

## Chapter 2

# Methanotrophs: Methane Mitigation, Denitrification and Bioremediation

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**Abstract** Methanotrophs are bacteria capable of using methane as a carbon source. They can lower atmospheric methane emissions, remove N in environmental and wastewater treatment systems and even transform organic pollutants in soils. Methanotrophic methane mitigation technologies have been demonstrated beyond the laboratories as adaptable field-scale systems that may be engineered to meet site-specific climatic variations and ensure minimal atmospheric methane emission. In agricultural sediments and soils, methanotrophs sequester methane but are affected by fertiliser applications, while in wastewater treatment systems they can lower the costs associated with N removal. Finally, the methanotrophs are particularly appealing as bioremediation agents in methane-containing environments, as their primary enzymes have a broad substrate range that can transform various hydrocarbons, including aromatic compounds and halogenated aliphatics. These diverse bacteria are an important global methane sink and this importance is set to increase as anthropogenic emissions increase over the coming decades.

**Keywords** Denitrification • Landfill • Methanotrophs • Methane • VAM

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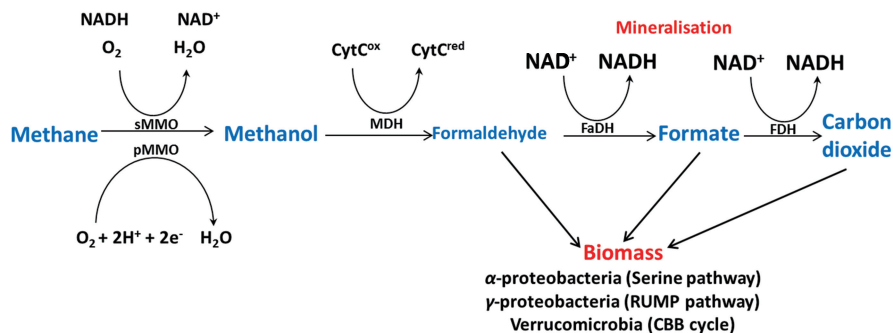
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## 2.1 Introduction

Methane is a potent long-lived highly radiative gas responsible for up to 20 % of the current warming induced by greenhouse gas emissions (Kirschke et al. 2013). Global annual methane emissions are estimated at approximately 550 Tg CH<sub>4</sub> year<sup>-1</sup>, and 60–70 % of this originates from biogenic sources and the rest from non-biogenic sources (IPCC 2013; Kirschke et al. 2013). Biogenic methane emissions are regulated by syntrophic microbial communities, which vary widely according to environmental factors such as temperature: moisture: salinity: pH: redox conditions: and available sulphate, nitrate and organic matter. Non-biogenic methane emission sources include geological settings, waste treatment facilities, fossil fuel industries and biomass burning, while biogenic sources include lakes, wetlands, rice cultivation, forests, livestock farming, oceans, wild animals, termites and permafrost (Kirschke et al. 2013; IPCC 2013; Karthikeyan et al. 2015; Strong et al. 2015).

Among the different microbes, methane oxidisers (primarily methanotrophs) and sulphate-reducing bacteria (SRBs) are the key microbial groups that degrade methane. Sulphate-reducing bacteria reduce sulphate into sulphide using methane as an electron donor. The SRBs are reported to be syntrophically associated with anaerobic methane-oxidising bacteria/archaea, but none of these anaerobic microbes have been isolated and the syntrophic mechanisms are still unclear (Knittel and Boetius 2009). Methanotrophs are capable of using methane as a carbon source. They are ubiquitous in nature, can be aerobic or anaerobic and serve as a global sink for methane (Hanson and Hanson 1996). Aerobic methane oxidation is well studied and many pure cultures have been isolated from various environments such as landfills, coal-bed rocks, rice fields, compost, forest soils, peat bogs, wetlands, soda lakes, thermal springs and marine sediments (Dunfield et al. 2003; Kalyuzhnaya et al. 2005; Kim et al. 2008; Hirayama et al. 2011; Lee et al. 2011; Antony et al. 2012; Saidi-Mehrabad et al. 2013). Their main enzymes for oxidising methane have broad substrate ranges (including ammonia), which allows their use in methane mitigation, bioremediation of organic pollutants and even N removal in wastewater treatment systems.

Methanotrophs were traditionally classified as Type I (gammaproteobacteria) or Type II (alphaproteobacteria), primarily according to their use of the ribulose monophosphate pathway (Type I) or serine pathways (Type II) for formaldehyde assimilation and arrangement of internal structures. They were further subdivided into a Type X group, consisting of gammaproteobacteria that had biochemical capabilities associated with Type II methanotrophs. The traditional classification scheme had its shortcomings, as the methanotrophic bacteria are more diverse and have greater biochemical capability than previously imagined. Methanotrophs are now predominantly classified according to whether they are gammaproteobacteria or alphaproteobacteria; Type X is regarded as a subdivision of Type I gammaproteobacteria. A recently discovered phylum that consists of thermophiles *Verrucomicrobium* (*Methylacidiphilum* and *Methylacidimicrobium* spp.) has also been added (Sharp et al. 2014; Kalyuzhnaya et al. 2015; Strong et al. 2015).



**Fig. 2.1** Generalised pathways for oxidising methane to carbon dioxide, or assimilating the intermediates as biomass. *sMMO* soluble methane monooxygenase, *pMMO* particulate methane monooxygenase, *MDH* methanol dehydrogenase, *FaDH* formaldehyde dehydrogenase, *FDH* formate dehydrogenase

Methanotrophs are able to consume methane because of an enzyme called methane monooxygenase, which uses  $O_2$  to oxidise methane to methanol. Methane monooxygenase (MMO) occurs commonly as particulate membrane-bound enzyme (pMMO) or as a soluble form (sMMO) that is synthesised in copper-deficient environments by some methanotrophs (Semrau et al. 2010). The methane monooxygenase enzymes (pMMO and sMMO) are unique functional enzymes of methanotrophs. The presence of the genes responsible (*pmoA* and *mmoX*) is particularly useful for molecular ecology studies (McDonald et al. 2008). The catalytic pathways that are initiated by the MMO enzyme are illustrated in Fig. 2.1. The pathways can split towards regenerating reducing equivalents or assimilation or into biomass. Essentially, MMO catalyses the  $O_2$ -coupled conversion of methane to methanol in methanotrophic bacteria that may be represented as follows:



The physiological reductant for pMMO has not been identified definitively but may involve quinones from the quinone pool reduced by a Type II NADH:quinone oxidoreductase or by methanol dehydrogenase (Culpepper and Rosenzweig 2012). The most likely physiological electron donor to pMMO is ubiquinol, but the source of electrons to reduce the resultant ubiquinone is not yet substantiated (Kalyuzhnaya et al. 2015). Artificial reductants such as duroquinol and NADH can be used to complete the oxidation (Shiemke et al. 1995). Methane monooxygenase (sMMO in particular) has a broad substrate range that includes various hydrocarbons and halogenated hydrocarbons (Jiang et al. 2010). Methane monooxygenase is also capable of oxidising ammonium, which means methanotrophs participate in the global cycling of nitrogen and methane. In natural systems, methanotrophs may play an important role in the nitrogen cycle and contribute significantly to nitrification in the rhizosphere. The relationships of methanotrophs within microbial communities are complex and can be affected by N type and availability; the complexity is compounded by their ability to fix  $CO_2$  (Chistoserdova et al. 2005; Jiang et al. 2010; Smith and Murrell 2010) and  $N_2$  (Pfluger et al. 2011; Singh and Strong 2015).

## 2.2 Methane Mitigation in Soils Associated with Agriculture, Coal Mining and Landfills

Globally, agricultural activities (including livestock farming); waste management (including landfilling); and fossil fuel retrieval, processing and delivery (including coal mining) are the three largest sources of anthropogenic methane (Hanson and Hanson 1996). Biological methane oxidation is vitally important to reduce these emissions. It is predicted that methanotrophs consume up to 40 Tg CH<sub>4</sub> year<sup>-1</sup> and sequester more than 50 % of the methane produced in soils (IPCC 2001; Reeburgh 2003; Reeburgh et al. 1993). The ability of the methanotrophs to lower methane emissions and degrade hazardous organic compounds has been reviewed (Hanson and Hanson 1996; Jiang et al. 2010; Semrau et al. 2010; Smith and Dalton 2004; Wendlandt et al. 2010). Methane oxidation rates may vary according to methane and oxygen concentrations. The following environmental variables (based on laboratory studies) regulate methane oxidation in soil:

- *Temperature.* Most methanotrophs are mesophilic and function optimally within a temperature ranging from 25 to 35 °C. Methane oxidation may cease at temperatures below 10 °C. Type I methanotrophs tend to have lower temperature optima and become more prolific under these conditions (Börjesson et al. 2004; Gebert et al. 2003).
- *Oxygen supply.* Methanotrophic bacteria are obligate aerobes that can achieve optimum methane conversion rates even at low oxygen concentrations. For bio-filters, methane oxidation only commenced when oxygen levels were above 1.7 %, and maximum methane oxidation rates were achieved at approximately 9 % oxygen content (Gebert et al. 2003).
- *Nutrients.* Inorganic N (ammonium/nitrate) might stimulate or inhibit methane oxidation in soils depending on N type and its concentration, methane concentration, pH and methanotroph species present. Methanotrophic bacteria have a relatively high N demand: 0.25 mole of N is required for every mole of assimilated carbon.
- *Moisture.* Soil pore volume strongly affects this parameter, but an optimum soil moisture content is generally between 10 and 20 % w/w. Too little moisture (<5 %) significantly lowers oxidation activity due to desiccation, while too much moisture inhibits gas transfer—molecular diffusion is approximately 10,000 times slower through water than air (Cabral et al. 2007).

### 2.2.1 Agriculture: Rice Paddy Soils

Modern agriculture has increased in scale and intensity and production is expected to double by 2050 because of greater food, feed and energy demands (Raja 2013). Meeting this growing demand will require more land and greater crop production efficiencies. Inevitably, this will lead to increased fertiliser use, which will impact the methane flux from agricultural soils as the microbially mediated production and consumption of methane is regulated by soil physico-chemical properties and strongly

impacted by fertiliser use, crop type, irrigation and organic amendment (Zheng et al. 2010). Nitrogen fertilisers containing ammonium or nitrate are widely recognised as one of the key factors affecting methane oxidation in agricultural soils (Kravchenko et al. 2002; Seghers et al. 2003). However, reports are contradictory due to unaccounted for variability of the sample sites. Ammonium-based fertilisers have caused inhibition (Hütsch et al. 1994), stimulation (Mohanty et al. 2006) or had no effect on methane oxidation (Delgado and Mosier 1996). Ammonium can inhibit methanotrophs by outcompeting methane oxidation by MMOs, generating hydroxylamine, which prevents assimilation and energy production. Ammonium inhibition was a common assumption applied to various ecosystems, until Bodelier et al. (2000) observed ammonium-stimulated methane oxidation and methanotroph growth in rice paddy soils. Methanotrophs are significant contributors to nitrification in the rhizosphere of model microcosms associated with rice plants (Bodelier and Frenzel, 1999). Generally, the short-term use of ammonium-based fertilisers may initially prevent enzymatic methane oxidation, while the long-term use affects various populations of the soil microbial communities and can impact methane production or oxidation (Bodelier and Laanbroek 2004; Ho et al. 2014), as it may also facilitate methane production by methanogens by providing an N source (Schimel 2000).

Rice production generates a large fraction of the agriculturally generated methane, which is troubling because production as this is anticipated to increase from 600 million tonnes in 2000 to 930 million tonnes by 2030 (Kubo and Purevdorj 2004). Simple strategies, such as adopting alternate wetting and drying cycles in rice production, have delivered promising results by reducing CO<sub>2</sub>-equivalent emissions up to 30 % (IRRI 2015). Alternatively, organic fertilisers or amendments may be incorporated into the soils. Using organic fertilisers may improve crop yields and the methane sink potential within agricultural systems, which may be further improved when combined with beneficial microbes (i.e. biofertilisers) that improve the activity of methane-oxidising bacteria such as methanotrophs. Biofertilisers may be an effective tool for agriculture that is environmentally beneficial compared to conventional inorganic fertilisers.

There are reports of the prospective role of biofertilisers with regard to methane mitigation (Singh and Strong 2015). Biofertilisers that contain aerobic photosynthetic organisms, such as *Azolla* (Yadav et al. 2014) or cyanobacteria (Mandal and Mitra 1982; Lakshmanan et al. 1994; Prasanna et al. 2002) or diazotrophs (Bhardwaj et al. 2014; Pingak et al. 2014) have lowered methane emissions from agricultural activity. Frequently, this is a result of improved dissolved oxygen availability. This has two significant effects on microbial communities. The first is that it provides oxygen that the methanotrophs require to oxidise methane, allowing for greater methane sequestration efficiencies—frequently in flooded soils poor in oxygen. Second, oxygen is toxic to the methanogens and may suppress the biological production of methane. These two outcomes have been noted to significantly lower overall methane emission normally associated with rice production. Additionally, incorporating nitrogen-fixing bacteria such as rhizobia (Rösch et al. 2002), methanotrophs (Hackl et al. 2004; Knief et al. 2003) or *Archaea* (Kemnitz et al. 2005) into a biofertiliser can increase N availability to paddy crops and lower N fertiliser requirements (Table 2.1).

**Table 2.1** Amendments that improve soil and sediment fertility or decrease methane emissions.

Amendment	Beneficial role	Soil type	References
Biochars	Improve methanotroph activity	Landfill soils	Sadasivam and Reddy (2015)
Farmyard manure (pressmud) combined with pyrite	Increase methanotroph population	Salinity-disturbed paddy soils	Singh et al. (2010)
Organic amendments combined with pyrite or fly ash	Improve methanotroph activity	Salinity-disturbed paddy soils	
Organic manure combined with fly ash	Increase methanotroph population	Dry tropical nutrient-poor saline soils	Singh and Pandey (2013)
Diazotrophs <i>Ochrobactrum anthropi</i> , <i>Azotobacter</i> and <i>Azospirillum</i>	Increased O <sub>2</sub> content—emit less CH <sub>4</sub>	Paddy fields	Pingak et al. (2014)
Biofertiliser <i>Azolla</i> and <i>Anabaena azollae</i>	Increased O <sub>2</sub> content—emit less CH <sub>4</sub>	Flooded paddy soils	Lakshmanan et al. (1994); Prasanna et al. (2002)
Biofertiliser	Promoted rice yields and emit less CH <sub>4</sub>	Paddy fields	Lakshmi et al. (2012)
Inoculating rice plant roots with <i>Azospirillum</i>	Increased O <sub>2</sub> in the rhizospheric region—emit less CH <sub>4</sub>	Paddy fields	Sahoo et al. (2014)
Cyanobacteria: <i>Synechocystis</i>	Increased O <sub>2</sub> content—emit less CH <sub>4</sub>	Paddy fields	Prasanna et al. (2002)

If agricultural output is to increase as steadily as the human population growth, sustainable and efficient tools are vitally required to mitigate methane emissions *via* natural soil microflora such as the methanotrophs, while simultaneously improving soil quality and crop yields. More research is still required to better understand the complex relationship between methane-oxidising bacteria and other soil microbes, microbially enriched organic amendments, N source, N concentration, phosphate availability, C:N ratio, to enhance the methane sink within agricultural soils.

## 2.2.2 Coal Mines

Coal bed or coal mine gas is a complicated gas mixture with a high methane content that is released during mining operations. Fugitive methane, emitted from coal mines around the world, represents approximately 8 % of the world's anthropogenic methane emissions (Su et al. 2005). The concentration of methane varies for different mining sites and varies locally according to coal quality and coal depth. Methane is emitted as it desorbs from coal during mining, crushing or inefficient combustion, or is actively diluted and pumped out of coal mines to prevent it reaching an explosive concentration. As with landfills, it is important to monitor, regulate and treat methane emissions from coal mines on-site. In gassy mines, the trapped methane is released as

either fugitive or as continuous emissions, with more than 70 % of the mines relying on dilution to obtain acceptable methane concentrations within the mine site (Heimann et al. 2013; Limbri et al. 2014). Here, methane is released or treated as follows:

- Ventilation air methane (VAM) is coal bed methane that is diluted with air to concentrations below 1 % (generally 0.1 to 0.7 % methane), or below the explosive limit;
- Gas drained from the coal seam or coal mine before mining at (60–95 % methane) that is generally collected for direct combustion and energy recovery; and
- Gas drained from worked, or partially worked, areas (30–95 % methane) that is either diluted or used either for energy recovery (Su et al. 2005).

There are implementation and cost barriers for biological treatment of VAM. Technically, using methanotrophic bacteria to remove methane is difficult because it is produced in large volumes with low methane concentrations (average of 0.65 %). There are issues associated with gas solubility, mass transfer, contaminant volatile organic carbon (VOCs) and particulate dust. Gas residence times represent a major hurdle for methanotroph-based biofilters. While 70 % of methane can be removed at a retention time of 15 min, longer retention times (30 min) are required for 90 % removal (Limbri et al. 2013; Sly et al. 1993). The low methane content is also problematic from a physiological perspective. Adding methanol, formate or other reducing equivalents, along with essential nutrients such as nitrogen or trace metals, is recommended to maintain cell activity (Dijk et al. 2012; Andreassen et al. 2013). Biological treatment is compounded by flow rates that fluctuate during operation because of fluctuating methane content with different coal quality and removal depths. Nonetheless, a handful of studies have assessed pilot-scale biofilters for methane removal from simulated VAM. Their results are difficult to compare directly because of differences in optimisation conditions, use of pure or mixed methanotrophs cultures, methane flow rates, gas residence times and reactor types, but high removal efficiencies of 85–98 % were achieved (Limbri et al. 2013).

Based on the operation, mine safety regulation and other methane mitigation system arrangements, the VAM flow and methane concentrations will be varied for different mine sites. It is very difficult to adapt biofilters for VAM treatment unless the methanotrophs are robust and optimised to withstand fluctuating environmental conditions. Currently, the scale of the biofilters required to treat the large volume of VAM is not economically feasible. If the carbon credits or other financial incentives are not imposed, the commercialisation of biofilter technology will struggle. However, there are potential alternatives such as the use of alternative filter packing material and the use of immobilised biocatalysts.

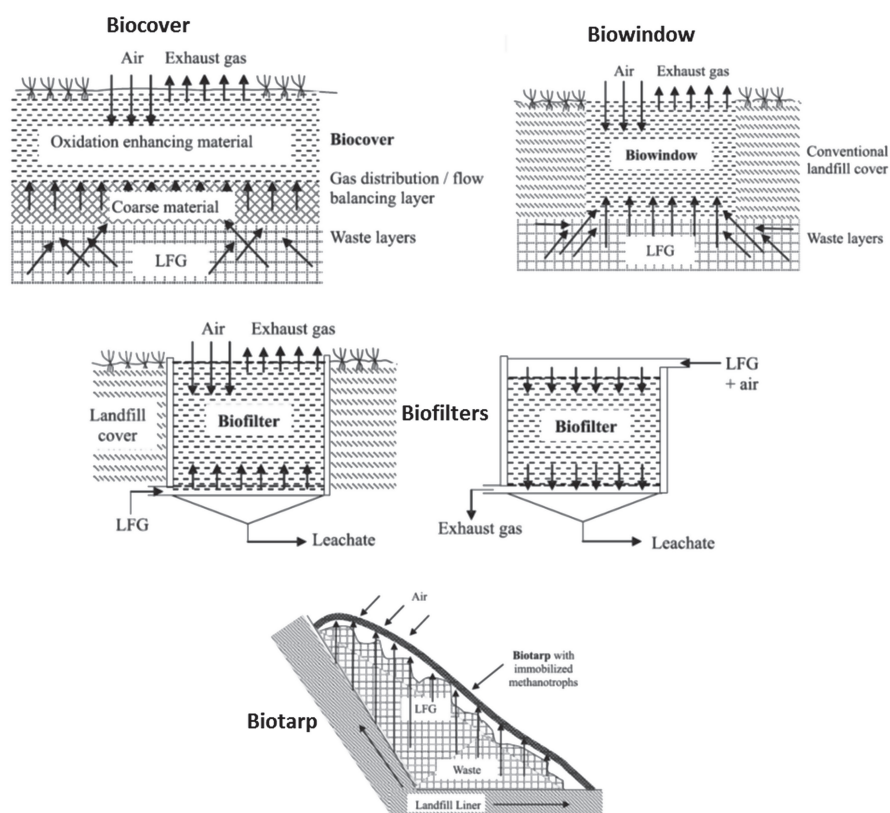
### 2.2.3 *Landfills*

Landfill gas (LFG) is mainly composed of methane and carbon dioxide, along with other trace gases or VOCs. Monitoring, control and treatment/prevention of LFG emissions are an integral part of landfill operations and maintenance.



The contribution of LFG emission to anthropogenic greenhouse effects has received considerable attention in recent years and much research has focused on emission control. The potential to exploit the microbial methane oxidation in bio-based engineered systems was recognised by various researchers for LFG treatment (Park et al. 2002; Börjesson et al. 2004; Haubrichs and Widmann 2006; Einola et al. 2007; He et al. 2008; Park et al. 2009; Rachor et al. 2011). Generally, the landfill methane is oxidised naturally by methanotrophs in the uppermost cover layers. Various factors govern methane oxidation in landfill cover soils, *viz.* methane flux, temperature, moisture content, oxygen distribution, extracellular polymeric substances formation, ammonium content and other VOCs. Further, when the top cover soils are vegetated, plant-mediated transport mechanisms may also affect the overall methane emissions from landfills (Chanton 2005).

Biobased methane mitigation systems mimic the landfill top soil cover systems with controlled environmental conditions that support methanotrophs. There are four types of biobased methane mitigation systems: biocovers, biowindows, biofilters and biotarps (Fig. 2.2). These are considered promising and cost-effective



**Fig. 2.2** Biobased methane mitigation systems mimic the landfill top soil cover systems (Huber-Humer et al. 2008) (This requires copyright permission)



systems that can provide methane mitigation for high or low levels of methane under prolonged conditions, i.e. during landfill operation/post-closure periods. Biobased systems can be readily configured to meet local conditions (topography and climatic conditions) and exploit naturally available materials.

### **2.2.3.1 Biocover**

The first prototype biocover system was proposed by Humer and Lechner (1999). It consisted of a layer of coarse gravel material to provide high gas permeability and a matured well-structured compost material to support methanotroph growth. Generally, biocovers offer the advantage of full landfill coverage, where the methane flux burden is spread over a large surface area and risk of LFG emission is minimised.

### **2.2.3.2 Biowindow**

These are similar to biocovers, but the difference is that they target relatively specific regions of landfill where point source emissions are observed. Biowindows are useful when covering the entire site is neither warranted nor economically feasible, and no gas collection system is available. Biowindow systems are generally arranged in discrete integrated structures in the top cover where LFG passively migrates through due to its increased permeability.

### **2.2.3.3 Biofilter**

Biofilters are engineered, self-contained, fixed bed systems, packed with materials that can support/sustain methanotroph growth. In contrast to biocovers, biofilters require either an active or passive gas collection system to feed through it and is suitable when active landfill extraction and subsequent energy recovery or flaring is no longer viable or not available. They require skilled operators and are more expensive than passively vented, robust open bed applications, but they have a small footprint and high gas removal capacity (Jiang et al. 2010).

### **2.2.3.4 Biotarp**

This is generally applied during the initial stages of landfilling to avoid early LFG emissions. It is similar to a daily cover and must be managed on a daily basis. It must be moist enough to support microbial growth but light enough to roll or fold. Its major advantage over other biobased systems is that the support matrix is inert and not subject to biochemical degradation.

The most commonly used biological solid substrates in biobased designs are mature compost, degraded or mechanically pre-treated municipal solid waste, wood chips and sludge. These biogenic materials naturally harbour methanotrophs and

are often locally available. Inorganic porous materials like gravel, clay pellets, glass beads, sands and soil are used as bulking agents in different layers. Substrate selection is important to ensure optimum conditions for microbial growth and efficient routing of LFG in biobased systems to support effective mitigation. Artificially designed and engineered media can also favour biobased systems, as they are homogeneous and have consistent physical and biochemical properties. However, in the construction of methane oxidation systems covering the large tracts of the landfill surface, huge amounts of suitable substrates are needed, and availability or costs incurred frequently limit application.

Methanotrophic methane mitigation technologies have been demonstrated as adaptable field-scale systems that may be engineered to meet site-specific climatic variations and ensure minimal atmospheric methane emission (Dever et al. 2007, 2011; Huber-Humer et al. 2008). Methane oxidation efficiencies as high as 100 % have been reported for field-scale applications (Nikiema et al. 2007; Gebert et al. 2009). Dever et al. (2011) conducted a field-scale trial at a landfill site (Sydney, Australia) investigating passive drainage and biofiltration of landfill gas as a means of managing landfill gas emissions from low to moderate gas generation landfill sites. Passively aerated biofilters operating in a temperate climate achieved maximum methane oxidation efficiencies greater than 90 % and average oxidation efficiencies greater than 50 % over 4 years of operation. Although temperature and moisture within the biofilter were affected by local climatic conditions, their effect on biofilter performance was overshadowed by landfill gas loading. A very interesting observation with implications for methane mitigation was that landfill loading and subsequent gas production was the primary factor governing the performance of passively aerated biofilters. Microbial methane oxidation was limited by outflowing biogas as it prevented diffusion of atmospheric oxygen into the biofilter.

A number of full-scale biobased research projects are underway in USA, Germany, Denmark, Australia and Canada. In Germany, the MiMethox (Microbial Methane Oxidation in landfill covers) developed a biocover system to reduce the methane emitted from landfills generating low-quality biogas. In Canada and Australia, biofilter test cells of different layering and materials have been constructed on landfills to evaluate the methane abatement under Nordic and arid climatic conditions, respectively. In the US, research towards applying biotarps, instead of daily topical applications of soil and wood chips, is underway for methane mitigation. The increasing use of gas collection systems bodes well for biofilters, their small footprint and high removal capacity. The IPCC 2007 assessment report lists biocovers and biofilters as key mitigation technologies that are projected to be commercialised before 2030.

## 2.3 Denitrification

The interaction between methane and nitrogen has been identified as one of the major gaps in carbon–nitrogen cycle interactions (Gärdenäs et al. 2011; Stein et al. 2012). Methanotrophs and autotrophic nitrifiers share many similarities. Methane oxidisers

and ammonium oxidisers are proposed to have a common evolutionary history as the enzyme systems are similar and the bacteria occupy similar ecological niches (Holmes et al. 1999; Stein et al. 2012). Genes that encode for pMMO or ammonia monooxygenase share high sequence similarities and, despite their different physiological roles, appear to be evolutionarily related enzymes (Holmes et al. 1999).

Methanotrophs can directly or indirectly participate in denitrification, especially in wastewater treatment systems. Modern wastewater treatment systems frequently supplement with costly external carbon sources, such as methanol, to achieve more stringent N discharge limits (Strong et al. 2011). Using methane as a low-cost carbon source to facilitate denitrification would be highly beneficial (Modin et al. 2007). Incorporating methane into the denitrification process was suggested by various researchers in the 1970s (Harremoes and Henze Christensen 1971; Davies 1973; Mason 1977), and four decades later, there have been striking discoveries and substantial progress regarding this coupled process. Methane-dependent denitrification can be divided into two categories according to oxygen availability: aerobic methane oxidation coupled to denitrification (AME-D) or anaerobic methane oxidation coupled to denitrification (ANME-D) (Modin et al. 2007). In spite of the functional differences between the microorganisms responsible for these two processes, the inherent mechanism is dependent on both microbes.

As alternatives are investigated to enable cheaper wastewater denitrification, there has been a recent increase in research published regarding aerobic methane oxidation coupled to denitrification (Zhu et al. 2011; Long et al. 2013; Sun et al. 2013; Liu et al. 2014). It simultaneously ameliorates two environmental issues: methane emissions and soluble nitrogen content in wastewaters. Methane-dependent denitrification appears to be an economical and environment-friendly technology to enable denitrification of nitrogen-contaminated wastewaters (including landfill leachate) by mixed microbial cultures using a cheap, sustainable carbon source (Long et al. 2013; Sun et al. 2013).

### ***2.3.1 Aerobic Methane Oxidation Coupled to Denitrification***

As early as the 1970s, it was hypothesised that the responsible agent in the mixed methanotrophic culture was a denitrifying methanol-consuming bacteria that used a methanotroph by-product to perform the initial reduction of nitrate to nitrite. Since then, AME-D has become an attractive focus for both atmospheric methane mitigation and nitrogen removal in wastewater treatment. Although the detailed process mechanisms remain unclear, two main pathways have been proposed. The first mechanism is direct nitrate/nitrite reduction by aerobic methanotrophic bacteria. Although no aerobic methanotroph has demonstrated ability of complete denitrification (i.e. releasing  $N_2$  as the terminal product), partial denitrification is possible. Certain aerobic methanotrophs can produce substantial amounts of nitrous oxide when exposed to high nitrite concentrations (Nyerges et al. 2010), and some of these methanotrophs contain functional denitrification genes (Stein and Klotz 2011). Very recently, *Methylomonas denitrificans* FJG1 directly reduced nitrate to

nitrous oxide (incomplete denitrification) under hypoxic conditions with nitrate as the electron acceptor and methane as the electron donor (Kits et al. 2015). In natural habitats such as lake sediments, incomplete denitrification can be performed by the cooperation of different types of aerobic methanotrophs with one or two denitrifying genes. Incomplete denitrification in the sediment of Lake Dagow (Brandenburg Germany) was initially catalysed by *Methylobacter tundripaludum* (*narG* and *nirS* genes) and completed by *Methylomonas methanica* or *Methylomicrobium alcaliphilum* (*norB* gene) (Dumont et al. 2013).

The second mechanism is indirect denitrification. Here, methanotrophs release soluble organic metabolites (methanol, formaldehyde, formate, acetate, etc.) that provide an electron donor for denitrifying bacteria (Modin et al. 2007). In wastewater treatment systems, nitrate/nitrite reduction is achieved by a consortium of aerobic methanotrophs and denitrifying bacteria. This syntrophic relationship, where one organism lives off the products of another organism, has been verified. Denitrifiers isolated from a methanotrophic environment exposed to an oxygen gradient were able to use methanol, formaldehyde and formate (i.e. methane oxidation intermediates) to achieve denitrification (Knowles 2005). Additionally, methanol- and acetate-consuming denitrifiers performed the denitrification in earlier research, where denitrification was achieved with methane as the carbon source under micro-aerophilic conditions (Costa et al. 2000).

### 2.3.2 Anaerobic Methane Oxidation Coupled to Denitrification

Nitrite-dependent anaerobic methane oxidation (n-damo) is a recently discovered process that couples anaerobic methane oxidation to nitrite reduction (Raghoebarsing et al. 2006). The novel mechanism for methane-dependent denitrification uses an intra-aerobic denitrification pathway and was performed by a new species with the proposed name: *Methylomirabilis oxyfera* (Ettwig et al. 2010). Even though it exists in a strictly anoxic environment, *M. oxyfera* encodes, transcribes and expresses all genes involved in aerobic methane oxidation. It was hypothesised to produce oxygen required in methane oxidation via dismutation of nitric oxide to dinitrogen gas and oxygen (Ettwig et al. 2010). It may also be a novel pathway to achieve complete denitrification from nitrite, instead of traditional process that requires nitrous oxide reductase. Since its discovery, the ecology of *M. oxyfera* and n-damo process has been intensely studied. The bacterium is widely distributed in sediments (Deutzmann and Schink 2011; Kojima et al. 2012), wetlands (Hu et al. 2014) and wastewater sludge (Luesken et al. 2011). More recently, the n-damo process was coupled with anaerobic ammonium oxidation to remove nitrogen (ammonium and nitrate) with high removal rates (Zhu et al. 2011; Hu et al. 2012; Shi et al. 2013), which has strong potential as a future wastewater nitrogen removal technology.

## 2.4 Bioremediation of Organic Contaminants

Methanotrophs are useful bioremediation agents because of the broad substrate range of their MMO enzymes, which allows their use in heavy metal removal (Al Hasin et al. 2010) and transformation of organic pollutants (Pandey et al. 2014). The sMMO and pMMO enzymes can transform a variety of hydrocarbons (summarised in Table 2.2), including alkanes, alkenes, alicyclic hydrocarbons,

**Table 2.2** Various hydrocarbons that can be oxidised by sMMO and pMMO enzymes and can transform a variety of hydrocarbons

Substrate	sMMO: major reaction products (relative molar proportions)	pMMO: major reaction products
<i>Alkanes</i>		
Methane	Methanol	Methanol
Ethane	Ethanol	Ethanol; Ethanal
Propane	Propan-1-ol (39); propan-2-ol (61)	Propan-1-ol; Propan-2-ol
Butane	Butan-1-ol (54); butan-2-ol (46)	Butan-2-ol
Pentane		Pentan-2-ol
Hexane	Hexan-1-ol (63); hexan-2-ol (37)	
Octane	Octan-1-ol (9); octan-2-ol (91).	
2-Methylpropane	2-Methylpropan-2-ol (70); 2-methylpropan-1-ol (30)	
<i>Alkenes</i>		
Ethene	Epoxyethane	
Propene	Epoxypropane/Propene oxide	Epoxypropane/Propene oxide
But-1-ene	1,2-Epoxybutane	1,2-Epoxybutane; 3-Buten-2-ol
<i>cis</i> -But-2-ene	<i>cis</i> -2,3-Epoxybutane (47); <i>cis</i> -2-buten-1-ol (53)	<i>cis</i> -2,3-Epoxybutane; Crotonaldehyde
<i>trans</i> -But-2-ene	<i>trans</i> -2,3-Epoxybutane (27); <i>trans</i> -2-buten-1-ol (73)	
1,3-Butadiene		1,2-Epoxybut-3-ene
<i>cis</i> -But-2-ene		<i>cis</i> -2,3-Epoxybutane; Crotonaldehyde
<i>trans</i> -But-2-ene		<i>trans</i> -2,3-Epoxybutane; Crotyl alcohol; Crotonaldehyde
<i>Alicyclic hydrocarbons</i>		
Cyclohexane	Cyclohexanol	
Methylene cyclohexane	1-Cyclohexane-1-methanol (13.7); methylene cyclohexane oxide (75.8); 4-hydroxymethylene cyclohexane (10.5)	
$\beta$ -Pinene	6,6-Dimethylbicyclo[3.1.1] hept-2-ene-2-methanol (72.3); $\beta$ -pinene oxide (27.7)	
Adamantane	1-Adamantol (50); 2-adamantol (50)	

(continued)

**Table 2.2** (continued)

Substrate	sMMO: major reaction products (relative molar proportions)	pMMO: major reaction products
<i>Halogenated aliphatics</i>		
Trichloroethene	Formate (35); CO (53); glyoxylate (5); dichloroacetate (5); chloral (6)	
1,1-Dichloroethene	Glycolate (80); dichloroacetaldehyde (3)	
Chlorotrifluoroethylene	Oxalate	
Tribromoethylene	Formate (80); bromal (5)	
<i>Mono-aromatics</i>		
Benzene	Phenol	
Toluene	Benzyl alcohol (60); cresol (40)	
Ethylbenzene	1-Phenylethanol (30); 4-hydroxyethylbenzene (70)	
Styrene	Styrene oxide	
Pyridine	Pyridine <i>N</i> -oxide	
<i>Di-aromatics</i>		
Naphthalene	1-Naphthol, 2-naphthol	
Biphenyl	2-Hydroxybiphenyl (9); 3-hydroxybiphenyl (1); 4-hydroxybiphenyl (90)	
2-Hydroxybiphenyl	Dihydroxybiphenyls	
2-Methylbiphenyl	Ring (56) and side chain (44) hydroxylated products	
2-Chlorobiphenyl	Hydroxychlorobiphenyls	
<i>Other compounds</i>		
Diethyl ether	Ethanol (47); ethanal (53)	
Carbon monoxide	Carbon dioxide	

Adapted from Jiang et al. (2010)

aromatic compounds and halogenated aliphatics (Colby et al. 1977; Schuetz et al. 2003; Smith and Dalton 2004). The enzymes can transform C<sub>1</sub>-C<sub>8</sub> n-alkanes into 1- and 2-alcohols, terminal alkenes into 1,2-epoxides and diethyl ether into ethanol/ethanal (Colby et al. 1977). Alkanes are hydroxylated mostly at the terminal and sub-terminal positions, while ring hydroxylation of aromatics occurs primarily at the *meta* position. The sMMO oxygenates alkenes to epoxides with retention of stereochemistry around the C=C double bond (Smith and Murrell 2009). Chlorinated compounds that are degradable by MMOs include chloroform (Alvarez-Cohen and McCarty 1991a), trichloroethylene (Alvarez-Cohen and McCarty 1991a, b; Henry and Grbic-Galic 1990, 1991; Koh et al. 1993; Smith et al. 1997), tetrachloro-ethene (Gerritse et al. 1995), hydrochlorofluorocarbons (Chang and Criddle 1995; DeFlaun et al. 1992), dichloroethene (Janssen et al. 1988) and even vinyl chloride (Nelson and Jewell 1993).

The oxidation of these substrates is termed co-metabolism. The broad range of the MMO enzymes allows for the catalysis, but unlike methanol, the oxidised products are essentially of no use to the cells energetically, as these compounds do not regenerate reducing equivalents that the MMO requires to remain functional for methane catalysis. High concentrations of co-substrates can starve the methanotrophs of energy needed to survive. Methane, methanol, formate or nutrients may be added to stimulate the methanotrophs and enhance biodegradation and biotransformation of contaminants. Biostimulation of methanotrophs according to the site-specific needs has even been demonstrated at a field scale *in situ* within contaminated aquifers and soils, and *ex situ* in bioreactors (McCarty and Semprini 1994; Semprini et al. 1994; Brigmon 2001; Jiang et al. 2010).

A variety of microbes have been genetically engineered to improve their remediative capacities (Morrissey et al. 2002; Liu et al. 2011; Villaceros et al. 2005; Azad et al. 2014). Genetic engineering may further enhance methanotrophs' tolerance to pollutants and degradation potential, the safety and the risk of genetic transfer, but will require close monitoring if applied in the natural environment (Morrissey et al. 2002; Singh 2011; Pandey et al. 2014). Alternatively, methanotroph–plant associations may be worth pursuing to create a stable methanotroph population in a soil environment—in a symbiotic relationship with plant roots. Even if the methanotrophs do not benefit the host greatly (as is normally the case with endophytes providing nutrients or secreting plant growth-promoting factors), as long as they are actively present in the environment it could be considered beneficial (Azad et al. 2014).

Although methanotrophs are capable of environmental detoxification, providing conditions to maintain an introduced methanotrophic culture, or enriching for methanotrophs may be difficult to implement and justify economically over large areas or dilute pollutant concentrations. Environmental remediation seldom has a commercial value other than avoiding enforced penalties, and the methanotrophs have too many specialised requirements to consider the catalytic whole-cell transformation as a useful tool for bioremediation. However, one avenue that could yield positive results without requiring intensive operational monitoring is using the plant–methanotroph symbiont relationship to enhance phytoremediation and bioremediation.

## 2.5 Conclusion

Methanotrophs are a diverse group of bacteria that are capable of mitigating anthropogenic methane emissions, removing N from environmental and wastewater treatment systems and can even transform organic pollutants in soils. Methanotrophic methane mitigation technologies have been demonstrated beyond the laboratories as adaptable field-scale systems that may be engineered to meet site-specific climatic variations and ensure minimal atmospheric methane emission. However, they are not without their limitations as methane is required to maintain cell activity and large volumes of gas with low methane content can be difficult to treat effectively



and cost efficiently. In agricultural sediments and soils, methanotrophs sequester methane, but are affected by fertiliser applications, while in wastewater treatment systems they can lower the costs associated providing an external carbon source to remove N. Methanotrophs are appealing as bioremediation agents in methane-containing environments, as their primary enzymes have a broad substrate range that can transform various hydrocarbons, including aromatic compounds and halogenated aliphatics. These bacteria are an important global methane sink and their importance will increase as anthropogenic emissions and environmental standards increase over the coming decades.

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