobic character, as evidenced by their association with the immiscible *n*-undecane phase (Table 4). The fraction of adherence for culture OF504 was high, due to its hydrocarbonoclastic property. Raiker *et al.*[40] observed the fraction of adherence for thraustochytrids to be high and ranging between 0.2 and 0.42. The fraction of adherence was however less for OF507 (0.0098), ES7 (0.178) and closer to that of OF401 (0.35).

The high fraction of adherence for OF401 (0.445) could be due to its hydrocarbonoclastic property. De Souza *et al.*[41] reported a high fraction of adherence (0.4–0.5) for culture P43, a phosphate-solubilizing bacteria. In the present study cultures ES6 and ES31 were hydrophilic, exhibiting an adherence of -0.23 and -0.055 respectively. The hydrophilic nature apparently reflects the presence of vegetative cells[42] and the probable presence of poly β hydroxybutyrate granules[43]. Hydrophobic property exhibited by methylotrophic bacteria makes them ideal candidates for the bio-remediation of hazardous environmental wastes like tricholoroethylene[44] and other xenobiotics.

Phenotypic characterization based on key biochemical (catalase, oxidase and modified oxidative fermentative tests, ability to use methane, methanol and *n*-undecane) and morphological traits (colony morphology, Gram stain, motility, size, etc.) showed that 33% of the offshore isolates and all the estuarine methylotrophs showed taxonomic affinities to *Pseudomonas* I and II sp. The offshore genera were more varied and belonged to *Flavobacterium*

Table 3. Molar growth yield constant (k) of estuarine and offshore MTB

	Methanol		
	4%	5%	
	Estuarine methylotrophs	Offshore methylotrophs	
	Ps. sp (n = 3)	Ps. sp (n = 2)	Fl sp (n = 1)
	0.041	0.024	0.018
Avg.	0.04	0.02	

Table 4. MATH assay

Culture	Initial turbidity (A)	Final turbidity (C)	Fraction of adherence (A–C)
ES6	0.200	0.246	−0.230
ES7	0.200	0.170	0.178
ES31	0.206	0.211	−0.055
OF401	0.203	0.133	0.350
OF504	0.200	0.111	0.445
OF507	0.203	0.201	0.0098

and *Pseudomonas* I and II sp. As these taxonomical groups are widespread in the marine environment and as the abundance of methylotrophs was as high as 1%, it is speculated that they could contribute significantly to the oxidation of methanol and its derivatives in nature. These groups could perhaps also participate in the oxidation of methane in the marine and estuarine environments. Experiments are underway to examine their methane-oxidizing activity in estuarine and marine sediments under simulated *in situ* conditions.

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Historic submergence and tsunami destruction of Nancowrie, Kamorta, Katchall and Trinket Islands of Nicobar district: Consequences of 26 December 2004 Sumatra–Andaman earthquake

M. G. Thakkar* and Bhanu Goyal

Department of Geology, R. R. Lalan College, Bhuj, Kachchh 370 001, India

The 26 December 2004 Sumatra–Andaman earthquake is one of the largest plate-boundary earthquakes in the recent seismic history of the world. This earthquake has also generated the greatest tsunami run-up and coastal devastation ever recorded. Our field study at four major islands of Nancowrie group of Nicobar District in Andaman and Nicobar archipelago has revealed that the islands are vertically subsided by 1.0–1.75 m with the submergence of coastal land area by thousands of square kilometers. It is also suggested based on our field observations that societal and socio-economic rejuvenation of the islands will need resurvey of entire topography of the islands, coastal bathymetry mapping and identification of newly developed ecological regimes. We have also prepared maps of the coastal submergence for these islands using field observations and remote sensing in this paper. Based on the present field study and geodetic studies by other workers, differential tilting of the Andaman micro-plate is also inferred.

Keywords: Coastal submergence, Nancowire group, Nicobar Islands, tsumani.

THE Sumatra–Andaman earthquake of 26 December 2004 occurred on 6:29 IST (0.58 UTC) at the subduction plate boundary where the Indian and Australian plates converge and plunge below the Sunda plate. The *Mw* 9.3 (revised magnitude) plate boundary earthquake is located at 3.7°N and 95°E off the Sumatra coast near the island of Simuelue with a focal depth[1] of ~15 km. The earthquake is considered as the second largest ever recorded on the globe, and it caused wobbling of the earth's axis[2]. Distribution of aftershocks reveals that the rupture plane is about 1200 km long extending to the north, up to the Andaman and Nicobar Islands[3,4]. Immediate observation by the satellite imageries confirms large-scale subsidence around the epicentral zone and many kilometers north of it, in the Andaman and Nicobar Islands. The present study on preliminary documentation of ground deformation and tsunami effects on the Nancowrie group of islands of Nicobar district, was carried out based on satellite imageries. These imageries provide information on inundation of the islands and site-specific details of subsidence and tsunami run-ups at each location. An attempt is also made to prepare preliminary maps that show the coastal area of subsidence on four islands – Nancowrie, Kamorta, Katchall and Trinket of the Nancowrie group. These inundation maps could be used in future planning of developmental activities in these islands. The coastal villages mentioned later and farm fields on all four islands can be identified as areas likely to be submerged in the future. A similar study on the other islands of Andaman and Nicobar groups could reveal the tectonic behaviour of the Andaman micro-plate.

The Andaman and Nicobar groups of 349 islands, situated in the Bay of Bengal, are separated by the ten-degree channel (Figure 1). The rocks of these islands are believed to have been formed from the sediments scraped off the descending Indian plate interleaved with ophiolites from the ocean floor beneath the Bengal Fan. Detailed geology of the Andamans has been described by Oldham[5] and Tipper[6]. The earliest rocks found in Andamans are Upper Cretaceous clastics with ultramafic and mafic intrusives. A complete succession of Tertiary rocks is found in the Andaman group of islands. Pleistocene sand beds,

*For correspondence. (e-mail: mgthakkar@rediffmail.com)