Water Practice & Technology

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Water Practice & Technology Vol 17 No 1, 329 doi: 10.2166/wpt.2021.101

Detection of methanotrophic microorganisms in sludge and sediment samples from sewage treatment systems

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ABSTRACT

This study investigated various groups of methanotrophic microorganisms, including the anaerobic methane-oxidizing archaea (ANME) group, aerobic methanotrophic bacteria, and bacteria of the phylum NC10 (*Candidatus Methylomirabilis oxyfera*). The polymerase chain reaction (PCR) technique was employed with specific primers for the *pmoA* gene from the aerobic methanotrophic and *M. Oxyfera* methanotrophic denitrifying (NC10 phylum) and for the anaerobic methanotrophic archaea 16S rRNA gene (ANME-1 and ANME-2). Sludge and sediment samples were analyzed from different sewage treatment systems installed at the Sanitation Research and Training Center (CePTS) at the Arrudas wastewater treatment plant (Arrudas WWTP), in Belo Horizonte, Brazil. ANME was shown to be present in sludges from the upflow anaerobic sludge blanket (UASB) reactor and activated sludge system, and in sediments from the polishing pond and wetlands. On the other hand, aerobic methanotrophic bacteria were detected only in the polishing pond samples, while no methanotrophic denitrifiers of the *M. oxyfera* genre were detected at all. Besides investigating and detecting different groups of methanotrophs in the treatment systems, the study provided support for another in which samples from the systems analyzed were selected for use as inoculum for methane anaerobic oxidation potential experiments and methanotrophic microorganism enrichment.

Key words: ANME, methane balance, methanotrophs, PCR, *pmoA* gene, wastewater treatment

HIGHLIGHTS

- Methanotrophs are essential to control methane fluxes in sewage treatment systems.
- The PCR technique was employed with specific primers to identify aerobic and anaerobic methanotrophs.
- Methane-oxidizing archaea were detected in all analyzed samples, whereas aerobic methanotrophs were found only in polishing pond sediments.
- Methanotrophic denitrifying microorganisms were not detected in any sample.

INTRODUCTION

Methane biological oxidation is a beneficial process that may control methane fluxes in the environment. The methane flux results from the balance between methane production by methanogenic archaea, and methane oxidation by bacteria (aerobic and/or anaerobic) and methanotrophic anaerobic archaea (Chowdhury & Dick 2013).

Several groups of bacteria widely distributed in the environment mediate aerobic methane oxidation, such as bacteria of the α- and γ-*Proteobacteria* classes (Knief 2015) and phylum *Verrucomicrobia* (Sharp *et al.* 2014; Carere *et al.* 2017). The first step in aerobic methane oxidation is the conversion of methane to methanol, catalyzed by the enzyme methane monooxygenase (MMO), which can be present in soluble (sMMO) or particulate (pMMO) forms (Lawton & Rosenzweig 2016). The pMMO alpha subunit, encoding the *pmoA* gene, is highly conserved and often used as a functional marker for analyzing methanotrophic microorganisms in the environment (Hakobyan & Liesack 2020).

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The most widely used primers for the *pmoA* gene are A189 and A682 (Cai *et al.* 2020). *pmoA* is a functional gene, so MMO primers are a tool that may provide data on the function and taxonomy of aerobic methanotrophic microorganisms in the environment (Luesken *et al.* 2011b; Guggenheim *et al.* 2019). Other primer pairs for aerobic methanotrophs, such as A189F/Mb601R and II223F/II646R, could also be used (Kolb *et al.* 2003; Cai *et al.* 2016).

A bacterium related to the phylum NC10 has also been described as capable of performing methane oxidation combined with nitrite reduction, a process so-called N-DAMO (nitrite-dependent anaerobic methane oxidation). These bacteria oxidize methane in an anaerobic environment by producing their own molecular oxygen from nitrous oxide – an intermediate compound in denitrification – yielding carbon dioxide (CO_2) and nitrogen (N_2) (Ettwig *et al.* 2009; Luesken *et al.* 2011a; Padilla *et al.* 2016). One candidate species within this phylum is *Candidatus Methylomirabilis oxyfera*. Despite living in an anaerobic environment, this species can perform aerobic methane oxidation via pMMO. However, it could not be amplified via polymerase chain reaction (PCR) with A189 and A682, the usual primers for aerobic methanotrophs (Luesken *et al.* 2011b). Furthermore, several failures were observed by aligning the usual primers for pmoA with the *M. oxyfera* sequence, especially with the reverse primers, which could explain the problems with PCR product detection.

Luesken *et al.* (2011b) thereby developed the degenerate primer A189_b (by modifying one nucleotide of the forward primer A189F), thus matching most methanotrophs present in the database, except for the genus *Methylacidiphilum*. Primer A682 also differed by eight nucleotides from the *M. oxyfera* sequence. Hence, primer cmo682 – specific for this bacterium – was developed. Another primer set – cmo182 and cmo568 – was also designed for *M. oxyfera,* and used for a nested PCR after amplification with primers A189_b and cmo682. Following Luesken *et al.* (2011b), the first primer set produced multiple bands in PCR, while nested PCR with primers cmo182 and cmo568 produced a single band of the correct size (389 bp).

In addition to bacteria of phylum NC10, anaerobic methane-oxidizing archaea (ANME) – subgroups ANME-1, ANME-2, and ANME-3 – also mediate anaerobic methane oxidation (Cui *et al.* 2015; Timmers *et al.* 2016). Studies of ANME diversity are usually based on the 16S rRNA gene or phylogenies of the gene encoding the enzyme methyl-coenzyme m-reductase (*mcrA*) (Ding *et al.* 2015; Vaksmaa *et al.* 2017). A widely used pair of primers for ANME detection – based on the 16S rRNA gene – are the ANMEF/907R primers, which allow the detection of the ANME 1 and ANME 2 groups (Thomsen *et al.* 2001; Timmers *et al.* 2017).

Methanotrophic microorganisms have been detected in natural environments, such as rice fields, wetlands and ruminant animals' intestines, as well as wastewater treatment sludges (Graef *et al.* 2011; Luesken *et al.* 2011a; Kalyuzhnaya *et al.* 2015). However, there is little knowledge about the presence and/or coexistence of various methanotrophic microorganisms, for example aerobic bacteria, anaerobic bacteria of the phylum NC10 (related to the N-DAMO process), and methanotrophic archaea of the ANME group, in sewage treatment systems. These microorganisms play an important role in reducing methane losses in anaerobic reactors, especially in sewage treatment plants with no biogas exploitation (Guerrero-Cruz *et al.* 2021). Microbiological monitoring may indicate the need for inoculation and cultivation of methanotrophs to ensure control of the methane balance in anaerobic systems, thus reducing greenhouse gas (GHG) emissions and the discharge of effluents containing dissolved methane (Guerrero-Cruz *et al.* 2021; Siniscalchi *et al.* 2021).

The aim of this study was to detect the presence of these methanotrophic groups, using PCR techniques, in samples from four domestic sewage treatment systems – an upflow anaerobic sludge blanket (UASB) reactor, an activated sludge system, a polishing pond, and constructed wetlands.

METHODS

Sampling locations

Sludge and sediment samples were collected from four different domestic sewage treatment systems within Arrudas wastewater treatment plant (Arrudas WWTP) – maintained by the Sanitation Company of Minas Gerais (COPASA) – in Belo Horizonte, Minas Gerais, Brazil.

The systems sampled were: a UASB reactor, an activated sludge system, a polishing pond, and wetlands. The activated sludge is a full-scale system comprising the secondary treatment at Arrudas WWTP, and the other units operate at pilot scale at the Center for Research and Training in Sanitation (CePTS) of the Federal University of Minas Gerais (UFMG), at Arrudas WWTP.

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Three of the systems are fed with raw sewage from Belo Horizonte. The fourth, the polishing pond, receives the treated effluent from the UASB reactor. Table 1 presents some characteristics of the sewage treatment systems investigated.

Table 1 | Main characteristics and operational conditions of the systems sampled

Treatment system	Main characteristics	Operational conditions	Reference	Sampling points
UASB reactor	Diameter = 0.30 m Height = 4.0 m Volume = 340 L	HRT = 7 h $Q = 48.6\ L\ h^{-1}$ Anaerobic	Souza *et al.* (2011)	20, 70, and 120 cm depth (digestion compartment)
Activated sludge	Width = 25 m Length = 115 m Height = 6 m Volume = 17,250 Area = 2,875 m^2	HRT = 5.4 h $Q = 2.25\ m^3\ s^{-1}$ $HLR = 3{,}3\ m^3\ m^{-2}\ h^{-1}$ $\Theta_c = 5.8$ d Aerobic	Data provided by COPASA	Recirculation line (outlet of secondary decanter tank and return to aeration tank)
Polishing pond	Width = 5.25 m Length = 25 m Height = 0.6–1.0 m Volume = 125 m^3	HRT = 4.9 d $Q = 29\ m^3\ d^{-1}$ $\Theta_c = >10$ yr Facultative	Data provided by CePTS	Pond bottom sediment
Vertical flow constructed wetlands	Width = 3.1 m Length = 9.4 m Side wall = 1.0 m Area = 29.1 m^2	$HLR = 0.60\ m^3\ m^{-2}\ d^{-1}$ $Q = 11.3\ m^3\ d^{-1}$ Anaerobic	Data provided by CePTS	10 and 30 cm (from the surface of planted and unplanted beds)

Q, flow rate; HRT, hydraulic retention time; HLR, hydraulic loading rate; Θ_c, sludge age.

Sample processing and preservation

- Pilot-scale UASB reactor: to form a composite sample, 1 L of sludge was collected at each reactor depth (20, 70, and 120 cm), totaling 3 L. The sludge was deposited in a bucket to facilitate sample homogenization. Subsequently, 100 mL aliquots were transferred to clean polyethylene flasks.
- Activated sludge system: approximately 2 L of sludge was collected in the recirculation line of the activated sludge system through a polyethylene collector installed there. The recirculation line receives the return sludge from the secondary decanters. Of the total sample, 100 mL was placed in flasks and transported to the laboratory.
- Polishing pond: the sediment at the treated effluent outlet from the first pond of the series was sampled into a 1 L flask, homogenized, and transferred to a 100 mL flask.
- Planted and non-planted vertical flow constructed wetlands: gravel samples – the constructed beds' supporting material – were collected at 10 and 30 cm depths below the surface of each bed (planted and non-planted). Samples from three random points in each bed were combined to form a composite. A 1 m^2 square was used to mark the collection points. The gravel collected was deposited in 500 mL flasks. For DNA extraction from the biofilm adhering to the gravel, the biofilm was detached by the methodology below:
  - the gravel was measured into 1 L beakers and transferred to a bucket;
  - 2 L of 1X PBS buffer was added to wash the gravel and mixed well to ensure that the biofilm was detached;
  - the procedure was repeated until the gravel was visually clear and no solids were adhering to it;
  - the liquid was transferred to 500 mL flasks and centrifuged at 4,000 rpm for 15 min;
  - the resulting pellet was transferred to 2 mL *Eppendorf* tubes and frozen at –20 °C for later DNA/PCR extraction.

Figure 1 shows the sampling points used.

All samples were identified (i.e., date and place), packed in polystyrene boxes with ice for preservation, and transported to UFMG's Sewage Microbiology Laboratory. In the laboratory, the material was centrifuged in 50 mL *falcon* tubes at 4,000 rpm for 15 minutes to remove any interstitial fluid. The supernatant was discarded, and the sludge and/or sediment resuspended in 50 mL of autoclaved phosphate-buffered saline (PBS 1X: 7.59 g-NaCl L^{-1}; 0.94 g-Na_2HPO_4 L^{-1}; 0.41 g-NaH_2PO_4 L^{-1}; pH = 7.2).

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Figure 1 | Sewage treatment systems investigated: (a) pilot-scale UASB reactor; (b) activated sludge system (return sludge); (c) polishing pond; (d) vertical flow constructed wetlands.

The samples were then homogenized in a vortex and centrifuged again to remove extracellular nucleic acids. The procedure was repeated once and the pellets transferred to 2 mL microtubes and frozen for further molecular analyses.

DNA extraction and PCR

Sample DNA extraction (described above) was performed according to Egli *et al.* (2003), with modifications. After extraction, the DNA's integrity was verified by submitting 2 μL aliquots of the samples to the agarose (1%) gel electrophoresis technique. TAE 0.5X was used as buffer solution (TAE 0.5X comprises 2.42 g of Tris base, 0.57 mL of glacial acetic acid, and 1 mL of 0.5M EDTA (pH = 8.0) adjusted to pH 7.5), and the solution made up to 1,000 mL with *Milli-Q* water. The *GeneRuler Express DNA Ladder* molecular weight marker (Thermo Scientific), containing 100–5000 bp, was used to verify the size of the nucleic acids extracted. The electrophoretic run ran for 30 minutes at 100 V, and the DNA fragments were then stained with *GelRed* and visualized using a transilluminator. The extracted total DNA was stored at −20 °C and then submitted to PCR using primers specific to ANME-1 and ANME-2 group methanotrophic archaea. Oligonucleotides specific to the *pmoA* gene of aerobic methanotrophic bacteria were also used to detect aerobic bacteria in the samples.

The methodology described by Ettwig *et al.* (2009) using primers for the *pmoA* gene of *Candidatus Methylomirabilis Oxyfera* was used to detect denitrifying methanotrophs. Aliquots from the first PCR cycle, which used

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primers A189bF and Cmo682R, were also employed (nested PCR) as templates in the second PCR amplification, using primers cmo182F and cmo568R.

The temperature profile consisted of an initial denaturation at 94 °C for 4 minutes, followed by 35 × 1 minute cycles at the same temperature, primer annealing for 1 minute, and extension for 1.5 minutes at 72 °C, with a final, 10 minute extension at 72 °C. The annealing temperatures were those specified for each primer. The primers used for all sludge and sediment samples are shown in Table 2.

Table 2 | Primer pairs used for PCR detection

Primer pair	Sequence (5′–>3′)	Target group	Fragment size (pb)	References
A189BF	GGNGACTGGGACCTTYTG	*pmoA* gene of *M. oxyfera*	500	Luesken *et al.* (2011b)
Cmo682R	TCGTTCTTYGCCGGRTTT			
Cmo182F	TCACGTTGACGCCGATCC		389	
Cmo568R	GATGGGGATGGAGTATGTGC			
A189F	GGnGACTGGGACTTCTGG	*pmoA* of aerobic methanotrophic	500	Holmes *et al.* (1995)
A682R	GAAsGCnGAGAAGAAsGC			
ANMEF	GGCUCAGUAACACGUGGA	16S rRNA gene of ANME-1 and ANME-2	817	Thomsen *et al.* (2001)
907R	CCGTCAATTCCTTTRAGTTT			
A189F	GGnGACTGGGACTTCTGG	Genera *Methylobacter* and *Methylosarcina*	432	Kolb *et al.* (2003)
MB601R	ACRTAGTGGTAACCTTGYAA			

The volumes, i.e., the reagent concentrations used in 50 μL reactions, were: 10 μL of 5X PCR buffer containing $MgCl_2$ (*Phoneutria*); 0.4 μL of dNTP at 25 mM each (*Fermentas*); 0.5 μL of each primer at 30 pmol μL^{-1} (*Fermentas*); 0.25 μL of Taq Polymerase at 5 U μL^{-1} (*Phoneutria*); 3 μL of BSA at 5 ng μL^{-1} (*Sigma*); 32.35 μL of sterile ultrapure water; and 3 μL of DNA at around 10–30 ng μL^{-1}. A negative control (containing only the PCR reagents and ultrapure water) was used for all reactions.

RESULTS AND DISCUSSION

Table 3 summarizes the PCR results with different primers for detecting aerobic and anaerobic methanotrophs in the sludge and sediment samples investigated. It is clear that methanotrophic archaea (ANME) were detected; that is, present, in all samples evaluated.

Table 3 | Methanotroph detection results

Treatment system	*pmoA* gene of denitrifying methanotrophic (A189BF-Cmo682R)	*pmoA* gene of denitrifying methanotrophs (Cmo182F-Cmo568R)	*pmoA* gene of aerobic methanotrophic (A189F-A682R)	Genera *Methylobacter* and *Methylosarcina* (A198-MB601R)	16S rRNA gene of anaerobic methanotrophic archaea (ANMEF – 907R)
UASB reactor sludge	–	–	–	–	+
Activated sludge system recirculation sludge	–	–	–	–	+
Polishing pond sediment	–	–	+	+	+
Wetland sediment	–	–	–	–	+

The symbols (+) and (–) indicate respectively positive and negative results for the detection of the groups via PCR. The amplicons were visualized on 1% agarose gel under ultraviolet light.

Studies have indicated global distribution of ANME-1 and ANME-2. ANME-3 seems to be restricted mainly to deep aquatic environments and volcanic muds, although it is found occasionally in other sediment types (Cui *et al.* 2015). It is almost always associated with *Desulfobulbus*, a genus of sulfate-reducing bacteria. Phylogenetic

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analysis of anaerobic methane oxidation in sediments, using the 16S rRNA gene, showed that the ANME groups are closely related to the orders *Methanosarcinales*, *Methanobacteriales*, and *Methanomicrobiales*, which include most of the cultivable methanogens (Zhou *et al.* 2015; Wagner *et al.* 2017; Leu *et al.* 2020).

Aerobic methanotrophs were detected only in the polishing pond sediment. Aerobic oxidation is essential to keep methane concentrations in equilibrium in freshwater sediments and soils, while anaerobic oxidation is the primary process occurring under anoxic conditions in marine sediments (Lee *et al.* 2012; Schubert & Wehrli 2018; Liang *et al.* 2019; Szal & Gruca-Rokosz 2020).

Denitrifying methanotrophs were not detected in the PCR tests, although Luesken *et al.* (2011a) detected them in nine WWTPs in the Netherlands. Those plants combine pre-denitrification; however, with an activated sludge system, and two were receiving tannery wastewater, combining partial nitrification and anaerobic ammonia oxidation (anammox) for treatment. All of them also received domestic and industrial wastewaters, except by one fed only with industrial wastewater. The systems evaluated by Luesken *et al.* (2011a) favored nitrite formation (partial nitrification), which is the precursor compound for the N-DAMO process and, consequently, the growth of denitrifying methanotrophs. On the other hand, the systems assessed in the present study (excluding the UASB reactor) promoted total nitrification, i.e., nitrate formation, thus leaving nitrite unavailable for denitrifying methanotrophs.

CONCLUSIONS

Methanotrophic microorganisms were identified from sludge and/or sediment samples collected from four different domestic sewage treatment systems operating on the same plant. Archaea of the ANME I and II groups, which perform reverse methanogenesis using sulfate as the electron acceptor, were detected in all samples. Aerobic methanotrophic bacteria were found only in the polishing pond sediment. Denitrifying methanotrophs of the *Candidatus Methylomirabilis oxyfera* genus (related to the phylum NC10) were not detected in this study.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 17 September 2021; accepted in revised form 11 October 2021. Available online 20 October 2021

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