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Housing Methane-Oxidizing Bacteria on Building Materials: towards a Sustainable Air Bioremediation and Building Materials Surface Protection

Ir. Giovanni Ganendra

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Science
Titel van het doctoraat in het Nederlands:
Methaan-oxiderende bacteriën op bouwmaterialen: naar een duurzame lucht bioremediatie en bescherming van de bouwstructuur.

Cover illustration by Tim Lacoere

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<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAC</td>
<td>Autoclaved Aerated Concrete</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>$\text{C}_{\text{CH}_4}$</td>
<td>Methane concentration ($1 % (v/v) = 10,000 \text{ ppmv} = 14,285 \text{ nM}$)</td>
</tr>
<tr>
<td>EC</td>
<td>Elimination Capacity</td>
</tr>
<tr>
<td>EBRT</td>
<td>Empty Bed Residence Time</td>
</tr>
<tr>
<td>EPS</td>
<td>Extracellular Polymeric Substance</td>
</tr>
<tr>
<td>EDS</td>
<td>Energy-Dispersive X-ray Spectroscopy</td>
</tr>
<tr>
<td>GHG</td>
<td>Greenhouse Gas</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>IL</td>
<td>Inlet Load</td>
</tr>
<tr>
<td>$K_{\text{so}}$</td>
<td>Solubility constant</td>
</tr>
<tr>
<td>$K_{m}$</td>
<td>Apparent half saturation constant</td>
</tr>
<tr>
<td>$k$</td>
<td>First order reaction constant</td>
</tr>
<tr>
<td>MOB</td>
<td>Methane-Oxidizing Bacteria</td>
</tr>
<tr>
<td>MMO</td>
<td>Methane Mono-Oxygenase</td>
</tr>
<tr>
<td>pMMO</td>
<td>particulate MMO</td>
</tr>
<tr>
<td>sMMO</td>
<td>soluble MMO</td>
</tr>
<tr>
<td>$\text{MMTCO}_2$-eq</td>
<td>Million Metric Ton of Carbon Dioxide equivalent</td>
</tr>
<tr>
<td>MICP</td>
<td>Microbiologically Induced Carbonate Precipitation</td>
</tr>
<tr>
<td>MBF</td>
<td>Methane Biofilter</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
</tr>
<tr>
<td>NMS</td>
<td>Nitrate Mineral Salt</td>
</tr>
<tr>
<td>OL</td>
<td>Outlet Load</td>
</tr>
<tr>
<td>OMM</td>
<td>Organic Molecular Matrix</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>PLFA</td>
<td>Phospholipid Fatty Acids</td>
</tr>
<tr>
<td>Notation</td>
<td>Description</td>
</tr>
<tr>
<td>----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>( P_{\text{CO}_2} )</td>
<td>Carbon dioxide production</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PHB</td>
<td>Poly-( \beta )-Hydroxybutyrate</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RF</td>
<td>Radiative Forcing</td>
</tr>
<tr>
<td>RuMP</td>
<td>Ribulose Monophosphate</td>
</tr>
<tr>
<td>RE</td>
<td>Removal Efficiency</td>
</tr>
<tr>
<td>SCP</td>
<td>Single Cell Protein</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acids</td>
</tr>
<tr>
<td>VAM</td>
<td>Ventilation Air Methane</td>
</tr>
<tr>
<td>VOC</td>
<td>Volatile Organic Compound</td>
</tr>
<tr>
<td>( V_{\text{max}} )</td>
<td>Apparent maximum methane consumption rate</td>
</tr>
<tr>
<td>XRD</td>
<td>X-ray diffractometer</td>
</tr>
<tr>
<td>( \mu \text{CT} )</td>
<td>X-ray micro-tomography</td>
</tr>
</tbody>
</table>
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CHAPTER 1
GENERAL INTRODUCTION
1. Methane

1.1 Methane as greenhouse gas

Industrial revolution in the 18th century was one of the turning point in the human history. Major transformation occurred where material production process exhibited a technological leap from manual (i.e., human or animal labor based) to automation processes. This included the advancement of steam engine, metallurgy and textile production system resulting in the increase of the production capacity of the respective industries. On the other hand, this innovation had also driven a concomitant growth in the fossil fuel consumption. Fossil fuel was increasingly utilized to produce energy needed to run the machinery. Fossil fuel combustion generated different gasses, some of which components that we know now as greenhouse gases. The release of greenhouse gases into the atmosphere has resulted in the surge of the atmospheric concentrations of these gasses from the industrial revolution era until now (Watson et al., 1990).

Greenhouse gas emission contributes to the warming of the climate by influencing the energy balance of the earth. The net energy balance, measured in terms of Radiative Forcing (RF), is a measure of the net change in the energy balance of the earth caused by external perturbations (Stocker et al., 2013). Positive RF means that there is a net positive energy balance resulting in the increase of the atmosphere temperature. Greenhouse gas absorbs and re-emits energy from the sun that is reradiated from the earth (Khilyuk & Chilingar, 2004). At higher atmospheric greenhouse gas concentration, higher amount of energy is absorbed/re-emitted by the gasses and positive RF is obtained. A 2.88 W m$^{-2}$ net RF was estimated from this increasing greenhouse gas concentration alone in 2011 (Stocker et al., 2013). Greenhouse gas emission is one of the contributors of the warming of the earth (combined land and ocean) by 0.74° C between 1906 and 2005 (Bernstein et al., 2007).

Main greenhouse gases are carbon dioxide, methane, nitrous oxide, chlorofluorocarbons, and water vapor. In terms of the level of atmospheric concentration and global emission rate, carbon dioxide is the highest greenhouse gas emitter to the atmosphere. Carbon dioxide contributes to ~77% of the global greenhouse gas emission with fossil fuel combustion being the main emission source (Bernstein et al., 2007). Due to this emission, carbon dioxide concentration in the atmosphere has increased from 280 ppmv in pre-industrial revolution era to 395 ppmv in 2013 (Blasing, 2014). Besides carbon dioxide, methane is the second most emitted greenhouse gas.

Methane, first discovered by Allessandro Volta in 1778 in Italy, is the most important organic greenhouse gas emitted to the atmosphere for its contribution to the global warming. The gas has a strong infrared absorbance (i.e., 25 times more efficient than carbon dioxide) which makes it a more effective greenhouse gas than carbon dioxide although having a shorter lifetime in the atmosphere (~9 years) (Bernstein et al., 2007; Lelieveld et al., 1998). Almost one fifth of the anthropogenic greenhouse gas RF growth since the industrial revolution era can be attributed to the methane emission (Nisbet et al., 2014). In 2009, atmospheric methane contributed to 0.5 W m$^{-2}$ (directly) and 0.2 W m$^{-2}$ (indirectly) out of the 2.77 W m$^{-2}$ total RF of all greenhouse gases (Dlugokencky et al., 2011). Direct contribution of methane to the positive atmospheric RF is related to the emission of the gas to the atmosphere whereas indirect contribution of methane to the atmospheric radiative force is due to its role in the atmospheric chemistry (Dlugokencky et al., 2011). Methane reacts with hydroxyl radical to produce ozone, carbon dioxide, carbon monoxide, and water vapor, another greenhouse gases (Hansen & Sato, 2004). The RF generated from these three components counts as the indirect RF contribution of methane. Methane therefore plays an important role in the climate warming regulation. As with other greenhouse gases, methane concentration in the atmosphere has increased since the industrial revolution. In fact, the growth
rate of atmospheric methane concentration has outweighed the one of carbon dioxide and this has been mostly driven by anthropogenic activities (Dlugokencky et al., 2011). From atmospheric concentration ~800 ppbv in the 1800’s, the concentration reached 1800 ppbv in 2011 (Etheridge et al., 1998; Kirschke et al., 2013).

Figure 1.1. Evolution of atmospheric methane concentration from 1980 until 2010. Dashed lines depict the atmospheric methane concentration whereas solid lines depict the concentration growth rate. Different types of lines indicate measurement from different agencies. NOAA = National Oceanic and Atmospheric Administration. CSIRO = Commonwealth Scientific and Industrial Research Organization. AGAGE = Advanced Global Atmospheric Gases Experiment. UCI = University of California Irvine. Graph was taken from Kirschke et al, 2013.

The dynamic of atmospheric methane concentration for the past three decades can be seen in Figure 1.1. Atmospheric methane concentration increased from around 1575 ppbv in the 1980’s to around 1700 ppbv in the 1990’s with growth rate varied between 10 to 15 ppbv yr\(^{-1}\) (Figure 1.1). This growth rate had decreased beyond 1990’s and stabilized until 2007. Afterwards, an increase of atmospheric methane concentration at a rate of ~5 ppbv yr\(^{-1}\) was observed until it reached ~1790 ppmv in 2010. The decline of the growth rate in 1990’s was contributed from the lower global fossil fuel consumption and natural occurrence (i.e. mount eruption) in that period (Etheridge et al., 1998). Higher methane emission from wetlands (natural and man-made) and fossil fuel consumptions have been the main contributors of the increasing atmospheric concentration from 2007 onwards (Kirschke et al., 2013). With global population set to reach 9.2 billion people in 2050, methane emission from anthropogenic activities is also set to increase in the forthcoming future (Jiang & Hardee, 2011). Therefore, methane emission mitigation is crucial to control the global warming effect of the gas. In order to have an efficient methane emission strategy, methane global budget from different sources and sinks should be understood.
1.2 Methane sources and sinks

Methane emission is contributed from the natural and anthropogenic sources (Table 1.1). The total emission has doubled from 215 Tg yr\(^{-1}\) in pre-industrial revolution era to 550 Tg yr\(^{-1}\) in 2009 (Dlugokencky et al., 2011).

### Table 1.1 Overview of the global methane budget

<table>
<thead>
<tr>
<th>Natural sources</th>
<th>Tg CH(_4) yr(^{-1})</th>
<th>Anthropogenic sources</th>
<th>Tg CH(_4) yr(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural wetland</td>
<td>217</td>
<td>Agricultural and waste</td>
<td>200</td>
</tr>
<tr>
<td>Fresh water (incl. lakes and rivers)</td>
<td>40</td>
<td>Biomass burning (incl. biofuel)</td>
<td>35</td>
</tr>
<tr>
<td>Wild ruminant animals</td>
<td>15</td>
<td>Energy</td>
<td>96</td>
</tr>
<tr>
<td>Wildfires</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Termites</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Geological (incl. oceans)</td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrates</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Permafrost</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total emission per source</strong></td>
<td>347</td>
<td><strong>Total methane emission</strong></td>
<td>678</td>
</tr>
<tr>
<td><strong>Sinks</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropospheric OH</td>
<td>528</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stratospheric loss</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropospheric Cl</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total methane loss</strong></td>
<td>632</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imbalance (source - sink)</td>
<td>46</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Data are taken from Kirschke et al, 2013 using bottom up approach. Inventories taken from the year 2000 to 2009*

Methane sources can be divided into three groups: biogenic (from methane generating microbes), thermogenic (from geological processes), and pyrogenic (from incomplete combustion of fossil fuel or biomass) (Kirschke et al., 2013). In wetlands and fresh water, methane is produced by methanogenic archaea from the anoxic part of the respective niches and subsequently diffuse up to the surface before the gas is released to the atmosphere (Conrad, 1996). In those environments, not all methane produced by microorganisms is released to the air. A high fraction of methane is oxidized by Methane-Oxidizing Bacteria (MOB) residing in the oxic part of the environment (Conrad, 1995; Conrad, 1996). The same process applies to the methane emitted from the oceans and hydrates. Methane emission from wetlands is dependent on both climate factor (e.g., temperature) or human activities (e.g., land conversion). Methane is also emitted from the gut of wild ruminants and termites. Methanogens in the rumen of cattle are the methane producers in ruminants’ gut. Methane formation by methanogens residing in both types of animal is the electron sink for hydrogen generated from feed digestion (Johnson & Johnson, 1995). Varying climate condition induces methane production from wildfire and permafrost. Methane is generated from wildfires due to the incomplete combustion of organic material whereas methane trapped in permafrost is released as a result of the warming of the region (Bousquet et al., 2006).

Anthropogenic methane emission is mainly contributed from the agricultural practice and waste (Table 1.1). Agricultural practice includes cattle breeding (i.e., ruminants) and cultivation of rice paddy field. Methane emission from ruminants is the emission sum from the animal digestive track (ruminants) and the animal manure from the animal barns (Jungbluth et al., 2001). Similar with wetlands, methane is released from the anoxic part of the waterlogged rice paddy field (Smith et al., 2008). Methane emitted from landfills counts as the main contributor from the waste sector. Active and finished landfills are covered by soil on their surfaces creating anoxic regions below the soil (Lou & Nair, 2009). Degradation
of waste from landfill generates methane that seeps through the top soil into the atmosphere. Methane is also emitted from the anaerobic degradation of organic matters in wastewater (Daelman et al., 2012). Biomass burning is a direct result of agricultural land clearing and crop waste burning (Dlugokencky et al., 2011). Additionally, biofuel incomplete combustion releases methane as by-products. Fossil fuel related emission is the one coming from natural gas piping system and processing equipment in the transmission and distribution process, oil extraction and gas flaring, and coal mines. Methane is released from the subterranean pocket of the earth once the coal is mined (Thakur et al., 1996).

Approximately 95% of atmospheric methane loss can be attributed to the methane reaction with different chemical species in the atmosphere. Methane reaction with hydroxyl radical accounts for ~90% of this total loss. The reaction occurs in the stratosphere and troposphere layers. Hydroxyl radical is generated from the photodissociation of ozone and water vapor and, as explained previously, the reaction of this radical with methane produces different other greenhouse gases (Sonnemann & Grygalashvily, 2014). Tropospheric methane loss is sensitive to the budget of hydroxyl radical in that space as the radical component reacts not only with methane but also with the resulting greenhouse gases (Collins et al., 2002; Lelieveld et al., 1998). This causes the prolongation of methane lifetime in the atmosphere and the global warming effect of the gas. Methane is also oxidized by MOB in aerated soil, however, this counts only for ~5% of the atmospheric loss.

From Table 1.1 it can be derived that the dynamic of global methane budget is influenced by the methane emission from both natural and anthropogenic sources and the methane atmospheric sink. This atmospheric mass balance of methane determines the global warming effect of the gas. For natural methane emission, the global emission is mainly influenced by the amount of available natural wetlands (Dlugokencky et al., 2011; Kirschke et al., 2013). Wet regional summer in the tropic area in 2009 and 2010 led to the expansion of the wetlands in the region (Nisbet et al., 2014). This has led to the growth of more than 10 ppbv yr⁻¹ of methane emission from the tropical wetlands alone. Methane emission from the permafrost in the artic rose significantly higher than the global growth rate in 2007 but has followed the global trend afterwards (Nisbet et al., 2014). Predicting the projection of natural methane emission is proven to be more laborious than the anthropogenic ones. Higher uncertainties were found in comparison with the anthropogenic emission (Kirschke et al., 2013). One factor that plays a role in this uncertainties is the occurrence of natural phenomena such as mount Pinatubo volcanic eruption in 1991 and El Niño southern oscillation in 1997. Mount Pinatubo eruption negatively impacted the aerosol on hydroxyl radical production whereas El Niño southern oscillation inflicted fire activity in tropics and boreal regions of Eurasia (Bernstein et al., 2007). Furthermore, the dynamic of hydroxyl radical in the troposphere greatly influenced the methane lifetime in the atmosphere. Due to its reaction with a numbers of atmospheric pollutants (e.g., phenol, benzene, alkene), the component is sometimes called the “atmospheric cleaner” (Sonnemann & Grygalashvily, 2014). A positive hydroxyl radical trend will decrease atmospheric methane lifetime as it increases the destruction rate of methane in the atmosphere. Kroll et al (1998) reported a decrease of methane lifetime from 9.2 to 8.6 years from 1978 to 1993 due to the increase of hydroxyl radical atmospheric budget of 0.46% yr⁻¹ in this period (Krol et al., 1998). In 2012, an increase of 3% yr⁻¹ of the atmospheric hydroxyl radical was predicted (John et al., 2012). Lelieveld et al. (1998) also predicted that the atmospheric methane lifetime will further increase by about 6% until 2050, which may translate to an atmospheric methane lifetime of 8.4 years (Lelieveld et al., 1998).

After calculating the whole methane budget, an imbalance of around 46 Tg yr⁻¹ net methane emission in average between the year 2000 and 2009 is found and this was driven mainly by the increase in the anthropogenic emission (Table 1.1). It is therefore important to estimate future anthropogenic methane emission in order to have an effective mitigation strategies.
1.3 Anthropogenic methane emission projection

United State Environmental Protection Agency (USEPA) has recently published reports projecting the anthropogenic methane emission between 2010 to 2030 that is summarized in Figure 1.2 (USEPA, 2013; USEPA, 2014). Total emission increase by 1259.2 MMTCO$_2$-eq (1259.2 Tg) in 2030 compared to 2010 is estimated and this equals to an annual growth of 62.96 Tg. Driven by technology development and population (economic) growth, the highest methane emission surge in that period is expected to come from the energy and agricultural sectors.

The highest among all sectors, the oil and natural gas industry might contribute to the methane emission increase of 436 MMTCO$_2$-eq (26 %) between 2010 and 2030 due to the higher production capacity and consumption. This increasing production will be carried out to meet the future fossil fuel demand of the industry (e.g., to produce steam in boiler) and household (e.g., for heating, automotive fuel) (Karakurt et al., 2012; Yusuf et al., 2012). Russia and USA will be the main emission contributors from this sector compared to the rest of the world in 2030. Contribution from unconventional emission sources from the energy sectors such as shale gas, tight gas, and coalbed methane will also be increasingly significant to the global methane emission in the future.

The second highest among all sectors, methane emitted from livestock will increase for 430.4 MMTCO$_2$-eq (20 %) between 2010 and 2030. Although higher animal productivity is expected in the future, demand for meat and dairy product will outyield this increased productivity due to the population growth in the developing countries (Godfray et al., 2010). This will push farmers to increase the number of livestock to meet the need. Over the past 50 years, the number of livestock has increased for 1.5, 2.5, and 4.5 times for cattle (incl. sheep and goat), pigs, and chicken, respectively and the trend will continue in the future (FAO, 2009). Hence, with higher number of livestock, higher amount of methane will be emitted. Region wise, the highest emission growth is projected in Asia (1.8 %), Middle East (1.6 %), and Africa (1.5 %) and this is correlated with the higher population growth in those regions compared to others (Jiang & Hardee, 2011).

Methane emitted from coal mining, landfill, and wastewater processing will grow for 195 MMTCO$_2$-eq, 112 MMTCO$_2$-eq, and 97 MMTCO$_2$-eq, respectively from 2010 to 2030. China, being the highest emitter, will contribute to 55% of the global methane emission from coal mining in 2030. Similar with oil and gas sector, coal production will increase in the future to meet the growing energy demand (Karakurt et al., 2012; Yusuf et al., 2012). The main driver of methane emission growth from landfill and wastewater sectors will be the increasing waste stream in developing countries as the human population and income will increase. This brings about higher buying power for goods resulting in the increasing waste generation. However, a decrease of emission of about 11 MMTCO$_2$-eq is predicted from the rice paddy fields in 2030 compared to in 2010 as there will be a shift in the global food demand towards livestock based food.
Overall, anthropogenic methane emission will increase in the future in almost all sectors. Emission from the oil and gas sector will exhibit the highest increment whereas emission from coal mines will give the highest growth rate. Several methane emission mitigation strategies have thus been applied in the previously mentioned sectors. Methane emission mitigation strategies have so far focus on the anthropogenic emission mitigation as they are cost-effective and can be design-driven (Dlugokencky et al., 2011).

1.4 Anthropogenic methane emission mitigation

An overview of anthropogenic methane emission mitigation strategies can be seen in Table 1.2. These strategies can be divided into three distinct approaches: emission prevention, waste gas remediation, and methane recovery. From the agriculture sector, strategies are applied to mitigate methane emission from the: (i) enteric fermentation system, (ii) livestock manure, and (iii) rice paddy field. For emission coming from the enteric fermentation system, mitigation strategies focus on the animal diet manipulation and innovative feeding management system. Both types of applications aim to control the digestive processes in the rumen so that lower amount of methane is produced. This can be achieved by direct or indirect manipulation of the microbial community composition and activities (e.g., modifications of rumen’s operating conditions such as pH).

Feed digestion in the rumen of ruminants runs through the following processes: (i) hydrolysis of polysaccharides, proteins, and other polymers into monomers, (ii) fermentation of those monomers into different Volatile Fatty Acids (VFA), hydrogen, and carbon dioxide, and (iii) methane production (methanogenesis) from hydrogen, acetate and carbon dioxide (Boadi et al., 2004). Methanogenesis is performed by methanogenic archaea and is the terminal step of the feed digestion process. Among other produced FVAs, acetate and butyrate are the precursors of hydrogen. Methane production is therefore controlled by suppressing acetate and butyrate production in the fermentation process and direct the process towards higher propionate selectivity (Cottle et al., 2011). Higher propionate production can be obtained, for example, by the supplementation of feed containing a high proportion of grain (Table 1.2).
Additionally, direct manipulation of methanogens activity or population has also been performed. For example, bromoethanesulphonate addition in feed is known to lower methanogenic activity.

Several other strategies have also been applied to reduce enteric methane emission (Table 1.2). Animal productivity is improved so higher meat or milk production per amount of methane released per animal is obtained (Patra, 2012). Breeding of livestock known to emit less methane was also performed by genetic engineering. Feeding management improvement aims to control the digestion residence time in the rumen (Boadi et al., 2004). For example, by increasing the level of intake, the food passes through the rumen at higher rate, thus lowering its digestibility.

The emission mitigation from livestock manure include improved manure handling and manure post-processing (Table 1.2) (Chadwick et al., 2011). When storage facility or slurry pit is sealed, it creates anoxic environment in the pit favoring methanogenesis of the manure. Manure handling modification is therefore aimed to minimize the creation of these anoxic regions whereas manure post-processing is performed to directly inhibit methanogens activity during storage. From the economical point of view, anaerobic digestion of manure is usually preferred. Produced biogas can be used to generate electricity or as the fuel for other processes. Global policies in mitigating methane emission from the agricultural sector mainly focus on the mitigation of manure emission (Key & Tallard, 2012). This is based on the argument that emission reduction from manure is more feasible to monitor. On the other hand, emission from enteric fermentation system is dependent on larger number of factors such as the animal age, geographical factor, genetic type, etc which make it more difficult to rationalize.

Methane emission mitigation from the rice paddy field mainly attempts to regulate the waterlogging period of the field over the whole rice cultivation process (Johnson et al., 2007; Uprety et al., 2011). Emission strategies aim to both optimize rice production or directly inhibit the methane emission. Applications include crop modification and chemical amendment in the fertilizer. Addition of biological agent, e.g., MOB, is also done to oxidize methane produced by the methanogens.

Emission mitigation from the energy sector aims to limit fugitive emission from fossil fuel (i.e., oil, natural gas, and coal) production facilities (Karakurt et al., 2012; Yusuf et al., 2012). In oil and natural gas facilities, mitigation effort is performed starting from the production, through the distribution and transmission system (Table 1.2). Modification is applied in the process production system by replacing the existing piping or process equipment or adding a process unit (e.g., catalytic converter to oxidize methane). Increased maintenance is also performed to monitor fugitive methane leaks. In coal mines, methane emission mitigation is realized to both limit the methane release to the atmosphere and to ensure the safety of the mine workers (Yusuf et al., 2012). This is based on the fact that methane released from the mined coal pocket can push the atmospheric methane concentration in the coal mine up to a dangerous level (i.e., explosive limit) (Kirchgessner et al., 1993). Emission mitigation in this sector include waste gas flaring or utilization as fuel for different processes.

From the waste sector, strategies are applied to mitigate methane emission from landfill and wastewater treatment plant. Gas produced from covered landfill, especially the new one (i.e., 25 years of age), contains a high amount of methane. Hence, methane can be recovered from this waste gas stream and be used for different purposes e.g., electricity generation and fuel for chemical processes (Karakurt et al., 2012; Yusuf et al., 2012). The waste gas can also be collected via vertical or horizontal wells and flared. In wastewater treatment plant, methane emission can be minimized by optimizing the aerobic processes in the facility (e.g., improved aeration system). In anaerobic digester, produced methane can be used to generate electricity or as an energy source to heat wastewater or sludge digestion tank.
<table>
<thead>
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<th>Sector</th>
<th>Source</th>
<th>Mitigation strategies</th>
<th>Example(s)</th>
</tr>
</thead>
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<tr>
<td>Agriculture</td>
<td>Enteric fermentation</td>
<td>(a) Additive supplementation in feed</td>
<td>(a.1) Addition of natural ingredients e.g., processed oilseed, sunflower oil, fat</td>
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<td>(a.2) Addition of microorganisms inhibitors e.g., bacteriocins, bacteriophage, methanogens and protozoa inhibitor</td>
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<td>(a.3) Addition of probiotics e.g., monensin, lasolocid</td>
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<td>(a.4) Addition of other chemicals e.g., production enhancing agent (bovine somatotropine), bromoethanesulphonate, halogenated compounds (chloroform)</td>
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<td></td>
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<td>(b) Selection of high quality feed</td>
<td>(b.1) Utilization of high concentrate level (at high level of intake)</td>
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<td>(b.2) Utilization of high quality forage</td>
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<td>(b.3) Utilization of a mixture of feed e.g., grains and whole plant silages</td>
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<td>(b.4) Utilization of high protein feed</td>
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<td>(b.5) Forage processing, grinding, pelleting</td>
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<td>(c) Feeding management modification</td>
<td>(c.1) Rotational grazing of animals / early grazing</td>
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<td>(c.2) Increase feed level of intake</td>
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<td>(c.3) Lower meal frequency</td>
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<td></td>
<td>(d) Other strategies</td>
<td>(d.1) Immunization</td>
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<td></td>
<td></td>
<td>(d.2) Animal genetic selection</td>
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<td></td>
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<td></td>
<td>(d.3) Addition of microorganisms e.g., MOB, acetogens</td>
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<td></td>
<td></td>
<td>(d.4) gas bioremediation</td>
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<tr>
<td>Manure</td>
<td>(a) Manure handling modification</td>
<td></td>
<td>(a.1) Lower manure piling in the storage facility</td>
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<td></td>
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<td>(a.2) Frequent removal of slurry from the slurry store</td>
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<td>(a.3) Minimizing slurry volume during summer months</td>
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<td>(a.4) Switch to dry manure management</td>
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<tr>
<td>Manure</td>
<td>(b) Manure post-processing</td>
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<td>(b.1) Manure dilution before storing</td>
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<td>(b.2) gas bioremediation</td>
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<td>(b.3) Manure slurry cooling</td>
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<td></td>
<td>(b.4) Enhancing crust formation</td>
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<td>(c) Anaerobic digestion of manure</td>
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<tr>
<td>Sector</td>
<td>Source</td>
<td>Mitigation strategies</td>
<td>Example(s)</td>
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<tr>
<td>Rice paddy field</td>
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<td>(a) Crop diversification</td>
<td>(a.1) Rice-potato-sesame rotational cropping system</td>
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<td></td>
<td>(b) Water management</td>
<td>(b.1) Field drainage during flowering period</td>
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<td>(b.2) Intermittent irrigation</td>
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<td></td>
<td></td>
<td>(c) Biological mitigation</td>
<td>(c.1) Addition of <em>Mycorrhiza</em> and MOB</td>
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<td>(d) Chemical amendment in the fertilizer</td>
<td>(d.1) Addition of nitrate, prilled urea, muriate of potash, green manure as chemical fertilizer additives</td>
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<td>(e) Cultivation selection and management</td>
<td>(e.1) Incorporation of rice straw compost before transplanting</td>
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<td>(e.2) Appropriate selection of rice cultivar and fertilizer</td>
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<td>(e.3) Late transplanting with comparatively aged seedlings</td>
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<tr>
<td>Energy</td>
<td>Oil facilities</td>
<td>(a) Equipment modifications/upgrades</td>
<td>(a.1) Replacing wet seals with dry seals in gas wells</td>
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<td></td>
<td></td>
<td>(b) Changes in operational practices</td>
<td>(b.1) Gas flaring in both offshore and onshore</td>
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<td></td>
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<td></td>
<td>(b.2) Offshore methane collection and use</td>
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<td>(b.3) Methane reinjection into the wells</td>
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<td>(c) Installation of new equipment</td>
<td>(c.1) Installation of vapor recovery unit</td>
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<td></td>
<td>(c.2) Installation of plunger systems in gas wells</td>
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<td>(c.3) Installation of surge vessel for capturing blow-down vent</td>
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<tr>
<td>Natural gas facilities</td>
<td></td>
<td>(a) Production abatement options</td>
<td>(a.1) Utilization of catalytic converters in selected well field engines</td>
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<td></td>
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<td>(a.2) Enhanced inspection and maintenance at inspection sites</td>
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<td></td>
<td>(a.3) Installation of flash tank separator in glycol dehydration system</td>
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<td>(b) Processing abatement options</td>
<td>(b.1) Retrofit fuel gas for reciprocating compressors and blow-down</td>
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<td></td>
<td></td>
<td></td>
<td>(b.2) Replace gas pneumatic controls to instrument air</td>
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<td>(c) Transmission abatement options</td>
<td>(c.1) Use pipeline pump-down techniques to lower gas line pressure before maintenance</td>
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<td></td>
<td>(d) Distribution abatement options</td>
<td>(d.1) Use hot taps in service pipelines connections</td>
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<td></td>
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<td>(d.2) Use composite wrap for non-leaking pipeline defects</td>
</tr>
</tbody>
</table>
### Table 1.2 Continued.

<table>
<thead>
<tr>
<th>Sector</th>
<th>Source</th>
<th>Mitigation strategies</th>
<th>Example(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coal mines</td>
<td>(a)</td>
<td>Degasification and pipeline injection</td>
<td>(a.1) Vertical well degasification</td>
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<td></td>
<td></td>
<td>(b) Oxidation of ventilation air methane</td>
<td>(a.2) Gob well degasification</td>
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<td></td>
<td></td>
<td>(c) Gas collection and flaring</td>
<td>(a.3) In-mine borehole degasification</td>
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<tr>
<td></td>
<td>(d)</td>
<td>Direct gas utilization</td>
<td>(b.1) Heat and carbon dioxide generation with an oxidizer technology</td>
</tr>
<tr>
<td>Waste</td>
<td>Landfill</td>
<td>(a) Gas collection and flaring</td>
<td>(d.1) Gas utilization for coal-fired power station, chemical plant feed (e.g., methanol)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b) Electricity generation</td>
<td>(a.1) Gas collection from vertical wells in old landfill</td>
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<tr>
<td></td>
<td></td>
<td>(c) Direct gas utilization</td>
<td>(a.2) Gas collection from horizontal wells in active landfill</td>
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<tr>
<td></td>
<td></td>
<td>(d) Change in waste management practice</td>
<td>(b.1) Electricity generation with gas turbine, microturbine, reciprocating engine</td>
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<td></td>
<td>(a)</td>
<td>Wastewater treatment process optimization</td>
<td>(c.1) Gas utilization as fuel to run leachate evaporators and liquid natural gas production</td>
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<tr>
<td>Wastewater</td>
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<td>(c.2) Gas utilization for industrial processes such as kiln operation</td>
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<td></td>
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<td>(d.1) Adding composting to landfill</td>
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</tbody>
</table>

Overall, the type of methane emission abatement can be divided according to the methane concentration level in the waste gas. At high atmospheric concentration (> 30 % (v/v)), methane can be recovered and used for other purposes (e.g., electricity generation). At low atmospheric concentration (< 1 % (v/v)), methane is remediated before being released to the atmosphere e.g., feeding the waste gas (e.g., ventilation air methane (VAM)) to an oxidation unit in coal mines. Mitigation strategies can also be divided to mitigation of point or diffusive (i.e., fugitive) emission sources. Point source mitigation is emission mitigation from a known emission point e.g., ruminant gut, gas collection point in landfill. Diffusive emission mitigation is emission mitigation of methane emitted from unspecific point or when the gas stream is not regulated. Example is the gas leak from the piping or equipment system in the oil and natural gas production facilities. Point source mitigation strategy can be the activity inhibition of methanogens in ruminant guts or gas flaring from coal mines gas collection point. Example of diffusive source mitigation strategy is the enhanced equipment maintenance in the oil and natural gas production facilities.

Beside the application of chemical oxidizer technology (e.g., thermal flow reverse reactor, catalytic monolith reactor, etc), the use of biochemical reactor can be an alternative to remediate methane emission at low concentration (< 1 % (v/v)). The principle difference of each reactor is the type of catalyst used to remediate methane. As explained in section 1.2, MOB is part of the methane sink in the atmosphere. In the biochemical oxidizer, MOB are used as the biocatalyst. Several advantages of using a biochemical oxidizer include the less extreme reactor operating conditions (at ambient temperature and pressure instead of ~500ºC in catalytic flow reversal (Salomons et al., 2003) or 400-500ºC & up to 7 atm in monolith reactors (Su & Agnew, 2006)), suitability in different environments (e.g., animal stable, landfill), and the absence of additional process units (e.g., pressure swing adsorption and membrane separation in catalytic flow reverse reactor (Karacan et al., 2011; Warmuzinski, 2008)). The use of much lower temperature and pressure will, for example, give rise to lower operational cost of biochemical oxidizer (e.g., the absence of air pre-heating to the reactor) compared to the chemical reactor (Limбри et al., 2013). Considering the previously mentioned advantages, a lot of researches have been performed in the application of methane bioremediation.

2. Methane bioremediation

2.1 Methane-Oxidizing Bacteria

MOB are part of methylotrophic bacteria, a group of bacteria capable of utilizing one carbon compounds as their carbon and energy sources (Lidstrom, 2006). All methylotrophs share the ability to utilize formaldehyde, a central intermediate metabolic compound to synthesize biomass (Anthony, 1982; Chistoserdova et al., 2005). However, only MOB possess the capability to oxidize methane among all methylotrophs to formaldehyde. MOB was first discovered by Dutch microbiologist Nicolaas Söhngen working in Beijerinck laboratory in Delft, The Netherlands, where he isolated the pink pigmented *Bacillus methanica* (later named *Methylobomonas methanica*) from aquatic plant material. However, not until the 1970's that significant advances on MOB research was made as Roger Whittenbury and colleagues isolated around 100 gram negatives and strictly aerobic MOB strains. Initially, MOB classification was made based on microbiological observations (e.g., cell morphology and growth characteristics) (Whittenbury et al., 1970). However, concurrent with the research development in the area of molecular biology (e.g., the use of 16S ribosomal RNA based phylogenetic analysis for classification), several categories for the classification had been added e.g., DNA base composition and dominant phospholipid fatty acids (PLFA) (Bowman et al., 1993). With numerous discoveries made in the MOB research for the past two decades, MOB classification has expanded significantly.
Nowadays, MOB are classified according to the arrangement of the intracytoplasmic membrane, metabolic pathway taken to synthesize biomass, and membrane fatty acids characterization among others. MOB classifications according to the different characteristics can be seen in Table 1.3. MOB are divided into two groups based on the membrane type of the bacteria: type I and type II. Previously, another group, type X, which had different characteristics such as the capacity to fix carbon dioxide, was added to accommodate *Methylococcus capsulatus* (Bath) strain (Murrell & Dalton, 1992). However, type X MOB has been included as the subset of type I MOB in the *Gammaphyoteobacteria* class (Bowman et al., 1993). MOB are typically known to have uniform intracytoplasmic membrane throughout the cells as bundles or vesicular discs (type I and X MOB) or along the periphery of the cell (type II MOB). Besides these two distinct characteristics, type I, X, and II MOB also possess signature membrane phospholipid fatty acids with 14-16, 16, and 18 carbons length, respectively (Hanson & Hanson, 1996). For the carbon assimilation step, MOB belonging to the type I and X undergo the ribulose monophosphate (RuMP) pathway whereas type II MOB follows the serine pathway (Anthony, 1982).

Type I and II MOB are confined within the *Gammaphyoteobacteria* (family *Methylococcaceae*) and *Alphaphyoteobacteria* (family *Beijieriinikiaeae* and *Methylocystaceae*) phyla, respectively (Table 1.3). Additionally, MOB from phylum *Verrucomicrobia* (family *Methyloadiphilium*) has also been discovered (Op den Camp et al., 2009). In total there are 18, 2, 3, and 1 genera within the *Methylococcaceae*, *Methylocystaceae*, *Beijieriinikiaeae*, and *Methyloadiphilium* families, respectively. Classification in Table 1.3 has included the most recent MOB isolations such as the filamentous *Clonothrix* and *Crenotrix*, and other novel genera (e.g., *Methylogobullus*, *Methylomarinovum*, *Methyloparacoccus*). Although typically known to be obligate methane oxidizer, numerous facultative MOB e.g., from the *Methylocella* genus, have been isolated as well (Dedysh et al., 2005). Facultative MOB can grow on multi carbon substrate, e.g., acetate, beside methane. It was hypothesized that facultative MOB convert those non-methane substrates into one of the intermediates of the serine cycle (e.g., malate) for biomass synthesis (Semrau et al., 2011). Lastly, although not confined as MOB, several anaerobic methane oxidizers: (1) *M. oxyfera* bacteria (nitrite-driven) from the NC10 phylum (Ettwig et al., 2010), (2) a consortium of methanotrophic archaea (sulphate-driven) from the *Euryarchaeota* phylum with Sulphate-Reducing Bacteria (Knittel & Boetius, 2009), and (3) a mixed culture using manganese or iron as electron acceptors (Beal et al., 2009), have also been discovered. Altogether, they constitute the majority of the methane sink in the anaerobic part of aquatic environments (e.g., river and sea).

MOB are often found in an environment with continuous supply of methane and oxygen (Bowman, 2006). Example being the one living in places with high methane emission (see Table 1.1) such as landfill (Kumaresan et al., 2009) or ruminant’s manure (Hoefman et al., 2014a). MOB also reside at the anoxic/oxic interface of soil or aquatic sediment where oxygen diffuses from the atmosphere and methane is produced from the anoxic part of the niche. These MOB can be divided into the ones residing in unsaturated (e.g., upland soil) or water-saturated soil (e.g., natural and man-made one such as peat bogs and rice paddy field, respectively). In rice paddy field, MOB inhabit, for example, the roots of the plant. Here is the place where oxygen is transported to the plant and methane, from the decomposition of root exudates or dead roots, is produced (Conrad, 1996). MOB populating upland soil i.e., the so-called high-affinity MOB, are known to consume atmospheric methane (Knief et al., 2003). As explained in section 1.2 and 1.4, soil MOB are central in the global methane budget by oxidizing methane released from the anoxic part of different environments (e.g., wetlands, landfills, and marine environment). A much higher methane flux to the atmosphere would most likely occur if the biological methane oxidation doesn’t take place (Higgins et al., 1981; Murrell & Jetten, 2009).
| Table 1.3. Methane Oxidizing Bacteria classification and their general characteristics |
|-------------------------------------------------|----------------------|----------------------|----------------------|
| Phylum                                          | Family              | Genera               | Gammaproteobacteria   | Alphaproteobacteria  | Verrucomicrobia    |
|                                                 |                     | Methylophaga         | Methylocystaceae      | Methyloferulla       | Methylophilum      |
| Family                                          |                      |                      |                      |                      |                    |
| Methylococcaceae                                |                      | Methylobacter, Methylophaga, Methylococcus, Methylocaldum, Methylohalobius, Methylothermus, Methylosarcina, Methylosoma, Methyloparacoccus |                      |                      |                    |
| Methylocystaceae                                |                      | Methylocystis, Methyloferulla |                      |                      |                    |
| Methyloferulla                                  |                      |                      |                      |                      |                    |
| Methylophilum                                   |                      |                      |                      |                      |                    |
| Intracytoplasmic membrane formation             |                      | Bundle of discs perpendicular to cell periphery | Parallel to cell periphery | Membrane vesicles parallel to long axis on one side of the membrane (Methylocapsa) or cytoplasmic membrane invaginations (Methylocella) | Vesicular membrane |
| Resting stage                                   |                      | Cysts, except Methylohalobius, Methyloparacoccus, Methylophaga, Methylocaldum, Methylohalobius, Methyloparacoccus, Methylophaga, Methylocaldum, Methylohalobius, Methyloparacoccus (no resting stage) | Cysts (varied between Methylocystaceae and Methyloferulla strains); exospores (Methylosinus) | Cysts (Methylocapsa); exospores (Methylocella) | Exospores |
| Carbon assimilation                             |                      | RuMP pathway         | Serine pathway        | Serine pathway        | Serine pathway      |
| CO₂ fixation                                    |                      | Only Methylococcus and Methylocaldum | -                      | -                     | +                    |
| sMMO¹                                            |                      | Only Methylococcus and Methylocaldum | + (Methylosinus); ± (Methylocystis) | - (Methylocapsa); + (Methylocella, Methyloferulla); - | +                    |
| N₂ fixation¹                                    |                      | - except Methylococcus, Methylophaga, Methylocaldum (+) and Methylosa (±) | ±                          | ±                     | +                    |
| pH growth range                                 |                      | Growth range: 5.5-9.5 (Mb), 3.5-8.5 (Ms), NR (Mc), 6.5-7.5 (Mb), 5.2-7.5 (Mc), 4.1-9 (Mn), 6.9-9 (Mr), NR (Msp), ~7 (Mg), (Cr), (Cl), 4.1-10.5 (Mv), 4.5-8.5 (Mmr), 5.3-6.9 (Mm), 6.3-6.8 (Mm) | 4.2 – 7.6                  | 3.5-7.2                | 0.8-6               |
| T growth range (°C)²                            |                      | 0-40 (Mb), 8-42 (Ms), 25-65 (Mc), 20-62 (Md), 15-42 (Mb), 37-72 (Mm), 4-35 (Mb), 4-30 (Mm), 10-30 (Mr), 0-21 (Msp), 30-35 (Mg), (Cr), (Cl), 4-32 (Mv), 15-45 (Mmr), 4-30 (Mgb), 30-55 (Mm), 20-37 (Mp) | 8-37                     | 4-33                   | 37-65               |
| Salt tolerance (%) NaCl²                        |                      | 0.3-4 (Mb), 0.1-2 (Ms), NR (Mc), 0.1-0.5 (Md), 1-15 (Mh), 0-1 (Mt), 1-15 (Mm), <1 (Mm), 0.3-12 (Mr), ~1.8-3.5 (Msp), ~0.5 (Mg), (Cr), (Cl), ~0.2 (Mv), <3 (Mmr), <0.6 (Mgb), 1-5 (Mm), <1 (Mp) | ~0.5                     | < 0.5                  | NR²                 |
| Motility¹                                        |                      | + (Md, Mb, Mr, Mmr, Mm); - (Mc, Mn, Mg, Mv, Mgb, Mp); ± (Mt, Mb, Mx) | + (Methylosinus); - (Methylophilum) | -                     | -                    |


¹, ², ³, and ± indicates the existence, non-existence or varying reports, respectively, of a specific characteristic
⁴ Abbreviations: Methylobacter (Mb), Methylophaga (Ms), Methylococcus (Mc), Methylocaldum (Md), Methylohalobius (Mb), Methylothermus (Mr), Methylosarcina (Ms), Methylosasma (Mm), Methyloparacoccus (Mp)
⁵ NR: not reported
⁶ Required sea water for growth
⁷ Range was taken from the minimum to the maximum value of all isolated strains from the specific family or genera
⁸ A variant of the serine cycle may operate
MOB also inhabit different bodies of water such as the freshwater (e.g., lakes) and the oceans (Hanson & Hanson, 1996). Similar to wetlands, methane is produced from the sediment of those environments (e.g., cold seep from the ocean floor) and it is partly removed by MOB residing in different strata of the water. Anaerobic methane oxidation, as explained previously, is responsible for another fraction of the methane oxidation in the marine sediment (Knittel & Boetius, 2009). Aerobic methane oxidation by MOB in the aquatic environment depends on the oxygen penetration depth into the water, thus, higher methane oxidation occurs at the water’s surface.

Besides the previously mentioned niches, MOB are known to inhabit a number of other environments. MOB have been discovered in environments at varying latitude ranging from the dry artic soil or boreal wetlands to the tropical forest soils (Teh et al., 2006; Torn & Chapin, 1993). They have also been found in an engineered system such as the wastewater treatment plant (Ho et al., 2013b). Besides the thermophilic type X MOB, the bacteria were initially known to be mesophilic and neutrophilic (Bowman et al., 1993; Whittenbury et al., 1970). However, findings from the past decades have shown that MOB can also be found in places exhibiting extreme ends of temperature, pH, or salt content. Examples are *Methylothermus thermales* (able to grow up to 67°C) and *Methylobacter psychrophilus* (able to grow at 3.5°C (Omelchenko et al., 1993; Tsubota et al., 2005). Type I haloalkaliphilic MOB, *Methylococcosium kenyense* was isolated from a soda lake in Kenya and they are able to grow at pH 10.5 (Kalyuzhnaya et al., 2008). The thermoaacidophilic *Verrucomicrobia*, are the most acidophilic MOB known and they are able to grow at pH 0.8 (Op den Camp et al., 2009). Finally, MOB can also form a “tight relationship” with animal (e.g., marine invertebrates and termites) or plants (Ho et al., 2013a; Petersen & Dubilier, 2009; Raghoebarsing et al., 2005).

### 2.2 Methane oxidation and the carbon assimilation pathway by MOB

MOB oxidize methane via the Methane Mono-Oxygenase (MMO) enzyme to methanol before further oxidation to formaldehyde takes place (Fig 1.3). MOB can oxidize methane with high selectivity (100% conversion to methanol) at ambient temperature and pressure (Murrell & Dalton, 1992). This is in stark contrast with the known chemical pathway where methane is converted through a series of reactions occurring at high temperature and pressure (e.g., methane hydrolysis to syngas takes place at 700 – 900 ºC and 1-25 bars) and producing a numbers of by-products (e.g., carbon monoxide, carbon dioxide, etc) (Dalton, 2005; Hermans et al., 2009). Due to these advantages, MOB have been promoted as a ‘model reactor’ for an efficient methane oxidation (Que & Tolman, 2008).

MMO is divided into two types: particulate MMO (pMMO) and soluble MMO (sMMO). These enzymes differ in their intracellular locations: pMMO being cell membrane-bound whereas sMMO is found in the cytoplasm of the cell. Almost all MOB can express pMMO with the exception of the ones from *Methyllocellla* and *Methyloferulla* genera (see Table 1.3) (Semrau et al., 2010; Vorobev et al., 2011). However, sMMO is found only in some subsets of MOB, namely, from *Methylcystis* and *Methlysinus* genera and a few strains of *Methylomonas, Methylococcosium* and *Methylococcus* genera (Lidstrom, 2006). For MOB capable of expressing sMMO and pMMO, the expression of each enzyme is dependent on the copper availability to the cell (a phenomenon called “copper switch”); pMMO predominated at a high copper/biomass ratio (>0.85 - 1 µmol (g dw)^{-1}) and sMMO is expressed at a low copper/biomass ratio (Collins et al., 1991; Stanley et al., 1983). pMMO grown MOB also has higher substrate to biomass conversion and affinity to methane (Trotsenko & Murrell, 2008). Both sMMO and pMMO require oxygen as the terminal electron acceptor to oxidize methane, however, they oxidize methane using different mechanism with copper involvement being the prime distinction.
Methane oxidation by sMMO involves three different regulatory proteins: protein A (hydroxylase), protein B (regulatory protein), and protein C (oxidoreductase). The enzyme has a carboxylate-bridged binuclear iron site that resides in the hydroxylase part and acts as the catalytic center (Green & Dalton, 1989b; Green et al., 1985). Complete description of the sMMO catalyzed methane oxidation can be seen in Murrell and Dalton, 1992 and Wallar and Lipscomb, 1994. Briefly, reaction starts with the binding of methane to the catalytic center of the enzyme. Subsequently, electron is transferred from NADH as reducing equivalent to protein A where the catalytic center is situated via protein B and C. The binding of oxygen to reduced protein A triggers further reaction which releases water and methanol. This will restore (re-oxidized) protein A to its initial state and a new oxidation reaction can be repeated with a new methane molecule. Among all reaction steps, the C-H bond breakage of methane in the enzyme – methane – oxygen complex is known to be the rate limiting step of the whole methane oxidation steps (Green & Dalton, 1989a). The key intermediate component in the sMMO catalyzed methane oxidation, called Q, is a complex molecule containing dinuclear FeIV cluster that reacts with methane to break the C-H bond of the molecule (Banerjee et al., 2015; Rosenzweig, 2015).

As explained previously, formaldehyde utilization to synthesize biomass in MOB follow two different pathways: the RuMP (type I) or serine (type II) pathways (Fig 1.3). When RuMP pathway is used, formaldehyde combines with ribulose-5-phosphate to produce hexulose-6-phosphate. Further metabolic reactions produce fructose-6-phosphate, acetyl-coA, and glutamate as the precursors to synthesize new cell materials (i.e., acetyl-coA and glutamate for lipid and protein based materials, respectively) via a series of anabolic reactions. Ribulose-5-phosphate is reproduced from the glyceraldehyde-3-phosphate as part of metabolic rearrangement reactions. In serine pathway, formaldehyde, together with glycine, are converted to serine. In the consecutive reactions, 3-phosphoglycerate, acetyl-coA, and glutamate are produced as the intermediates for biomass synthesis. To complete the cycle, glycine is produced again from glyoxylate coming from the reaction between isocitrate (produced from the citric acid cycle) and malyl-coA (produced from 2-phosphoglycerate downstream reactions). For MOB possessing the serine cycle, in a nutrient deficient environment (e.g., nitrogen), malyl-coA can be converted to acetyl-coA, an intermediate to produce poly-β-hydroxybutyrate (PHB) (Asenjo & Suk, 1986). PHB accumulation by MOB can be seen as a strategy to store “energy” whenever there is a lack of nutrient supply. PHB degradation can provide reducing equivalents (e.g., NAD(P)H) needed to fulfill the energy requirement of the bacteria (Pieja et al., 2011a). To determine if MOB possess either (or both) metabolic cycle, an assay of key enzymes from each cycle is typically performed. These are hexulosephosphate synthase and hexulosephosphate isomerase for RuMP pathway and hydroxypyruvate reductase or glycerate-2-kinase for serine pathway (Trotsenko & Murrell, 2008).

MOB have been used in several biotechnological applications. This is due to the fact that pMMO and sMMO have low substrate specificity and are known to oxidize a number of other organic compounds such as different aliphatic alkanes to aromatic compounds. sMMO is also able to degrade more substrates (up to C-8 and aromatic) than pMMO (up to C-5 without aromatics) (Semrau, 2011). However, for the compounds that can be degraded by both enzymes, pMMO can degrade the pollutant at higher concentration tolerance and longer activity period. As sMMO transforms the pollutant at higher rate than pMMO, when pMMO is used, the toxicity effect of the metabolic product is negatively effecting the bacteria at a slower rate. The capacity of sMMO to degrade different hydrocarbons was firstly investigated using M. capsulatus (Bath) (Colby et al., 1977). Due to its success, the number of research of the co-metabolic degradation of pollutants using the MMOs has increased since.
Figure 1.3. Simplified metabolic pathway(s) of methane oxidation by MOB (Methane Oxidizing Bacteria). For type I MOB, formaldehyde enters the Ribulose Mono-Phosphate (RuMP) (Type I and X) or serine (Type II) pathway before the synthesis of new cell materials. Under nutrient deficient environment, Type II MOB can synthesize Polyhydroxybutyrate (PHB) from acetyl-coA as intermediate. Illustration is gathered from different sources: Anthony, 1982; Asenjo and Suk, 1986; Trotsenko and Murrell, 2008; Solomon et al, 2014.
MOB have been utilized to remediate polluted environments. Bioremediation strategy can be divided into: (1) in-situ biostimulation of autochthonous MOB at the polluted site by injecting methane and air into the site or (2) ex-situ remediation using a bioreactor. Example of in-situ application is the bioremediation of an aquifer contaminated with trichloroethylene (Baker et al., 2001). For this application, the provision of other nutrients can come from the remediation site (i.e., the soil). Several advantages of using MOB as biocatalysts for bioremediation include the use of methane as growth substrate and the higher pollutant removal capacity of the bacteria (Lontoh & Semrau, 1998). Methane is a cheaper carbon source compared to other carbon sources (e.g., glucose) that is typically added when other bacteria are used. The gas can be obtained for example from the anaerobic digester of a wastewater treatment plant. Despite the advantages, several challenges that need to be considered when using MOB for bioremediation are: (1) the competitive inhibition between methane and the pollutant for the active site of MMO, (2) consumption of extra reducing equivalents (e.g., NADH2) for the pollutant degradation, and (3) MMO degradation over time due to the pollutant toxicity effect (Jiang et al., 2010; Wendlandt et al., 2010). Solution of these challenges can be to add formate into the site to produce more reducing equivalents or to continuously supplying methane/air gas mixture to sustain new cell reproduction (thus regeneration of enzyme). This leads to the potential use of facultative MOB where other carbon sources can be provided for biomass synthesis whereas MMO enzyme can be exclusively used for pollutant degradation (Semrau et al., 2011).

Beside pollutant bioremediation, MOB have also been utilized for a few other biotechnological applications. MOB can be used as an alternative single cell protein (SCP) source for the protein supply of animal and human especially in developing countries (Hamer, 2010; Trotsenko et al., 2005). The use of MOB as SCP source provides a more sustainable solution than the use of other bacteria as methane is utilized as the raw material rather than agricultural products for human food. Another application of MOB is the use of the bacteria to produce PHB. This biopolymer can replace the petrochemically produced polymers as the raw materials of plastic. PHB produced by MOB is known to have similar properties (e.g., melting temperature or mechanical strength) as polypropylene (Zhang et al., 2011). PHB production from methane also holds higher economical value than the use of the gas for electricity generation. M. parvus OBBP can intracellularly store PHB up to 70% of its biomass weight (Asenjo and Suk, 1986). Another MOB strains, M. alcaliphilum, can also produce ectoine, one of the chemicals used for cosmetic production (Trotsenko et al., 2005). Recently, a proposal of methane bioconversion to biofuel such as butanol using MOB was made. It was claimed that a more competitive (in terms of energy and carbon efficiencies) biofuel production compared to the currently used bioprocess can be achieved. The innovation being the use of an alternative enzyme such as benzene dioxygenase to oxidize methane instead of MMO as methane oxidation by MMO is energy inefficient (Haynes & Gonzalez, 2014). As suggested by the authors, to provide such feature for MOB, development in genetic and metabolic engineering should be made.

Despite the previously mentioned biotechnological applications, the biotechnological application of MOB still largely focus on the methane bioremediation. The technology has been applied in different niches such as landfills, coal mines, agricultural sites, or gas pipe leak sites. As the concentration level in those places varies, kinetic study of methane removal by MOB is central for the methane bioremediation research.

### 2.3 Kinetics of methane oxidation by MOB

Methane oxidation by MOB is dependent on a numbers of different environmental conditions (e.g., pH, T) or available nutrients (e.g., N source). For example, to synthesize the MMO enzyme, trace metals (e.g., copper; see section 1.5.2)), nitrogen, and vitamin are needed. If all these variables are factored in,
it will render the reaction kinetic model too complex. However, in order to have a practical understanding of the system, the kinetic of the methane oxidation can be simplified according to the Michaelis-Menten enzymatic model. This model defines the hyperbolic relationship between the concentration of a limiting substrate (in this case methane) to the rate of the substrate consumption by the bacteria (equation 1.1). Derivation of this equation can be seen in Prats and Forestier, 1988.

\[
V = \frac{V_{\text{max}} \times C_{\text{CH}_4}}{K_m + C_{\text{CH}_4}} \tag{1.1}
\]

From equation 1.1, \( V \) and \( C_{\text{CH}_4} \) are the methane consumption rate and the methane concentration, respectively. Two important parameters of the kinetic model are the maximum methane consumption rate (\( V_{\text{max}} \)) and the half saturation constant (\( K_m \)). \( V_{\text{max}} \) is the rate when the amount of substrate molecules is significantly higher than the available enzyme whereas \( K_m \) is the ratio of the reaction rate constant between the product (methanol) release to the one of the methane binding. There are two cases that characterize this kinetic equation. Firstly, at low methane concentration, it can be assumed that \( K_m \gg S \) and the kinetic equation will become a first order equation as follows:

\[
V = \frac{V_{\text{max}} \times C_{\text{CH}_4}}{K_m} \tag{1.2}
\]

Secondly, at high methane concentration, it can be assumed that \( K_m \ll S \) and the kinetic equation will become a zero order equation as follows:

\[
V = V_{\text{max}} \tag{1.3}
\]

\( V_{\text{max}} \) and \( K_m \) represent the true properties of an enzyme. However, MOB activity is not always observed using the MMO enzyme extracted from the bacterial cell. The kinetic of the bacterial methane oxidation can be observed from whole MOB cells or an environmental sample (e.g., soil). When the enzymatic extract is not used, the Michaelis-Menten kinetic parameters obtained are the apparent ones and the kinetic symbols used are therefore \( V_{\text{max(app)}} \) and \( K_{m(app)} \).

Another important parameter, the specific affinity constant (\( a_s \)), is inversely proportional to the \( K_m \) and is defined as the ratio of \( V_{\text{max}} \) to \( K_m \). The specific affinity is a parameter that indicates the degree of likeliness between an enzyme (i.e., MMO) to the substrate (e.g., methane). From the Michaelis-Menten kinetic model, the specific affinity is the slope of the first order kinetic (equation 1.2). MOB exhibiting high specific affinity consume methane at a relatively higher rate at low methane concentration. The concept of high-affinity activity by MOB has been widely investigated for the past decades. This property is said to be confined in pMMO expressing MOB (Lopez et al., 2013). It was initially suggested that MOB capable of expressing high-affinity activity are the ones consuming and thriving on atmospheric methane as part of the global methane sink (section 1.2).

From the Herbert-Pirt substrate relationship, assuming \( V_{\text{max}} \) and maintenance energy requirements of 250 mmol CH4 (C-mol biomass)\(^{-1}\) h\(^{-1}\) and 4.5 kJ (C-mol biomass)\(^{-1}\) h\(^{-1}\), respectively, MOB can only maintain themselves solely on atmospheric methane when they exhibit \( K_m \) value of around 110 nM (Knief & Dunfield, 2005). Indication of high-affinity activity by MOB was firstly pointed out by Bender and Conrad when they obtained \( K_m \) of around 30-51 nM when investigating the kinetics of different soil samples (Bender & Conrad, 1992). Thereafter, several other researches of high affinity activity by MOB were also reported in different soil samples (i.e., upland soil clustered USC-\(\alpha\) and USC-\(\gamma\)) or enrichment thereof (Benstead & King, 1997; Dunfield et al., 1999; Knief et al., 2003). Among those detected MOB, only two strains from *Methylcystis spp.* isolated from upland soil could maintain their methane removal capacity when they were incubated at atmospheric methane for at least three months (Knief & Dunfield,
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2005). One of the strains tested for that study, Methylocystis sp. strain SC2, was apparently capable to express two pMMOs depending on the associated growth methane concentration (i.e., lower or higher than 600 ppmv) and this resulted in the different kinetic behavior by the bacteria (Baani & Liesack, 2008; Dam et al., 2012). This observation supports the results from other researches where the specific affinity of the MOB was apparently dependent on the MOB growth/enrichment condition of the samples. Growth at a high methane concentration (in % range) resulted in the higher $K_m$ by the bacteria (Dunfield et al., 1999). Recently, some other reports of high affinity activity were also published (Kravchenko et al., 2010; Martineau et al., 2014). Nevertheless, up until now attempts to isolate high affinity MOB capable to survive solely on atmospheric methane have so far been futile. The other school of thought says that, supported by the current finding of facultative MOB, these high-affinity MOB might thrive due to the consumption of other carbon source (e.g., acetate) existing in the soil (Belova et al., 2011; Pratscher et al., 2011) or provided by other bacteria (heterotrophs) (Ho et al., 2014).

Numerous kinetic studies of the methane oxidation by MOB have been reported up until now (Table 1.4). They were performed to investigate: (1) the influence of a parameter (e.g., temperature), a condition (e.g., starvation), or a component (e.g., hydrocarbon pollutant) to the activity of the bacteria, or (2) the MOB activity in a specific environmental niche (e.g., landfill soil). These studies were therefore conducted in a wide range of parameters and using different methods. Kinetic studies were typically conducted using MOB pure culture, mixed culture, or environmental sample (Table 1.4). The kinetic of an MOB strain has also been reported several times and significantly different kinetic parameters were obtained due to the difference of the employed methods. The $K_m$ value of $M. trichosporium$ OB3b by Yoon et al (9.4 ± 1.2 µM; 20 ºC) and Calhoun and King (1.0 ± 0.3 µM; 25 ºC) were different due to the difference in the incubation temperature (Calhoun & King, 1998; Yoon & Semrau, 2008). The presence of another component in the bacterial culture can also influence the kinetic expression by the bacteria. Halogenated alkanes can be co-metabolized by the MMO enzyme (section 1.5.2) and therefore the kinetic model should be adjusted to the competitive inhibition one (Prats & Forestier, 1988). From Table 1.4, the lowest $K_m$ among all pure culture studies was generally obtained within the type II MOB group whereas samples taken from upland soil exhibited the lowest $K_m$ among environmental samples (Knief & Dunfield, 2005)). Within a category, for example the kinetics of landfill soil, the value difference of a parameter can be three folds (Bogner et al., 1997). In this case, incubation at high methane concentration prior to the kinetic test might induce the enrichment low-affinity MOB causing the higher calculated $K_m$. Overall, any comparison of kinetic parameters should take into account the previously mentioned factors.
<table>
<thead>
<tr>
<th>Samples</th>
<th>( V_{\text{max(app)}} )</th>
<th>( K_{m(app)} )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Pure culture</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Methylocystis</em> sp LR1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10&lt;sup&gt;9&lt;/sup&gt; nmol CH(_4) cell(^{-1}) h(^{-1})</td>
<td></td>
<td>(Dunfield &amp; Conrad, 2000)</td>
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<tr>
<td><em>Methyllobacter albus</em> BG8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.7–27.8</td>
<td>2.2–12.6</td>
<td></td>
</tr>
<tr>
<td><em>Methylococcus capsulatus</em> (Bath)</td>
<td>3710</td>
<td>23</td>
<td>(Carlsen et al., 1991)</td>
</tr>
<tr>
<td><em>Methylococcus</em> sp.</td>
<td>NR&lt;sup&gt;†&lt;/sup&gt;</td>
<td>44</td>
<td>(Benstead et al., 1998)</td>
</tr>
<tr>
<td><em>Methylosinus trichosporium</em> OB3b&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.0 ± 1.5</td>
<td>1.0 ± 0.3</td>
<td>(Calhoun &amp; King, 1998)</td>
</tr>
<tr>
<td><em>Methylocapsa acidophila</em> B2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100 ± 10 – 167 ± 25</td>
<td>1.00 ± 1.00 – 2.03 ± 0.45</td>
<td>(Dedysh et al., 2001)</td>
</tr>
<tr>
<td><em>Methylomonas albus</em> NCIMB 11123&lt;sup&gt;e&lt;/sup&gt;</td>
<td>310 ± 50</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td><em>Methylococcus luteus</em>&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Methylocystis</em> sp L6&lt;sup&gt;e&lt;/sup&gt;</td>
<td>680 ± 20</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Methylocystis</em> sp SC2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>110 ± 10 – 2,410 ± 140</td>
<td>0.11 – 2.2 (pmoA 2)</td>
<td>(Baani &amp; Liesack, 2008)</td>
</tr>
<tr>
<td><em>Methylocystis</em> sp DWT&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>1,280 ± 110</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td><em>Methylocystis</em> sp SC2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>340 ± 20</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td><em>Methylocystis</em> sp DWT&lt;sup&gt;e,f&lt;/sup&gt;</td>
<td>280 ± 20</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td><em>Methylocystis</em> sp SC2&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1,860 ± 60 – 2,000 ± 110</td>
<td>9.2–9.3 (pmoA 1)</td>
<td></td>
</tr>
<tr>
<td>(b) Enrichment mixed culture</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil enrichment culture</td>
<td>83.4 ± 8.2</td>
<td>1.9 ± 0.3 (1% (v/v) CH(_4) incubation)</td>
<td>(Dunfield et al., 1999)</td>
</tr>
<tr>
<td>Activated sludge enrichment</td>
<td>6,875 ± 1,937 – 16,875 ± 3,500&lt;sup&gt;n&lt;/sup&gt;</td>
<td>4.8 ± 0.5 – 16.0 ± 0.8</td>
<td>(Lopez et al., 2014)</td>
</tr>
<tr>
<td>(c) Environmental sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic soil (cambisol and humisol soil)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forest soil</td>
<td>0.7 – 3.4</td>
<td>0.03 – 0.05</td>
<td>(Bender &amp; Conrad, 1992)</td>
</tr>
<tr>
<td></td>
<td>60.2 – 1016&lt;sup&gt;i&lt;/sup&gt;</td>
<td></td>
<td>(Dunfield &amp; Knowles, 1995)&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>Forest soil</td>
<td>1</td>
<td>0.01</td>
<td>(Benstead &amp; King, 1997)</td>
</tr>
<tr>
<td></td>
<td>0.18</td>
<td>0.03</td>
<td>(Roslev et al., 1997)</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 0.2 – 5.8 ± 0.3</td>
<td>6.1 ± 0.9 – 25.6 ± 4.9</td>
<td>(Gulledge &amp; Schimel, 1998)</td>
</tr>
<tr>
<td></td>
<td>7 – 25.9</td>
<td>0.04 – 0.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.08 – 6.2</td>
<td>5.2 – 510</td>
<td>(Gulledge et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>28.1</td>
<td>15&lt;sup&gt;i&lt;/sup&gt;</td>
<td>(Saari et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>28.1</td>
<td>15&lt;sup&gt;i&lt;/sup&gt;</td>
<td>(Tate et al., 2012)</td>
</tr>
<tr>
<td>Samples</td>
<td>$V_{\text{max(app)}}$</td>
<td>$K_{\text{m(app)}}$</td>
<td>Reference</td>
</tr>
<tr>
<td>------------------------------</td>
<td>------------------------</td>
<td>----------------------</td>
<td>--------------------------------</td>
</tr>
<tr>
<td>(c) Environmental sample</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upland soil</td>
<td>0.01 – 3.1$^k$</td>
<td>2 – 196$^i$</td>
<td>(Knief et al., 2003)</td>
</tr>
<tr>
<td>Landfill soil</td>
<td>5.6 (low CH$_4$ incubation); 45$^i$ (low CH$_4$ incubation) 25.380$^i$ (high CH$_4$ incubation) 1.4 ± 0.1 – 25.8 ± 0.5 7.0 ± 0.5 – 2056 ± 118 300 – 2,387$^m$ 0.009 79,500$^j$</td>
<td>(Albanna et al., 2007) (Knief et al., 2003) (Bogner et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>Freshwater sediment</td>
<td>0.6</td>
<td>32$^i$</td>
<td>(Bender &amp; Conrad, 1994)</td>
</tr>
<tr>
<td>Grassland soil</td>
<td>6.8</td>
<td>19.9$^i$</td>
<td>(Czepiel et al., 1995)</td>
</tr>
<tr>
<td>Agriculture (corn) soil</td>
<td>3.2 – 5.3</td>
<td>79.5 – 141.7</td>
<td>(Prajapati &amp; Jacinthe, 2014)</td>
</tr>
<tr>
<td>Flooded rice paddy soil</td>
<td>1.9 – 25</td>
<td>6.2 – 81.1$^j$</td>
<td>(Dubey, 2003)</td>
</tr>
<tr>
<td>Mineral soil</td>
<td>0.0001 – 0.0006</td>
<td>5.9 – 30.6</td>
<td>(Walkiewicz et al., 2012)</td>
</tr>
<tr>
<td>Artic lake sediment slurry</td>
<td>7.7 ± 1.4 – 201.4 ± 16.1$^i$ 4.5 ± 2.4 – 10.6 ± 2.0</td>
<td>(Lofton et al., 2014)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Unstarved cells  
$^b$ Kinetic parameter ranges are taken from both batch and chemostat (methanol limited) tests  
$^c$ Kinetic parameter ranges are taken from 1 week starved cell and 24 hours reincubated cell under 2% (v/v) methane/air atmosphere after 1 week starvation period  
$^d$ Kinetic parameters are taken from trial 1  
$^e$ $V_{\text{max}}$ was recalculated according to the following formula: $V_{\text{max}} = a^0 * K_{\text{m}} * 0.1 \text{ L} * 1/0.4 \text{ L} * 1/0.03395 * 10^{-18}$; 0.1 L = liquid volume; 0.4 L = gas headspace volume; 0.03395 = Ostwald constant at 25°C  
$^f$ $\text{NH}_4^+$ and NO$_2^-$ concentration ranges from 3.4 – 138 µM and 4.1 – 78.1 µM, respectively  
$^g$ Concentration in the liquid phase  
$^h$ in ppmv  
$^i$ in nmol CH$_4$ ml$^{-1}$ h$^{-1}$  
$^j$ in µg g dw$^{-1}$  
$^k$ in nmol g wet w$^{-1}$ h$^{-1}$  
$^l$ NR not reported  
$^m$ NR not reported

NR not reported
2.4 Methane biofiltration

As explained in section 1.4, methane bioremediation utilizes a biochemical oxidizer (bioreactor) with MOB as the biocatalyst. For gaseous waste having low solubility like methane, the typical bioreactors used are the biotrickling filter and biofilter where MOB are immobilized on a carrier material. An advantage of bacterial immobilization is the absence of a biomass separation unit in the reactor system. Two different types of bacterial immobilization are the attached growth (i.e., bacterial inoculation and growth on the material) and the artificial immobilization (e.g., entrapment using polymer beads, microencapsulation) (Cohen, 2001). In entrapped immobilization the biomass system can be engineered to exhibit higher activity by entrapping MOB strains known to possess such property. However, the methane removal rate can be significantly lowered due to the additional transfer limitation created by the entrapment layer (e.g., the capsules). Therefore, immobilization using the attached bacterial growth on the carrier material is normally chosen. Furthermore, biofilter is typically preferred than biotrickling filter to treat methane gas waste. In both types of filters, the waste gas flows through the carrier materials and methane is transformed by MOB before exiting the filter. The main difference between both filters is the continuous liquid feeding set in biotrickling filter whereas the liquid is only intermittently fed in a biofilter. Biofilter also provides higher surface area than biotrickling filter and the use of this reactor results in lower diffusion limitation due to the thinner water layer surrounding the biofilm. The use of a biofilter also minimize the creation of anaerobic zone that might present in biotrickling filter due to the higher water load.

A numbers of different biofilter designs have been reported so far. Methane biofilter can be designed as an open or closed system. In an open biofilter, there is no separation between the filter and the surrounding environment. Open biofilter is typically applied in an old and small landfill where methane valorization is not possible anymore (Nikiema et al., 2007). The advantage of having open biofilter is the cheaper construction cost whereas the disadvantage is the limited control of operating parameters such as temperature. Although depending on the niche, the majority of existing methane biofilters is the closed ones as optimization of the filter performance by varying the operating parameters to obtain maximum methane removal can be performed. Additionally, both gas and liquid streams can be fed into the filter in a con- or countercurrent manner. In a concurrent flow, both streams are fed at the top of the filter whereas in the counter-current system, the gas is fed from the bottom of the filter. Selection of the fluid directional feeding is usually a degree of freedom chosen by the researchers as the filter performance is typically not influenced by this factor (Menard et al., 2012). Liquid feed can also be directly introduced into the filter via a sprinkler or by passing the waste gas into a humidification tower prior to the filter. Liquid is fed into the reactor to provide essential nutrients (e.g., nitrogen), control the filter temperature, and keep the moisture content on the filter bed (Cohen, 2001). The use of humidification tower is feasible when supplementation of essential nutrients is not substantial i.e., when organic carrier material is used.

A biofilter consists of solid (the biofilm containing MOB and the carrier material), liquid (the liquid fed into the biofilter and the one surrounding the biofilm), and gas phase (the waste gas fed into the biofilter) (Figure 1.4). In the biofilter, methane undergoes several processes before being transformed by the bacteria (Malhautier et al., 2005). Methane is transported via a convective flow from the biofilter inlet to the filter bed using the power generated by a compressor. The component is then transferred to the liquid phase surrounding the biofilm. Methane concentration in the liquid phase is dependent on the solubility of the component (i.e., Henry coefficient). In the liquid phase, methane diffuses until it reaches the boundary layer of the biofilm. After subsequent transfer into the biofilm phase, methane diffuses within the biofilm before reaching the bacteria wherein the transformation process (i.e., biological oxidation) actually takes place. Additionally, the bacteria can also coexist in the water phase due to the
biofilm detachment process (Kreft et al., 2001). In this case, the transformation process can also occur in the liquid phase. The time required for these processes are as follows: diffusion (20-1000 s), convection (0.5-20 s), reaction (0.001-20 s) (Picioreanu et al., 1999). The methane biodegradation rate is then dependent on the methane transport process in the biofilter and the biotransformation rate by the bacteria. The typical methane concentration profile in a biofilter can be seen in Figure 1.4.

Figure 1.4. (a) Illustration of a biofilter setup with counter current gas and liquid feeding. The carrier material is a fixed bed system with biofilm layer attached on the material. (b) Magnified carrier material in the biofilter. (c) Typical methane concentration profile in the biofilter. Illustration by Tim Lacoere.

Biofilter performance is evaluated based on the methane Elimination Capacity (EC) and Removal Efficiency (RE) in the filter. Both parameters represent the capacity of the bacteria to consume methane fed into the biofilter. To obtain the EC, the volumetric (or surface) Inlet Load (IL) and Outlet Load (OL) need to be calculated first. Furthermore, when the biofilter is applied in an environment at low methane concentration < 1 % (section 1.4), the methane removal rate by MOB typically follows a first order kinetics (Melse & Van der Werf, 2005). This assumption is based on the fact that methane has low solubility in the liquid phase and thus, the methane transfer into the liquid phase is rate limiting. Assuming that the reactor behaves like an ideal plug flow reactor, then the first order reaction constant, $k$, can be calculated using equation 1.4 as follows:

$$\frac{Q}{V} = \frac{k}{\ln\left(\frac{C_{\text{CH}_4,\text{in}}}{C_{\text{CH}_4,\text{out}}}\right)}$$

(1.4)

Q and V from equation 1.4 represent the inlet gas flow rate and the effective filter bed volume, respectively. $C_{\text{CH}_4,\text{in}}$ and $C_{\text{CH}_4,\text{out}}$ represent the inlet and outlet methane concentrations from the biofilter, respectively. Another important parameter to evaluate a biofilter is the produced carbon dioxide ($P_{\text{CO}_2}$). This parameter is important to evaluate the greenhouse gas potential of the technology. As seen in Figure 1.3, carbon dioxide is produced from the bacterial metabolism and this should be taken into account when evaluating the biofilter. Overview of engineering parameters to evaluate the biofilter performance and how to calculate them can be seen in Table 1.4.

A biofilter was initially employed to remove odor and volatile organic / inorganic pollutants (Delhomenie & Heitz, 2005). The use of biofilter to remove methane was firstly used for the treatment
of coal mines fugitive emission (Apel et al., 1992; Apel et al., 1991). Afterwards, numerous other methane biofilter researches have been conducted either in the lab scale or field test. A few examples of methane biofilters that have been reported so far can be seen in Table 1.5.

**Table 1.5. Overview of different methane biofilter parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Bed Residence Time (EBRT) [s]</td>
<td>$EBRT = \frac{V}{Q}$</td>
</tr>
<tr>
<td>Volumetric Inlet Load (IL) [g m⁻³ h⁻¹]</td>
<td>$IL = \frac{C_{in} \times Q}{V}$</td>
</tr>
<tr>
<td>Removal efficiency (RE) [-]</td>
<td>$RE = \frac{C_{CH₄,in} \times C_{CH₄, out}}{C_{CH₄, in}} \times 100%$</td>
</tr>
<tr>
<td>Elimination Capacity (EC) [g m⁻³ h⁻¹]</td>
<td>$EC = IL \times \frac{RE}{100}$</td>
</tr>
<tr>
<td>Carbon dioxide production (PCO₂) [g m⁻³ h⁻¹]</td>
<td>$PCO₂ = \frac{(C_{CO₂, out} \times C_{CO₂, in}) \times Q}{V}$</td>
</tr>
<tr>
<td>First order reaction constant (k) [-]</td>
<td>$k = \frac{EC \times ln\left(\frac{C_{CH₄,in}}{C_{CH₄, out}}\right)}{\left(\frac{C_{CH₄,in}}{C_{CH₄, out}}\right)}$</td>
</tr>
</tbody>
</table>

The symbols in the equations are: $V =$ filter bed volume (m³); $Q =$ Inlet gas flow rate (m³ h⁻¹); $C_{CH₄, in} =$ inlet methane concentration (g m⁻³); $C_{CH₄, out} =$ outlet methane concentration (g m⁻³); $C_{CO₂, in} =$ inlet carbon dioxide concentration (g m⁻³); $C_{CO₂, out} =$ outlet carbon dioxide concentration (g m⁻³).

For surface inlet load, then the volume term in the equation is replaced by the biofilter bed cross section (m²). For the OL, then $C_{in}$ is replaced by $C_{out}$.

Optimum biofilter performance can be obtained by varying a lot of different parameters which in themselves can be interdependently connected. For example, the type of inoculum can determine the microbial community composition in the filter and therefore the overall biological activity. The inoculum can be taken from an environment known to contain MOB with high affinity such as the soil. Thus, when soil is used as the carrier material, inoculation step is usually not necessary. Low RE (0-10%) is typically observed at the start of the filter run as bacterial activation period is needed. The RE will subsequently increase once the bacteria are enriched (Nikiema et al., 2007). A high RE can be reached faster when MOB exhibiting high growth rate are present due to the higher biomass concentration obtained the longer the filter is run. However, this increases the risk of clogging in the filter.

Besides the type of MOB present in the filter, the liquid and gas feeding operational settings have a major influence to the biofilter performance. The liquid feeding frequency in the liquid is important to control the biomass growth in the filter (Kennes & Veigas, 2002). Likewise, for the gas feeding, increasing the IL can increase the EC as it induces biomass growth. An almost linear relationship between the IL and EC was observed in previous reports (Melse & Van der Werf, 2005; Sly et al., 1993). However, the EC usually decreases beyond a specific IL when Q is continuously increased as the contact time between methane and the bacteria lowers and the risk of carrier material desiccation increases. Addition of organic solvent in the liquid feed has been proposed as an optimization strategy (Kennes et al., 2009). Methane is known to have a high solubility in silicone oil (Rocha-Rios et al., 2010). By adding silicone oil in the liquid feed, methane will be upconcentrated in the liquid phase surrounding the biofilm and hence higher activity can be exhibited by the bacteria (see equation 1.1). However, silicone oil supplementation will increase the operating cost significantly (€ 161 L⁻¹; www.sigmaaldrich.com) and add further waste processing problem.
When controlling the liquid feeding, the nutrient concentration inside the liquid can also be varied. Among different nutrients, copper and nitrogen are essential for the MMO enzyme expression whereas phosphorus is important for the bacterial growth (Huang et al., 2011; Nikiema et al., 2007). Besides the nutrient concentration, the molecule form of the added nutrient also determines the bacterial activity. Nitrogen is typically added as nitrate or ammonium and the supplementation of both components are known to improve RE (Limbri et al., 2013; Nikiema et al., 2005). However, there is a threshold of ammonium addition as the component can inhibit the methane oxidation by MOB by competitively bind to MMO (Bedard & Knowles, 1989). A maximum 0.2 g N-NH₄⁺ L⁻¹ addition was allowed when the biofilter was operated at an IL of 20 g CH₄ m⁻³ h⁻¹ (Menard et al., 2012).

Temperature and pH are another parameters influencing the biofilter performance. The temperature in the reactor needs to be controlled as methane oxidation by MOB is an exothermic reaction releasing 880 kJ mol⁻¹ of methane (Hanson & Hanson, 1996). The released heat causes the creation of temperature gradient that can go up to 4 °C difference within the biofilter (Limbri et al., 2013). This leads to higher water evaporation rate from the reactor. The lowering water content on the carrier material combined with the increasing operating temperature will eventually lower the activity of the bacteria (Limbri et al., 2013). To prevent this problem, higher liquid feeding frequency / flow rate or lower gas flow rate are usually set. However, it should be noted that too high moisture content can risk creating anaerobic zone around the biofilm preventing the oxidation of the methane (Kennes et al., 2009). The pH on the water phase is also important to maintain the bacterial activity. Carbon dioxide production as a result of methane oxidation will acidify the water phase as part of the carbonate equilibrium. To tackle the problem, buffer components is provided in the liquid feed.

When designing a biofilter, carrier material selection is arguably the most crucial step. The preferred carrier materials possess a high porosity and surface area to provide space for the bacteria to grow and to increase the contact area between the bacteria and methane, respectively (Menard et al., 2012; Nikiema et al., 2007). Additionally, the material should also have good water retention capacity, heat resistant, provide buffering capacity, and inexpensive (Delhomenie & Heitz, 2005). Carrier material can be an organic or inorganic ones. The advantage of using organic material as biofilter carrier material is the provision of intrinsic essential nutrients whereas inorganic material gives less compaction problem. Filter bed compaction can lead to higher pressure drop inside the filter and thus higher pump operational cost. Examples of organic carrier material are soil, compost, and pine bark. Among different organic carrier materials, compost provides a rich bacterial community, nutrients, and water holding capacity (Menard et al., 2012). Examples of inorganic carrier materials are different types of rock, activated carbon, and clay. Activated carbon is known to support a high methane removal by MOB and adsorb other pollutant components that might exist in the waste gas (Menard et al., 2012). Although inorganic material is relatively more expensive, it provides longer life time and better performance. It should also be considered that the nutrient provision from the organic material is limited thus the advantage of using organic material is temporary. More importantly, the use of inorganic material typically gives better biofilter performance (Josiane & Michele, 2009; Nikiema et al., 2005; Nikiema & Heitz, 2010). Based on the previously mentioned criteria, building materials has the potential to be a good carrier material for a methane biofilter. For example, several building materials also possess high porosity and surface area (e.g., Autoclaved Aerated Concrete (AAC)). Although some biofilters tested some of the raw materials for building materials production (e.g., stone, perlite, tobermorite ; Table 1.5), no studies have been performed using “ready-made” building materials as the biofilter carrier material. Some advantages of using “ready-made” building materials compared to their raw material are the absence of competition between building material manufacturer, the possibility of recycling used building material, and the possibility of using constructed building wall as a biofilter.
<table>
<thead>
<tr>
<th>Filter bed material</th>
<th>Soil mixture</th>
<th>Carrier material</th>
<th>C \text{[mixture]} \ (% (v/v))</th>
<th>Q \text{[m]} \ h^{-1} \text{p}</th>
<th>IL \text{[g m}^{-3} \text{h}^{-1} \text{p} \text{n}]</th>
<th>EC \text{[g m}^{-3} \text{h}^{-1} \text{p} \text{n}]</th>
<th>RE \text{(%)}</th>
<th>EBRT \text{(min)}</th>
<th>\text{Pco}2 \text{[g m}^{-3} \text{h}^{-1} \text{p} \text{n}]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil mixture</td>
<td>Soil mixture</td>
<td>Soil mixture</td>
<td>2.5</td>
<td>0.15</td>
<td>165</td>
<td>64</td>
<td>39</td>
<td>6</td>
<td>NRb</td>
<td>(Streese &amp; Stegmann, 2003)</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>Compost / perlite</td>
<td>Compost</td>
<td>0.85</td>
<td>0.75</td>
<td>25</td>
<td>8</td>
<td>32</td>
<td>13.5</td>
<td>NRb</td>
<td>(Melse &amp; Van der Werf, 2005)</td>
</tr>
<tr>
<td>Landfill soil</td>
<td>Landfill soil</td>
<td>Landfill soil</td>
<td>0.1</td>
<td>0.03</td>
<td>24</td>
<td>24</td>
<td>100</td>
<td>90</td>
<td>Elevating</td>
<td>(Pratt et al., 2012b)</td>
</tr>
<tr>
<td>NRb</td>
<td>Gravel stone</td>
<td>Gravel stone</td>
<td>0.13</td>
<td>0.33</td>
<td>18.6</td>
<td>8</td>
<td>43</td>
<td>8.8</td>
<td>20</td>
<td>(Nikiema &amp; Heitz, 2010)</td>
</tr>
<tr>
<td>NRb</td>
<td>Stones</td>
<td>Stones</td>
<td>0.43</td>
<td>0.25</td>
<td>9</td>
<td>3.9</td>
<td>43</td>
<td>4.2</td>
<td>5</td>
<td>(Girard et al., 2011)</td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Stones</td>
<td>Stones</td>
<td>0.7</td>
<td>0.25</td>
<td>61.8</td>
<td>18</td>
<td>59</td>
<td>20</td>
<td>NRb</td>
<td>(Ramirez et al., 2012)</td>
</tr>
<tr>
<td>Soil mixture</td>
<td>Hyuga stone and activated carbon</td>
<td>1.5</td>
<td>0.08 (d) ; 1 (h)</td>
<td>0.015</td>
<td>30.4</td>
<td>18</td>
<td>59</td>
<td>20</td>
<td>NRb</td>
<td>(Kim et al., 2013)</td>
</tr>
<tr>
<td>Pine bark</td>
<td>Pine bark and perlite</td>
<td>0.5</td>
<td>0.17 (d) ; 0.3 (h)</td>
<td>0.04</td>
<td>400.6</td>
<td>70</td>
<td>30</td>
<td>NRb</td>
<td>(du Plessis et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>Compost</td>
<td>Compost</td>
<td>Compost</td>
<td>6.6 - 10.8</td>
<td>0.5 (d) ; 1.5 &amp; 2 (h)</td>
<td>0.5 - 2.8</td>
<td>5 - 20b</td>
<td>27.5</td>
<td>95</td>
<td>100 - 450</td>
<td>-58 ( % )</td>
</tr>
<tr>
<td>Type II MOB mixed culture</td>
<td>Mixture of gravel, clay, sand, and soil</td>
<td>0.1</td>
<td>0.45 - 22.5</td>
<td>0.3 - 15</td>
<td>46.4 - 80b</td>
<td>80</td>
<td>100</td>
<td>24 - 1200</td>
<td>NRb</td>
<td>(Gebert &amp; Grongroft, 2006)</td>
</tr>
<tr>
<td>Municipal solid waste</td>
<td>Municipal solid waste</td>
<td>0.1</td>
<td>0.3 (d) ; 0.5 (h)</td>
<td>0.005-0.02</td>
<td>2.5 - 6b</td>
<td>5.6</td>
<td>85</td>
<td>240 - 840</td>
<td>-2.75 ( % )</td>
<td>(Einola et al., 2008)</td>
</tr>
<tr>
<td>Landfill soil and earthworm cast</td>
<td>Landfill soil and earthworm cast</td>
<td>5 - 25</td>
<td>0.14 (d) ; 0.5 &amp; 1 (h)</td>
<td>0.006 - 0.22</td>
<td>31 - 560</td>
<td>100</td>
<td>4.2</td>
<td>72</td>
<td>-10</td>
<td>(Park et al., 2009)</td>
</tr>
<tr>
<td>Soil</td>
<td>Gravel or compost</td>
<td>0.7</td>
<td>0.15 (d) ; 1.35 (h)</td>
<td>0.25</td>
<td>71.2 / 65.8</td>
<td>29.2 / 12.5</td>
<td>41 / 19</td>
<td>4.3</td>
<td>65</td>
<td>(Nikiema et al., 2005)</td>
</tr>
<tr>
<td>NRb</td>
<td>Gravel</td>
<td>Gravel</td>
<td>0.3</td>
<td>0.18</td>
<td>16.2</td>
<td>54</td>
<td>6</td>
<td>11.5 - 30</td>
<td>NRb</td>
<td>(Veillette et al., 2012b)</td>
</tr>
<tr>
<td>Compost</td>
<td>Compost</td>
<td>Compost</td>
<td>NRb</td>
<td>235</td>
<td>188</td>
<td>80</td>
<td>NRb</td>
<td>(Humer &amp; Lechner, 1999)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. foninorum</td>
<td>Glass tubes</td>
<td>Glass tubes</td>
<td>0.25 – 1</td>
<td>NRb</td>
<td>750</td>
<td>735</td>
<td>10</td>
<td>0.7b</td>
<td>(Sly et al., 1993)</td>
<td></td>
</tr>
<tr>
<td>Peat, landfill and agricultural soils</td>
<td>Peat, landfill and agricultural soils</td>
<td>1.73</td>
<td>0.15 (d) ; 1 (h)</td>
<td>0.009</td>
<td>320</td>
<td>160</td>
<td>50</td>
<td>0.6</td>
<td>10</td>
<td>(Stein &amp; Hettiaratchi, 2001)</td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Gravel stone</td>
<td>Gravel stone</td>
<td>0.13 – 1</td>
<td>0.15 (d) ; 1 (h)</td>
<td>0.25</td>
<td>12 - 95</td>
<td>49.5</td>
<td>90</td>
<td>Up to 4.2</td>
<td>70</td>
</tr>
<tr>
<td>NRb</td>
<td>NRb</td>
<td>NRb</td>
<td>0.13 – 1</td>
<td>0.15 (d) ; 1 (h)</td>
<td>0.25</td>
<td>95</td>
<td>36</td>
<td>38</td>
<td>Up to 4.2</td>
<td>90</td>
</tr>
<tr>
<td>NRb</td>
<td>NRb</td>
<td>NRb</td>
<td>0.7</td>
<td>0.15 (d) ; 1 (h)</td>
<td>0.25</td>
<td>67</td>
<td>39</td>
<td>58</td>
<td>4.2</td>
<td>NRb</td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Stone</td>
<td>Stone</td>
<td>0.08 – 1</td>
<td>0.15 (d) ; 1.35 (h)</td>
<td>0.25</td>
<td>75</td>
<td>44.7</td>
<td>59.6</td>
<td>5.7</td>
<td>77.5</td>
</tr>
<tr>
<td>NRb</td>
<td>Inorganic material</td>
<td>0.3</td>
<td>0.15 (d) ; 1 (h)</td>
<td>0.18</td>
<td>20</td>
<td>13</td>
<td>65</td>
<td>6</td>
<td>22.59</td>
<td>(Veillette et al., 2011)</td>
</tr>
<tr>
<td>NRb</td>
<td>Gravel stone</td>
<td>Gravel stone</td>
<td>3.3 ( % )</td>
<td>0.15 (d) ; 1 (h)</td>
<td>0.25</td>
<td>46.7 ( \pm 0.9 )</td>
<td>18.8 ( \pm 1 )</td>
<td>( \leq 60 )</td>
<td>4.2</td>
<td>NRb</td>
</tr>
<tr>
<td>Soil</td>
<td>Soil / perlite</td>
<td>Soil / perlite</td>
<td>4.4 ( % )</td>
<td>0.35 (d) ; 1 (h)</td>
<td>NRb</td>
<td>30</td>
<td>16</td>
<td>53</td>
<td>( &gt; 180 )</td>
<td>64.1 ( % )</td>
</tr>
<tr>
<td>NRb</td>
<td>NRb</td>
<td>NRb</td>
<td>4.8 ( % )</td>
<td>0.15 (d) ; 1 (h)</td>
<td>0.25</td>
<td>71.2 ( \pm 4.5 )</td>
<td>30.0 ( \pm 2.4 )</td>
<td>42 ( \pm 1 )</td>
<td>4.25</td>
<td>75</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>Polyurethane foam</td>
<td>15.3 ( \pm 0.5 )</td>
<td>0.08 (d) ; 1 (h)</td>
<td>1.1</td>
<td>( \sim 229 )</td>
<td>24</td>
<td>11</td>
<td>4</td>
<td>60</td>
<td>(Ramírez et al., 2012)</td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Polyurethane foam</td>
<td>5</td>
<td>0.08 (d) ; 1 (h)</td>
<td>0.015</td>
<td>106.5 ( \pm 10.4 )</td>
<td>65.1 ( \pm 15.2 )</td>
<td>43 - 88</td>
<td>180</td>
<td>0.56 * 0.07 ( % )</td>
<td>(Kim et al., 2014a)</td>
</tr>
<tr>
<td>NRb</td>
<td>Inorganic material</td>
<td>0.2 – 0.9</td>
<td>18 ( % )</td>
<td>0.25</td>
<td>18.5 - 83.3</td>
<td>39.4</td>
<td>47.2</td>
<td>4.3</td>
<td>2.26</td>
<td>NRb</td>
</tr>
<tr>
<td>M. methanica</td>
<td>Glass rings</td>
<td>Glass rings</td>
<td>0 – 50.9</td>
<td>0.08 (d) ; 0.7 (h)</td>
<td>0.012</td>
<td>0.35</td>
<td>0.32</td>
<td>90.4</td>
<td>17.6</td>
<td>2.5</td>
</tr>
</tbody>
</table>

\( \text{Q} \text{[m]} \text{h}^{-1} \text{p} \text{n} \) is the filter bed volume (\text{m}) per unit time (\text{h}) at each feed rate (\text{p}). The standard deviation for each value is \( \pm 1 \% \).
3. Building materials

3.1 Building materials for biological air treatment

Building materials have been utilized previously as a direct or indirect support of the biological agents in a biofilter. Indirectly, building materials, when constructed as a building wall, provides a surface area where the biofilter system can be built on. To create the biofilter, a synthetic surface is assembled on the wall for the biological agents (i.e., the “filter”) to reside. There is a gas passage in between the synthetic material and the building wall, hence, the biofilter is not in direct contact with the building wall. In this closed system, air passes through the “filter” using forced force created by a fan. Biodegradation of pollutant takes place on the “filter” and the “clean air”, after passing through the “filter”, is recirculated back via the gas passage to the place where the air was taken. An example of this system is the use of the biofilter to maintain indoor air quality of a building. The biofilter can be placed inside the building or built separately outside the “treated” building. This type of biofilter has been applied to remove pollutants such as different VOCs (e.g., formaldehyde), particulates, and monoaromatics (e.g., toluene, ethylbenzene) among others (Darlington et al., 2000; Lu et al., 2012). These components are known to have adverse effects on human health (e.g., sick building symptoms) (Jones, 1999; Wallace, 2001). The use of a biofilter to improve indoor air quality has also proven to be more efficient compared to other technologies such as filtration and adsorption (Guieysse et al., 2008; Soreanu et al., 2013).

The biological agents in the biofilter are different type of plants and microorganisms known to degrade various pollutants. Examples are mosses (e.g., Plagiomnium cuspidatum), hydroponic, and pseudo wetlands (Darlington et al., 2001). The microbial community mostly resides within the plant network (e.g., on the plant roots) and together they act as the biocatalyst to break down the pollutants. Compared with traditional biofilter that houses only microorganisms, the addition of plant gives an extra advantage of providing the bacteria with carbon and energy sources such as acetate from the exudates (Guieysse et al., 2008; Sandhu et al., 2007). Thus, the technology can be applied to degrade pollutants that cannot be degraded by the plant at a very low concentration as there is enough nutrients for the bacteria to survive. The plant leaves also increases the surface area of the filter thus making the degradation process more efficient. However, indoor biofiltration can also give some disadvantages, for example, by increasing the probability of mold growth in the building due to the higher moisture content in the recirculated air (Soreanu et al., 2013).

Other application of indirect biofiltration is the creation of “living wall” (Ottele et al., 2011; Perini et al., 2011). This is a modular system containing plants and soil attached on a synthetic layer on a wall. The system can be placed either inside a building or on external wall of a building. For outdoor application, the main aim of the “wall” is to reduce the heat effect of the sunlight during the summer time and provide insulation during winter time (Fernandez-Canero et al., 2012). The plant leaves reflects and absorb the sunlight directed to the building thus reducing the heat effect in the building. Additionally, for both indoor and outdoor applications, in line with the biofiltration concept explained previously, the “wall” can act as an air purifier and provide aesthetic added value (Feng & Hewage, 2014). For outdoor application, beside reducing pollutants, the plants also use carbon dioxide for photosynthesis process.

Similar to the classical biofilter, building materials can also act as a direct support for the biological agent. An example is the creation of green façade system (Ottele et al., 2011; Perini et al., 2013). This is basically the attachment of biological agents on existing building surface. The attachment can be formed using a “climber” planted on the ground (e.g., plants such as evergreen). The “climber” grows vertically and attaches directly to the wall or by means of steel cable or trellis (Perini et al., 2011). The
application of green façade is aimed to reduce heat accumulation in the building, be an air purifier, and provide aesthetic value.

Besides the use of a climber, the building wall can also undergo direct colonization by one or several groups of living organism (Guillitte, 1995; Miller et al., 2012). These organisms can be different kind of plants, algae, fungi and bacteria. After successful biological colonization and growth, a “green layer” consisting of those organisms acts similarly to the organisms on the surface of the green façade. To provide good colonization surface, building materials need to possess several properties that makes the material susceptible for the organisms to grow. These include suitable pH, chemical composition, porosity, and surface roughness of the materials (Manso et al., 2014a; Manso et al., 2014b). Example of the use of building material as the support for air biofiltration can be seen in Figure 1.5. Overall, based on the previously mentioned studies, it can be concluded that building materials have the capability to be the carrier materials for a typical air biofilter. Hence, it can also be a good candidate for the carrier material of a methane biofilter.

![Figure 1.5](image)

**Figure 1.5.** (a) direct outdoor greening system. (b) indirect outdoor greening system. (c) indirect outdoor greening system combined with planter boxes. All three systems function as air biofilters on top of other functions. Source: Perini et al, 2013

### 3.2 Biotechnological applications in the construction industry

Building material colonization by microorganisms can have a negative impact on building material. These microorganisms have proven to be contributing to the weathering of building material by producing deleterious substances (Gaylarde et al., 2003; Saiz-Jimenez, 1997). As a result, building material deteriorates and the material lifetime decreases (Gaylarde et al., 2003; Warscheid & Braams, 2000). However, as explained in section 3.1, when used as a biofilter, these microorganisms have proven to be beneficial. Microorganisms have also been used for some other applications in the construction industry. The two main applications that have been studied extensively are the use of the bacteria as the biocatalysts for building material surface protection and the self-healing of concrete (see PhD thesis of Willem De Munyck (2009) and Jianyun Wang (2014)). In both applications, the capacity of the bacteria to induce mineral precipitation is used as a mean to conserve building materials.

#### 3.2.1 Microbiologically Induced Carbonate Precipitation (MICP)

Microbiologically Induced Carbonate Precipitation (MICP) is the production of carbonate mineral driven by environmental condition (e.g., pH) alteration as a result of microbial activity. Beside MICP, biomineralization can also be biologically controlled. Here, the microorganism completely regulates the mineralization process regardless of the environmental conditions and this normally takes place in the intracellular of the cell (i.e., within the organic matrices or vesicles inside the cells). An example being the formation of iron minerals by *Geobacter metallireducens* and *Shewanella putrefaciens* (Baeuerlein, 2006; Skinner, 2005). MICP forms one of the biogeochemical processes existing in the nature and for
In the past two decades, its engineering applications have been studied extensively (Le Metayer-Levrel et al., 1999; Stocks-Fischer et al., 1999). For example, the carbonate minerals from MICP plug cracks in rock layers naturally or by human intervention at an oil reservoir (Adkins et al., 1992; Cunningham et al., 2013). In the marine system, MICP occurs as a result of a change in the carbon dioxide mass balance in the seawater (e.g., carbon dioxide production from the bacterial anaerobic degradation or organic matter) (Morita, 1980). In the area of environmental biotechnology, MICP was applied for the removal of calcium from the wastewater treatment or the sequestration of metal (e.g., copper, strontium) from soil and groundwater (Achal et al., 2011b; Hammes et al., 2003b; Warren et al., 2001). MICP has also been utilized for soil strengthening process (Harkes et al., 2010; Whiffin et al., 2007).

MICP is driven by the imbalance of the carbonate equilibrium (equation 1.5). This occurs as a result of the pH shift or the production / consumption of one of the carbonic species. Microbial activity basically influences those two factors.

\[ \text{CO}_2(g) \leftrightarrow \text{CO}_2(aq) + \text{H}_2 \text{O} \leftrightarrow \text{H}^+ + \text{HCO}_3^- \leftrightarrow 2\text{H}^+ + \text{CO}_3^{2-} \]  

(1.5)

\( \text{H}_2\text{O} \) represents the dissolved gaseuous carbon dioxide and the carbonic acid itself. The \( pK_a \) and \( pK_c \) of carbonic acid (including the dissolved carbon dioxide) and bicarbonate, respectively, are 6.35 and 10.32 at 25º C and 1 atm.

MICP can be influenced by the microorganism autotrophic or heterotrophic metabolic processes. The main difference between these two pathways is the type of component in the carbonate balance that is utilized by the microorganism. The autotrophic pathway induces carbonate precipitation due to the (gaseous) carbon dioxide utilization by microorganisms (e.g., methanogenesis and photosynthesis) using the carbonate anhydrase enzyme (Ehrlich, 1998). The transport of carbon dioxide to the cell’s internal environment is coupled with the active transport of bicarbonate ions. This bicarbonate ions subsequently react with calcium ions to form calcium carbonate (Zavarzin, 2002).

The heterotrophic pathway is performed by bacteria and the MICP driven by this pathway can be divided into passive and active precipitations (Castanier et al., 1999). Active precipitation occurs due to the ionic exchange through the cell membrane. Passive precipitation takes place as a result of the bacterial organic matter degradation. This passive precipitation involves the bacterial nitrogen and sulphur cycles. These are: (a) the ammonification of amino acid, (b) the dissimilatory reduction of nitrate, (c) the degradation of urea or uric acid, and (d) dissimilatory reduction of sulphate (Castanier et al., 1999). The ammonification of amino acid and the degradation of urea or uric acid occurs at aerobic condition whereas dissimilatory reduction of nitrate takes place at anaerobic or microaerophily condition.

All heterotrophic pathways induce calcium carbonate precipitation by directly producing carbonate ions or by influencing (i.e., increasing) the pH of the environment. For example, in the degradation of urea, pH is increased due to the ammonium ions production (Stocks-Fischer et al., 1999). This pH increase occurs locally at the microenvironment of the bacteria as a result of the electrochemical calcium and hydrogen ions gradients in concomitant with the organic compound degradation (Hammes & Verstraete, 2002). At high (local) pH, the carbonate equilibrium shifts to higher carbonate ions speciation among other dissolved inorganic compounds (equation 1.5). In the presence of calcium ions, the produced carbonate ions react with the calcium ions to form calcium carbonate and when the saturation state (\( \Omega \)) of the mineral is overcome (i.e., the ion activity product is higher than the solubility constant (\( K_{\text{sat}} \)), then calcium carbonate is precipitated (Stocks-Fischer et al., 1999).
\[ \text{Ca}^{2+} + \text{CO}_3^{2-} \leftrightarrow \text{CaCO}_3 \]  
\[ \Omega = \frac{a(\text{Ca}^{2+}) \times a(\text{CO}_3^{2-})}{K_{\text{so}}} \]  

\( \text{a} \) represents the activity coefficient of the ion and the numerator in equation 1.7 is the ion activity product. For simplification, ideal condition is assumed and the activity coefficient is equal to 1 and the activity can be approximated into the concentration of the ion. \( K_{\text{so}} \) of calcite at 25 \(^\circ\text{C} \) is \( 4.8 \times 10^{-9} \)

Besides influencing the pH, and carbonate balance, the bacteria can also act as the nucleation site of the calcium carbonate crystallization process (Ferris et al., 1987). Due to the electronegative charge of the bacterial membrane as a result of the presence of different charged groups, the cell wall can bind to the calcium ions and the precipitation can take place on the surface of the bacteria. Moreover, biofilm and Extracellular Polymeric Substance (EPS) produced by the bacteria may also play a role in binding the calcium ions and act as the nucleation site (Rodríguez-Navarro et al., 2003). Illustration of the heterotrophic (i.e., urea degradation) induced calcium carbonate precipitation can be seen in Figure 1.6.

**Figure 1.6.** (a) Illustration of calcium carbonate precipitation induced by the bacterial urea degradation. Illustration by Tim Lacoere

Another bacterial metabolic process that can drive MICP is the conversion of an organic acid into weaker acid. This process resulted in the production of bicarbonate and carbonate ions and when calcium ions are available, then calcium carbonate is formed. Soil bacteria are known to convert acetate and oxalic acid to weaker carbonic acid (Braissant & Verrecchia, 2002; Braissant et al., 2002). Looking at the metabolic process of MOB (Figure 1.3), the bacteria are capable to transform formate to carbon dioxide (carbonic acid in a solution; equation 1.5). The process, together with the availability of the bacteria as nucleation site and provision of external calcium, ticks all the requirements needed for calcium carbonate precipitation. MICP by MOB has not been investigated before and this can open up a new MICP alternative. Overall, due to the capacity of bacteria to induce calcium carbonate precipitation, both civil and environmental engineers saw the process to be potentially applied for the consolidation of building material. Henceforth, for the past two decades, several researches have dedicated their work in this topic.

### 3.2.2 Building material surface protection by MICP

When applied as building material surface protectant, the mineral precipitate from MICP is deposited on the surface of the material to decelerate the weathering processes by giving waterproofing and strengthening effects (Barabesi et al., 2003; Tiano, 2004). MICP has been applied on different types of stone- and cementitious-based building materials (e.g., concrete and ornamental stone). Previously, the application of inorganic and organic chemical-based treatment using, for example, synthetic resin such as epoxy, proved not to be effective due to the incompatibility of the new protective film surface, unstable molecular structure of the film, high service cost, and its contribution to the environmental pollution (Rodríguez-Navarro et al., 2003). Moreover, the use of limewater (\( \text{Ca(OH)}_2 \)) solution also gave
insufficient consolidating effect (Price et al., 1988). Alternatively, MICP exhibited higher consolidating effect due to the presence of the biomaterial (e.g., the cells, EPS, and biofilms) in the protective mineral layer (Dick et al., 2006; Zamarreno et al., 2009). This biologically induced mineral precipitate was apparently also less soluble than the chemically produced ones (Rodriguez-Navarro et al., 2003). Hence, compared to other treatments, MICP is a relatively more effective method to conserve building materials. Example of the resulting carbonate mineral precipitation on building materials can be seen in Figure 1.7.

Investigation of MICP on building materials have been conducted using different metabolic pathways, bacteria, media, and delivery methods (For extensive review, see De Muynck et al, 2010a and references therein). As explained in section 3.2.1, MICP can be driven using different metabolic pathways. For building surface protection, then applicable pathways are the aerobic urea hydrolysis, organic acid utilization, and amino acid oxidation. Previous studies were therefore conducted using strains or cultures that possess these pathways.

Several bacterial strains have been tested for their capacity to induce carbonate mineral precipitation. *B. cereus* was capable to drive 0.6 gram of calcium carbonate to precipitate per gram of organic matter metabolized (Castanier et al., 1999). *Myxococcus xanthus* has also been tested to conserve ornamental stone (Jroundi et al., 2010). Dick et al tested the capacity of six different *Bacillus* strains (i.e., five *Bacillus sphaericus* and one *Bacillus lentus*) to consolidate Euville limestone (Dick et al., 2006). These authors also indicated that among different bacterial parameters, the bacterial ζ potential (i.e., measure of the surface potential), specific initial urea degradation, and tendency to form continuous dense calcium carbonate layers are the most important properties for their effectiveness to restore limestone. Several other bacterial strains that have been tested for their capacity to induce carbonate mineral precipitation and applied on building materials are the ones from *Pseudomonas, Arthrobacter, Pantoea, Cupriavidus, Acinetobacter*, and *Halobacillus* genera (Daskalakis et al., 2013; Jroundi et al., 2012; Park et al., 2010; Rivadeneyra et al., 2004; Zamarreno et al., 2009). Besides the use of pure cultures, De Munynck et al tested the capacity of ureolytic mixed culture for their capacity to treat concrete surface (De Munynck et al., 2008a). However, they concluded that *B. sphaericus* was a better biocatalyst compared to this mixed culture.

Beside external addition of bacterial culture on the building material, several researches have tried different approaches. Sterile medium was added on non-sterile stone-based building material to activate the capacity of autochthonous bacteria to induce carbonate mineral precipitation (Jimenez-Lopez et al., 2011).
2008). Jroundi et al found that the bacterial community inhabiting the target stone of the conservational building was dominated by the ones from the *Gamma* - and *Betaproteobacteria* phyla (e.g., from the *Rhizobium*, *Brevundimonas*, and *Comamonas* genera) (Jroundi et al., 2010). Using this bacterial community, they successfully induced MICP on the stone and consolidate the material. They also considered the practice safer in terms of limiting the growth of acid producing bacteria and economically more feasible as they didn’t have to grow specific bacterial strain known to induce the precipitation. However, a drawback of using this method, when depending solely on the activity of indigenous bacteria, is the lower mineral production rate as the community is not enriched. Hence, it takes significantly more time for the mineral precipitate to effectively consolidate the building material and this renders the process inefficient. The effectiveness of this method is also dependent on the nature of the indigenous bacterial community of the stone. Thus, when different community is present or the community is altered (e.g., when using different stone or by applying biocide, a common practice prior to the treatment), different consolidation effect might be obtained from the one reported by those authors. Organic Molecular Matrix (OMM) extracted from the sea shell or bacterial cell wall have also been tried as the biocatalyst of MICP on building material (Pei et al., 2013; Tiano et al., 1999). However, due to the practicality issue for the extraction process and the low precipitate yield, the use of OMM is regarded to be inefficient.

Selection of organic matter and microorganism strain determines the MICP pathway taken. Oxidative deamination of amino acid by *M. xanthus* was induced due the presence of bacto-casitone (i.e., pancreatic digest of casein) in the medium (Jroundi et al., 2012). The bacteria utilized the component as their carbon and energy sources. Furthermore, the medium matrix determines the mineral phase of the precipitates (Zamarreno et al., 2009). For example, the presence of chloride ions during the precipitation process increases the probability of rhombohedral crystals formation (De Muynck et al., 2008a). Rhombohedral calcite is known to exhibit higher consolidating effect than, for example, the acicular vaterite crystals (Rodriguez-Navarro et al., 2003). Vaterite is a metastable crystal phase formed at significantly higher mineral supersaturation than calcite. Due to this crystal thermodynamic instability, vaterite may convert into calcite over a prolonged period. On the other hand, the addition of chloride based salt is not preferred when applied on concrete due to its detrimental effect (i.e., ingestion of chloride ions into the concrete leads to the corrosion of the reinforced steel) (Van Tittelboom et al., 2010). Carbohydrate is also preferably not added as a carbon source to avoid bacterial acid production which can inhibit the alkalinization of the environment (Daskalakis et al., 2013). Different type of media that have been used for the concomitant bacterial growth and precipitation process for the surface treatment of building material are, among others, M3 and M3P (Barabesi et al., 2003; Jimenez-Lopez et al., 2007), CC (Jimenez-Lopez et al., 2008), B4 (Tiano et al., 1999), and nutrient media (Dick et al., 2006).

Besides the medium matrix, the organic matter starting concentration also influences the protective effectiveness of MICP. Logically, a high starting concentration is preferred for a high mineral precipitate production to give a better consolidating effect. However, this linear correlation is not necessarily obtained. A high calcium salt concentration could induce stress condition to the bacteria which limits their capacity to induce carbonate mineral precipitation (Rivadeneyra et al., 2004). Moreover, when higher amount of calcium carbonate is produced, the bacterial activity can be inhibited as the substrate transport rate into the cell is lowered. This occurs as a result of the mineral crystal presence around the cell. Considering the size of the bacteria and the pore network of the building material, there is only limited penetration depth for the bacteria into the material and limited room for the resulting crystal to fill. Hence, there is a maximum substrate starting concentration for the bacteria to metabolize to produce the necessary mineral amount to fill the pore. Le Metayer-Levrel et al could only obtain several
micrometer thick of bacterial penetration depth into the building material (Le Metayer-Levrel et al., 1999) whereas De Muynck et al could only reach 35-50 µm deep (De Muynck et al., 2008b). A 1 mm depth of newly formed carbonate was obtained by Rodriguez Navarro et al and this was attributed to the gliding motility of M. xanthus (Rodriguez-Navarro et al., 2003). De Munynck et al observed 2 mm biomineralization penetration depth in Savonnières and Euvillé limestone as those two types of stone exhibited a high porosity thus the bacteria could penetrate deeper into the material (De Munynck et al., 2011). In conclusion, there is an optimum substrate concentration to obtain an effective protective effect on building material by means of MICP. De Munynck et al found that for a given amount of bacterial cells, when using the urea hydrolysis pathway, the optimum calcium and urea dosage to give an effective waterproofing and consolidating effect are 20 g L\(^{-1}\) and 50 g L\(^{-1}\), respectively (De Munynck et al., 2010b).

The method to apply the bacterial culture on the building material is also important to have an effective building material protection. For proof of principle experiments, the material is typically immersed in the bacterial culture (De Munynck et al., 2011; Dick et al., 2006). More specifically, the bacteria are grown until specific cultural density is reached. The culture is subsequently separated from the medium supernatant before redissolving it in the precipitation medium. The building material is then immersed in this solution. However, for practical purpose, especially for existing building material, different method should be employed. Brushing and spraying the bacterial culture are the two main practical applications (Le Metayer-Levrel et al., 1999). Jroundi et al concluded that based on the industrial and economical constraints, the building material may be sprayed twice every day for seven days to obtain effective consolidating effect (Jroundi et al., 2010).

Starting culture density is a critical parameter to obtain sufficient mineral precipitate to fill building material pores. Tiano et al and Jimenez et al used approximately 10^6 cells ml\(^{-1}\) of B. subtilis and M. xanthus, respectively (Jimenez-Lopez et al., 2007; Tiano et al., 1999). Dhami et al concluded that a starting culture density of 10^7 cells ml\(^{-1}\) B. megaterium was sufficient to improve the durability of concrete (Dhami et al., 2013). Achal et al and Chahal and Siddique investigated the influence of starting culture density of Sporosarcina pasteurii (10^3, 10^5, and 10^7 cells ml\(^{-1}\)) to the improvement of the compressive strength of fly ash concrete (Achal et al., 2013; Chahal & Siddique, 2013). Achal et al observed that by applying 10^5 cells ml\(^{-1}\), the highest increase (22 %) of compressive strength of the concrete could be obtained.

Besides the microbiological and chemical aspects of MICP, the effectiveness of the biological surface treatment is influenced by the characteristic of the applied material and the environmental conditions in which the process takes place. Temperature influences bacterial activity and therefore affects the capacity of bacteria to drive MICP. B. sphaericus was the most suitable biocatalyst among other tested strains for limestone consolidation at 10 °C, 20 °C, 28 °C, and 37 °C (De Munynck et al., 2013). Temperature also influences the morphology of the precipitated crystals (Zamarreno et al., 2009). The pore structure of the building material is found to have a great effect on the surface treatment. De Munynck et al found that the biodeposition treatment exhibited greater effect on macroporous stones (De Munynck et al., 2011). Rodriguez Navarro et al also concluded that the type of building material is the overruling factor in determining the type of deposited biogenic crystal polymorph (Rodriguez-Navarro et al., 2012). Thus, based on previous studies, the operating conditions, the type of building material, and the medium matrix (as previously explained) are the decisive parameters determining carbonate crystal polymorphs for the MICP application. The overview of MICP approaches for building material surface treatment can be seen in Table 1.6.

If MICP driven by the formate oxidation by MOB as explained in section 3.2.1 can be proven, that MICP can give a more environmentally friendly approach for building material surface treatment compared to
the urea-based approach as there is no potential by-products that can harm the environment. The bacteria can also be used to remove methane from the air.

### 3.2.3 Self-healing of concrete by MICP

Other than being used as the driver to produce surface protectant for building material, MICP is also utilized to heal crack in concrete. Concrete, as they aged, exhibits cracks that are caused by multiple factors (e.g., weathering process, material stress, etc). These cracks hamper the mechanical integrity of the material and subsequently leads to the material failure. In order to repair the crack, the concrete structure needs to be maintained after a certain period of its installation. This gives rise to higher maintenance cost due to the additional man hours and other variable cost. Additionally, concrete production process is considered as non-sustainable as it contributes to 7% of the global CO$_2$ emission (Worrell et al., 2001). Considering these two factors, producing a concrete that has the capacity to autonomously heal its own crack might help in decreasing the maintenance cost caused by the concrete crack and the need to produce additional concrete due to the concrete aging related failure.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic pathway (substrate(s) used)</td>
<td>Oxidation of amino Acid (proteins and sodium carbonate), Organic acid utilization (calcium lactate, calcium acetate), Urea hydrolysis (urea and calcium chloride)</td>
</tr>
<tr>
<td>Biocatalysts</td>
<td>Pure strains from <em>Pseudomonas</em>, <em>Arthrobacter</em>, <em>Pantoaea</em>, <em>Cupriavidus</em>, <em>Acinetobacter</em>, <em>Bacillus</em>, <em>Myxococcus</em>, and <em>Halobacillus</em> genus; Ureolytic mixed culture; extracted organic molecular matrix; bacterial cell wall</td>
</tr>
<tr>
<td>Applications method</td>
<td>Immersion of building material in bacterial culture, bacterial culture spraying (with/without subsequent sterile medium spraying), brushing, paste application on building material, activation of indigenous bacterial community</td>
</tr>
<tr>
<td>Starting culture density</td>
<td>$10^3$ – $10^9$ cells ml$^{-1}$</td>
</tr>
<tr>
<td>Tested building material</td>
<td>Different limestone (e.g., Euville, Maastricht limestone), concrete</td>
</tr>
</tbody>
</table>

Self-healing concrete is a type of concrete that can heal the material’s crack autonomously by means of healing agent. The healing agent is incorporated into the cement mixture when constructing the concrete specimen. When crack appears, the healing agent is activated and fills the crack. Different polymer based components, have been investigated as the healing agent. For example, the use of expansive additive such as calcium sulfoaluminate-based agents and crystalline admixture, where upon ingression of water into the crack, reacts with water to form ettringite crystals which fill the crack (Van Tittelboom & De Belie, 2013). Another approach is to incorporate the healing agent in a capsule based material using two parts epoxy resin. When crack impacts the capsule and ruptures it, the healing agent leaks out from the capsule and fills the crack (Mihashi & Nishiwaki, 2012).

The use of bacteria to induce calcium carbonate precipitation for the self-healing concrete has been investigated for almost a decade (Jonkers & Schlangen, 2008; Van Tittelboom & De Belie, 2013). Here, the resulting calcium carbonate precipitate is used as the “healing agent” of the crack. Urea hydrolysis and *Bacillus* spp. are the typical bacterial metabolic pathway and bacterial strains, respectively, utilized to drive the precipitation process. This is due to the high carbonate specific production rate, the alkali-tolerant nature of the bacteria, and the capacity of the bacteria to form endospore (Hammes et al., 2003a; Jonkers et al., 2010). Hence, for this self-healing concrete application, the possibility of MOB to be used as the biocatalyst is excluded due to the incapacity of the bacteria to produce endospores.
The bacterial spores are incorporated into the concrete matrix during concrete processing. Due to the harsh environment of the process (i.e., high temperature and pH) and the need to maintain the spores’ viability / functionality, these spores are typically immobilized in an entrapping agent. Wang investigated different types of *B. sphaericus* spores immobilization material such as silica gel, polyurethane, and diatomaceous earth that gives the highest healing effectivity (Wang, 2013). From her PhD thesis it was concluded that a high concrete strength regain and lower water permeability reduction were obtained when the spores were immobilized in polyurethane (Wang et al., 2012a). Currently, research of self-healing concrete focuses on how to lower the production of cost of self-healing concrete and the investigation of the use of anaerobic MICP (i.e., denitrification) for self-healing concrete.

4. Objective of the thesis

The objective of this PhD thesis is to fill the research gaps pointed out previously in this chapter. The aim of this research is to investigate whether development on biotechnological applications using MOB can be made when the bacteria are immobilized on building materials. This work is divided into two parts. Three chapters deal with the exploration of the capacity of the immobilized MOB to remediate methane gas. The other two chapters deal with the investigation of the bacteria as the biocatalyst for building material surface protection. The chapters of the thesis are as follows:

**Part 1: Housing Methane-Oxidizing Bacteria on building materials for methane bioremediation**

**Chapter 2**

Main goal: to investigate the capacity of MOB to remove methane when the bacteria are immobilized on building material.

MOB pure strains and mixed culture were selected and their capacity to remove methane when immobilized on different building materials at high (~20 % (v/v)) and low (~100 ppmv) methane concentrations was investigated. MOB culture selection was based on the previous kinetic studies in liquid culture and the biotechnological applicability of the culture. Building material selection was based on the application niche and the material characteristics.

Outcome: The MOB-building material combination that exhibited the highest methane removal was selected for the biofilter study (Chapter 3)

**Chapter 3**

Main goal: to investigate the capacity of MOB to remove methane when the bacteria are immobilized on building material in a biofilter setup.

The selected MOB-building material combination in Chapter 2 was used as the engineering basis to design methane biofilter. A methane biofilter equipped with building material was inoculated with MOB and fed with methane at low concentration (~1000 ppmv) for four months. The biofilter performance was evaluated. using different engineering parameters (Table 1.4)

Outcome: The methane biofilter that had been operated in the lab was used to remediate methane livestock gas waste (Chapter 4).

**Chapter 4**

Main goal: to investigate the capacity of MOB in a methane biofilter setup (Chapter 3) to remove methane emitted from livestock.
In cooperation with ILVO Vlaanderen, the biofilter in Chapter 3 was set in a respiration chamber that was typically used to monitor methane emission from dairy cows. In a two days study, methane emitted from dairy cows that had been placed in the chamber was fed into the filter and the methane removal capacity of the MOB in the filter was studied. Several distinguished operating conditions differed from the lab scale ones were: (a) a much lower methane concentration (~100 ppmv), (b) a dynamic methane Inlet Load (IL) that was dependent on the cow’s feeding and ruminating period, and (c) the presence of other components (e.g., ammonia) in the effluent gas.

**Outcome:** The feasibility of the methane biofilter for bioremediation application using building material as the carrier material was given

**Part 2: Housing Methane-Oxidizing Bacteria on building material for building material surface protection**

**Chapter 5**

**Main goal:** to investigate the capacity MOB to drive calcium carbonate precipitation from the dissimilatory formate oxidation pathway.

The MOB culture that gave the highest methane removal from the test conducted in Chapter 2 was selected as the biocatalyst to drive MICP. Due to the capacity of the bacteria to oxidize formate to carbon dioxide, calcium formate was used as the substrate.

**Outcome:** Optimized MICP condition(s) was used as the basis for the application of the process to treat building material surface (Chapter 6).

**Chapter 6**

**Main goal:** to investigate the effectiveness of MOB based MICP as an alternative building material surface protection.

The building material giving the most suitable carrier material for the highest methane removal by MOB in Chapter 2 was selected as the model material and the MOB culture used in Chapter 5 was selected as the biocatalyst. A proof of concept of MICP on building material and the influence of the process on building material characteristics were given. Furthermore, the effectiveness of the surface treatment was evaluated.

**Outcome:** The feasibility of formate-driven based MICP using MOB as the biocatalyst as the basis for building material surface treatment was given.

**Chapter 7**

A general outlook of the research outcome from this thesis is presented. Conclusions are drawn and suggestions for future research are given.
PART 1

HOUSING METHANE-OXIDIZING BACTERIA ON BUILDING MATERIALS FOR METHANE BIOREMEDIATION

Overview

Part 1 deals with the exploration of building material utilization as the carrier material for methane biofiltration. Part 1 is divided into three chapters. It started with a study of the selection of the best possible combination of building material-Methane Oxidizing Bacteria (MOB) culture that exhibited the highest methane removal rate (Chapter 2). The results from this study were used as the basis for a methane biofilter engineering design and test in the lab scale environment (Chapter 3). After this test, the lab scale biofilter was used in a field test to remove methane from livestock effluent gas where different conditions than the ones of the lab were found (Chapter 4). The results obtained from studies in Chapter 2 to 4 were examined and the application suitability of building material as a carrier material for a methane biofiltration technology was assessed in Chapter 7.
CHAPTER 2
ATMOSPHERIC METHANE REMOVAL BY METHANE-
OXIDIZING BACTERIA IMMOBILIZED ON POROUS BUILDING
MATERIALS

Abstract

Biological treatment using Methane-Oxidizing Bacteria (MOB) immobilized on six porous carrier materials have been used to mitigate methane emission. Experiments were performed with different MOB inoculated on building materials at high (~20 % (v/v)) and low (~100 ppmv) methane concentrations. *Methylocystis parvus* in Autoclaved Aerated Concrete (AAC) exhibited the highest methane removal rate at high (28.5 ± 3.8 µg CH$_4$ g$^{-1}$ building material h$^{-1}$) and low (1.7 ± 0.4 µg CH$_4$ g$^{-1}$ building material h$^{-1}$) methane concentrations. Due to the higher volume of pores with diameter > 5 µm compared to other materials tested, AAC could likely adsorb more bacteria which might explain for the higher methane removal observed. The total methane and carbon dioxide-carbon in the headspace was decreased for 65.2 ± 10.9 % when *M. parvus* in AAC was incubated for 100 hours. From this study it was shown that immobilized MOB on building materials could be used to remove methane from the air and also act as carbon sink.

This chapter has been redrafted after:


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1. Introduction

Methane is the second most important greenhouse gas (GHG) after carbon dioxide contributing to atmospheric global warming. With global emissions above 600 Tg per year, methane represents approximately 14% of the total anthropogenic GHG emissions (Bernstein et al., 2007; Dlugokencky et al., 2011). Average methane concentration in the atmosphere is around 1.8 ppmv (Figure 1.1), but higher values are observed in places close to the emission sources, e.g., ~100 ppmv in livestock barns (Jungbluth et al., 2001). Due to the increasing global population, and hence, food demand, methane emission from livestock sector will experience one of the highest growth between 2010 to 2030 (Chapter 1 section 1.3). Therefore, strategies need to be implemented to mitigate methane emission from this sector.

Methane gas waste can be treated by direct combustion (i.e., flaring) or be used as a source of biofuel (Chapter 1 section 1.4). However, a high methane concentration is needed for these solutions to be viable. Considering the low methane concentration in livestock barns, biological treatment using methane-oxidizing bacteria (MOB) is a viable option due to the low investment, energy and operating cost (Nikiema et al., 2007; Veillette et al., 2012a).

MOB are able to utilize methane via the methane monoxygenase (MMO), a key enzyme in biological methane oxidation (Chapter 1 section 2.2). Briefly, MMO is responsible for the first step of methane oxidation to methanol. There are two types of MMO: the soluble MMO (sMMO) and the particulate MMO (pMMO). Located in the cytoplasmic membrane, pMMO has been detected in almost all MOB, except within the Methylocella and Methyloferula genus. On the other hand, sMMO, which is located in the cytoplasm, has only been found in several MOB species (e.g., M. trichosporium OB3B). Both MMOs are known to be nonspecific and have been used to degrade pollutants such as different alkanes and aromatic compounds (Semrau et al., 2010) with sMMO having broader range of substrates than pMMO. Overall, MOB have a potential to be used in biotechnology not only as the biocatalyst to reduce methane emissions but also to degrade a wide range of pollutants.

MOB have been used in a biofilter or biotrickling filters system to remove methane emission in landfill-cover soil, livestock barns and manure storages (Chapter 1 section 2.4). Methane emission treatment using immobilized bacteria has several advantages compared to when suspended bacteria were used (e.g., higher conversion rate). The most important factors to have a high methane removal rate by the immobilized MOB are the high surface area and porosity of the bacterial support (Chapter 1 section 2.4). Among different types of materials, building materials having a high porosity and surface area may provide a niche for these MOB.

In the last decade, bacteria have been applied on building materials for different purposes (for detailed explanation, see Chapter 1 section 3.2). Briefly, bacteria capable of producing calcium carbonate have been applied on stone as a surface treatment and to infer self-healing properties in concrete. For example, biodeposition on the surface of concrete could improve the durability of the material (De Muynck et al., 2008b) and bacteria immobilized on diatomaceous earth were capable to fill cracks in concrete by means of precipitation (Wang et al., 2012b).

This study initiated the first part of this thesis and it was aimed to select the best possible MOB-building materials combination where MOB could exhibit the highest methane removal capacity. More specifically, this study reported the kinetics of methane removal by MOB enriched on different inorganic materials. Firstly, selection of MOB having the highest methane removal capacity on building materials
was done. Secondly, an optimization of methane removal by the selected MOB culture was performed by varying different parameters (i.e., immersion time and starting culture density).

2. Materials and Methods

2.1 Building Materials

Experiments were performed on six types of building materials: (i) Autoclaved Aerated Concrete (Ytong, Belgium), (ii) Maastricht and (iii) Euville limestone, and three types of bricks ((iv) Safari Geel Bezand (Brick A), (v) Rocher rood (Brick B), and (vi) Tenere (Brick C) (Wienerberger, Belgium)). Selection was based on their application and porosity data. Ytong, Autoclaved Aerated Concrete (AAC), exhibits high porosity which makes it a good candidate for the experiment. Bricks are one of the most used building materials in livestock barns, especially in places that lack natural stones (e.g., in Flanders, Belgium). Maastricht and Euville limestones are described in detail elsewhere (De Muynck et al., 2010b). Building materials were stored at 28 °C prior to use.

2.2 Porosity analysis

Before analysis, six types of building materials (as mentioned previously) with dimensions of: 1 cm x 1 cm x 1 cm were dried at 70 °C until the weight losses were less than 0.1% (w/w). The porosity of building materials was analyzed in duplicate by Mercury Intrusion Porosimetry (MIP) using Pascal 140 and 440 porosimeters (Thermo Fischer Scientific, Belgium) according to the method described previously (Aligizaki, 2006).

2.3 Microorganisms

The selection of MOB was based on several criteria. Due to the alkaline nature of chosen building materials (Table S1), two alkali-phobic MOB were selected: Methylophilum alcaliphilum DSM 19304T and Methylophilum kenyense DSM 19305T. They are both type I MOB and grow optimally at pH 9 (M. alcaliphilum) and pH 10 (M. kenyense) (Kalyuzhnaya et al., 2008). As part of the experiments were performed at low methane concentration, two type II MOB were selected: Methylosinus trichosporium NCIMB 11131T and Methylocystis parvus NCIMB 11129T. Based on kinetic studies by Knief and Dunfield (Knief & Dunfield, 2005), M. trichosporium required a minimum of ~100 ppmv methane concentration necessary for cell maintenance, which is within the range of the methane emission level observed in dairy farm (Jungbluth et al., 2001). Thus, this strain could have a better survivability when applied on building material in livestock barns. M. parvus are known to accumulate Poly-3-hydroxybutyrate (PHB) (Pieja et al., 2011a) and MOB accumulating PHB could survive better at low methane concentration (Knief & Dunfield, 2005). Methylomonas methanica NCIMB 11130T (Type I) was also tested as this strain has been successfully immobilized in a biofilter setup to treat methane emission in coal mines (Apel et al., 1991). Additionally, an MOB mixed culture was tested. It was enriched from a moderately alkaline (pH 7.9) cropland clay soil originating from Gent, Belgium. Mixed culture enrichment was performed in Nitrate Mineral Salt (NMS) medium (Whittenbury et al., 1970). Pure cultures were grown in NMS medium and modified NMS medium (Khmelenina et al., 1997) for non-alkaliphilic (pH 6.8) and alkaliphilic MOB (pH 9), respectively. Bacterial enrichment for both pure cultures and the mixed culture was always performed in serum bottles under ~20% (v/v) methane concentration and the bottles were incubated on a shaker (120 rpm) at 28° C.
2.4 Bacteria cell counts

The culture density was measured by means of a CyAN™ ADP LX flow cytometer (Dakocytomation, Belgium) according to the live/dead staining protocol as described previously (De Roy et al., 2012). For each bacterial culture, a relationship was made between the culture density and the Optical Density (OD) value ($\lambda = 610$ nm). The OD value of the culture was measured using an SIS 9000 MDA spectrophotometer (Dr Lange GmbH, Germany). To obtain the OD value and culture density relationship, a two-fold dilution series (until $2^{-6}$ dilution) was made from an enriched (OD$_{culture} > 0.7$, mid exponential phase) bacterial culture. The OD and the culture density values for each dilution were measured and plotted. A relationship between the OD and the culture density for each culture was obtained from linear regression of the plot. For each bacterial enrichment, the bacterial culture density was calculated from the measured OD. With the exception of the influence of the starting culture density on MOB activity tests, MOB were cultured to a density of $2 \times 10^8$ live cells ml$^{-1}$ before the liquid was poured into a serum bottle containing a building material.

2.5 MOB incorporation on building materials

Building material blocks were cut into prisms with the following dimensions: 1 cm x 2 cm x 5 cm. Each specimen was glued on the bottom of a 250 ml serum bottle using epoxy glue (Pattex®, Belgium) and autoclaved (Figure 2.1). The following procedure was performed under sterile condition except for the mixed cultures. MOB liquid culture (~150 ml) was poured into the bottle until the specimen was immersed. The bottle was then closed and incubated at 28 °C under atmospheric air and at static conditions for 24 hours. Afterwards, the liquid was poured out from the bottle. The inside wall of the bottle was wiped with a paper towel to eliminate any bacterial effect from the bottle wall. Finally, the bottle was sealed with a butyl rubber stopper and screwed with an aperture cap.

![Figure 2.1.](image)

**Figure 2.1.** Glued building material inside 250 ml serum bottle closed with butyl rubber septa and screwed with an aperture cap

2.6 Activity tests of MOB on building materials

MOB activity is defined in this study as the methane removal capacity of MOB immobilized on building materials. Experiments were performed to investigate the MOB activity at high (~20% (v/v)) and low (~100 ppmv) methane concentrations. Serum bottles were injected with methane (99.5% (v/v), Air Liquide, Belgium) before measurements and incubated at 28 °C under static condition. Gas composition and pressure were measured in the headspace of the bottles to calculate the Methane Oxidation Rate (MOR) over 100 (high methane concentration) or 200 hours (low methane concentration) of incubation.

Three types of additional experiments for each material and methane concentration were performed to
confirm the biological nature of the methane removal. These were activity tests using: (1) only building material, (2) sterile culture medium (no bacteria) on building material, and (3) autoclaved bacterial culture on building material. Sterile medium and autoclaved bacterial culture were poured into serum bottles containing specimens for control (2) and (3), respectively. The liquid was then separated after 24 hours of immersion. Each experiment was performed in triplicate.

Based on the porosity analysis (Figure 2.2), AAC, Maastricht limestone, and Euville were chosen to be represented in the MOB activity test results because they possessed three different characteristics: (1) high porosity and specific surface area (AAC), (2) high porosity but low specific surface area (Maastricht limestone), and (3) low porosity and specific surface area (Euville limestone).

2.7 Influence of the bacterial starting culture density and the building material immersion time on MOB activity

Experiments were performed to optimize the methane removal efficiency of MOB at low methane concentration by varying: (1) the bacterial starting culture density ($8 \times 10^8$, $4 \times 10^8$, $1 \times 10^8$, and $5 \times 10^7$ live cells ml$^{-1}$) and (2) the immersion time of the specimen in the bacterial culture after the liquid culture was poured into the bottle (1, 5, 10, 20 hours of immersion time). When varying the starting culture density, immersion time was kept at 24 hours and when varying the immersion time, the starting culture density was kept at $2 \times 10^8$ live cells ml$^{-1}$.

2.8 MOB kinetic evaluations

To assess the methane removal kinetics of MOB, the Michaelis-Menten constants (apparent half saturation constant ($K_{m(app)}$) and apparent maximum specific rate ($V_{max(app)}$)) were evaluated. To obtain Michaelis-Menten constants, serum bottles containing Maastricht limestone inoculated with bacteria were injected with methane to reach ~20% (v/v) methane concentration in the headspace. The kinetic test was performed on Maastricht limestone due to the fact that more numbers of MOB cultures exhibited significant methane removal rates at both methane concentrations (see Results section; Table 2.3: 4 MOB cultures ($M. \text{alcaliphilum}$, $M. \text{trichosporium}$, $M. \text{parvus}$, and the mixed MOB culture) on Maastricht limestone whereas only 2 ($M. \text{alcaliphilum}$ and $M. \text{parvus}$) on AAC). Over the incubation period, the headspace methane concentration decreased due to the methane consumption by MOB. MOR was afterwards calculated from the evaluation of the methane concentrations in the headspace over time. MOR was then plotted against the initial methane concentration. The plot for each culture was fitted using hyperbolic non-linear regression to get Michaelis-Menten constants.

2.9 Gas composition analysis

Oxygen, carbon dioxide, and methane (~20% to 0.1% (v/v)) were measured using a Compact Gas Chromatography (GC) (Global Analyser Solution, The Netherlands) which was equipped with a Thermal Conductivity Detector. Methane below 0.1% (v/v) was measured using a Trace GC Ultra (Thermo Fisher Scientific, Belgium) which was equipped with a Flame Ionization Detector. Trace GC Ultra had a minimum detection limit of 100 ppbv of methane concentration. For the gas composition measurements, 1 ml (Compact GC) or 0.1 ml (Trace GC Ultra) of gas sample was taken from the headspace of the bottle using a gas tight syringe (Hamilton, Belgium) and directly injected to the GC. When the oxygen level in the headspace of the bottle was within 5-8% (v/v) range, new oxygen was added to the headspace until it reached ~15% (v/v) oxygen concentration. This was done so that oxygen would not become the limiting substrate for the MOB.
2.10 pmoA-based diagnostic microarray analysis

Total DNA was extracted from the mixed cultures using the Q-Biogene soil extraction kit (MP, Germany) in duplicate according to the manufacturer’s instructions. The DNA extract was used as a template for the diagnostic microarray analysis which was performed as described previously (Bodrossy et al., 2003) with minor modifications (Ho et al., 2011). The pmoA gene amplicons were derived using the A189f/T7_A682r primer combination (Bodrossy et al., 2003). The microarray analysis was performed in R ver. 2.10.0 (R Development Core, 2012), and visualized as a heat map using heatmap.2 as implemented in gplots ver. 2.7.4. The intended probe specificity have been given elsewhere (Ho et al., 2013b).

2.11 Statistical analysis

Except for kinetic tests, values are the mean of triplicate measurement values. Error bars represent the standard deviation. Comparison of means, assuming normal distribution, within one experiment (e.g., one building material and a methane concentration) was done using one way ANOVA test (p=0.05). Subsequent pairwise multiple comparisons tests (Holm-Sidak procedure) were performed to compare the differences between two mean values in the experiment (α=0.05). Statistical analyses were carried out in SigmaPlot v12.0 (Systat Software Inc, USA).

3. Results

3.1 Porosity analysis of building materials

As porosity is one of the important criteria for the immobilization of MOB (Chapter 1 section 2.4), this property of chosen materials was analyzed prior to the activity test. Porosity measurements of the building materials were performed using the MIP analysis. The following symbols were used for the different types of bricks: Safari Geel Bezand (Brick A), Rocher rood (Brick B), and Tenere (Brick C). Autoclaved Aerated Concrete (AAC) (46.8 % ± 1.8 % (v/v)) and Maastricht limestone (41.6 % ± 6.9 % (v/v)) exhibited the highest porosity among the different building materials tested (Table 2.1). AAC (0.36 ± 0.04 ml g⁻¹) and Maastricht limestone (0.28 ± 0.05 ml g⁻¹) also exhibited the highest macropores (pores with diameter > 5 µm) volume per gram of building materials (Table 2.1 and Figure 2.2). However, between the two, only AAC exhibited a high volume of pores with diameter < 0.5 µm (Figure 2.2). AAC also possessed a significantly higher specific surface area (45.8 ± 3.9 m² m⁻³) compared to other building materials which had specific surface areas lower than 6 m² m⁻³ (Table 2.1). Euville limestone and Brick B exhibited the lowest porosity (Euville limestone 11.8 % ± 1.5 % (v/v) ; Brick B: 10.3 % ± 1.2 % (v/v)) and specific surface area (Euville limestone 5.8 ± 1.7 m² m⁻³; Brick B: 5.8 ± 0.5 m² m⁻³) among other building materials.

Table 2.1. Pore structure characteristics of different building materials obtained by means of Mercury Intrusion Porosimetry (MIP) analysis. Error values indicate the standard deviation (n=3). The following symbols were used for the different types of bricks: Safari Geel Bezand (Brick A), Rocher rood (Brick B), and Tenere (Brick C).

<table>
<thead>
<tr>
<th>Properties</th>
<th>AAC</th>
<th>Maastricht limestone</th>
<th>Euville limestone</th>
<th>Brick A</th>
<th>Brick B</th>
<th>Brick C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porosity (% v/v)</td>
<td>46.8 ± 1.8</td>
<td>41.6 ± 6.9</td>
<td>11.8 ± 1.5</td>
<td>23.9 ± 4.5</td>
<td>10.3 ± 1.2</td>
<td>21.0 ± 11.4</td>
</tr>
<tr>
<td>Macropores volume (ml g⁻¹)</td>
<td>0.36 ± 0.04</td>
<td>0.28 ± 0.05</td>
<td>0.03 ± 0.01</td>
<td>0.08 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>0.09 ± 0.07</td>
</tr>
<tr>
<td>Specific surface area (m² m⁻³)</td>
<td>45.9 ± 3.9</td>
<td>2.6 ± 1.3</td>
<td>5.8 ± 1.7</td>
<td>3.6 ± 0.5</td>
<td>5.8 ± 0.5</td>
<td>2.3 ± 0.9</td>
</tr>
</tbody>
</table>

*Pore volume with diameter above 5 µm
Atmospheric methane removal by methane-oxidizing bacteria immobilized on porous building materials

Figure 2.2. The porosity of different building materials as determined by Mercury Intrusion Porosimetry (MIP) analysis. The porosity is given as the pores volume per gram of building material. For each building material, values in the graphs were represented from two measurements. The area within the square is the macropores (pore diameter > 5 µm) area. The following symbols were used for the different types of bricks: Safari Geel Bezand (Brick A), Rocher rood (Brick B), and Tenere (Brick C).

3.2 MOB on building materials activity tests

The activity tests were performed to investigate the activity of chosen MOB cultures when they were immobilized on building materials at both high (~20 % (v/v)) and low (~100 ppmv) methane concentrations. MOB were immobilized by immersing the material in the bacterial culture for 24 hours. Afterwards, the culture was separated and activity test was performed. The pH of the MOB cultures before and after the immersion period was also measured. After 24 hours of immersion, there was no appreciable increase of pH in all MOB cultures for all building materials (Table 2.2), with the exception of M. trichosporium in AAC (from 6.7 to 7.5). There was no change of pH in the culture of M. kenyense in Maastricht limestone and Brick B and in the culture of M. methanica in Brick A.

Table 2.2. pH values of mixtures of bacterial culture with building material before and after immersion period of 24 hours. The following symbols were used for the different types of bricks: Safari Geel Bezand (Brick A), Rocher rood (Brick B), and Tenere (Brick C).

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>AAC</th>
<th>Maastricht limestone</th>
<th>Euville limestone</th>
<th>Brick A</th>
<th>Brick B</th>
<th>Brick C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH start</td>
<td>pH end</td>
<td>pH start</td>
<td>pH end</td>
<td>pH start</td>
<td>pH end</td>
</tr>
<tr>
<td>M. alcaliphilum</td>
<td>8.7</td>
<td>8.8</td>
<td>8.7</td>
<td>8.8</td>
<td>8.7</td>
<td>8.8</td>
</tr>
<tr>
<td>M. kenyense</td>
<td>8.6</td>
<td>8.8</td>
<td>8.6</td>
<td>8.6</td>
<td>8.7</td>
<td>8.8</td>
</tr>
<tr>
<td>M. trichosporium</td>
<td>6.7</td>
<td>7.4</td>
<td>6.6</td>
<td>6.8</td>
<td>6.6</td>
<td>7.0</td>
</tr>
<tr>
<td>M. parvus</td>
<td>6.8</td>
<td>7.2</td>
<td>6.9</td>
<td>7.0</td>
<td>6.8</td>
<td>6.9</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>6.8</td>
<td>7.2</td>
<td>6.8</td>
<td>6.9</td>
<td>6.8</td>
<td>7.0</td>
</tr>
<tr>
<td>M. methanica</td>
<td>6.8</td>
<td>7.2</td>
<td>6.8</td>
<td>7.1</td>
<td>6.8</td>
<td>6.9</td>
</tr>
</tbody>
</table>
Table 2.3. Methane removal rates of Methane Oxidizing Bacteria (MOB) in Autoclaved Aerated Concrete (AAC), Maastricht limestone, and Euville limestone at high (~20 % (v/v)) and low (~100 ppmv) starting methane concentration along with the three control series for each building material. Values that appear in bold indicate significant methane removal by MOB relative to the control series for each type of the specimen (p<0.05). Error values are standard deviation (n=3).

<table>
<thead>
<tr>
<th>Methane removal rates</th>
<th>M. alcaliphilum</th>
<th>M. kenyense</th>
<th>M. trichosporium</th>
<th>M. parvus</th>
<th>Mixed culture</th>
<th>M. methanica</th>
<th>Control seriesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>High methane concentration (µg CH₄ (g building materials h)⁻¹) b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAC</td>
<td>21.7 ± 1.3</td>
<td>4.1 ± 3.5</td>
<td>2.9 ± 0.3</td>
<td>28.5 ± 3.8</td>
<td>20.6 ± 2.1</td>
<td>2.2 ± 0.3</td>
<td>-0.4 ± 0.6</td>
</tr>
<tr>
<td>Maastricht limestone</td>
<td>13.6 ± 0.9</td>
<td>1.4 ± 1.4</td>
<td>18.2 ± 0.7</td>
<td>9.6 ± 0.3</td>
<td>10.1 ± 0.8</td>
<td>0.6 ± 0.1</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Euville</td>
<td>4.2 ± 2.5</td>
<td>1.8 ± 1.2</td>
<td>1.4 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>1.2 ± 0.3</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td>Low methane concentration (10⁻³ µg CH₄ (g building materials h)⁻¹) b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAC</td>
<td>1.6 ± 0.3</td>
<td>0.7 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>0.5 ± 0.3</td>
<td>0.7 ± 0.5</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Maastricht limestone</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.9 ± 0.2</td>
<td>1.2 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>-0.2 ±0.1</td>
</tr>
<tr>
<td>Euville</td>
<td>0.9 ± 0.4</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.3</td>
<td>0.4 ± 0.1</td>
<td>1.0 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>

aControl series: Only stone (A), Medium in stone (B), Autoclaved MOB in stone (C).
bRate calculations were performed over 100 hours (high starting methane headspace gas concentration) or 200 hours (low starting methane headspace gas concentration) of incubation.
M. alcaliphilum, M. trichosporium, and M. parvus in Maastricht limestone exhibited significant methane removal both at high (M. alcaliphilum : 13.6 ± 0.9 µg CH₄ (g building materials h)⁻¹; M. trichosporium : 18.2 ± 0.7 µg CH₄ (g building materials h)⁻¹; M. parvus : 9.6 ± 0.3 µg CH₄ (g building materials h)⁻¹) and low (M. alcaliphilum : 0.5 x 10⁻³ ± 0.1 x 10⁻³ µg CH₄ (g building materials h)⁻¹; M. trichosporium : 0.9 x 10⁻³ ± 0.2 x 10⁻³ µg CH₄ (g building materials h)⁻¹; M. parvus : 1.2 x 10⁻³ ± 0.2 x 10⁻³ µg CH₄ (g building materials h)⁻¹) methane concentrations compared to the control series (P <0.05) (Table 2.3). The methane amount in the headspace of the control incubations varied during the experiment (i.e., negative or positive methane removal). Aseptic practice was performed during the preparation of the control and all materials (i.e., building materials, medium, MOB culture) were sterilized prior to the experiment. The varying methane amount in the headspace of the incubators was most likely due to experimental error (e.g., injection volume to the GC, different calibration) during analysis. Moreover, this effect is more pronounced at low methane concentration (Table 2.3).

Lower removal rates (i.e., three magnitudes lower) were observed for all MOB cultures at low methane concentration. No significant methane removal was observed when bacteria were inoculated in all type of bricks (data not shown). M. parvus in AAC exhibited the highest methane removal both at high (28.5 ± 3.8 µg CH₄ (g building materials h)⁻¹) and low methane concentrations (1.7 x 10⁻³ ± 0.4 x 10⁻³ µg CH₄ (g building materials h)⁻¹). M. kenyense did not exhibit significant methane removal when immobilized on any of the materials tested. M. methanica only showed activity in Maastricht limestone at low methane concentration.

The total gas C-content (originating from methane and carbon dioxide) in the headspace of serum bottles containing AAC or Maastricht limestone inoculated with M. parvus was decreasing over the incubation period at high methane concentration (Figure 2.3). After 100 hours of incubation, The total gas C-content in the bottles with M. parvus in Maastricht limestone decreased from 24.8 ± 2.2 mg-C to 16.3 ± 2.7 mg-C (34.1 % ± 8.1 % carbon removal). The decrease was more pronounced (from 23.0 ± 0.4 mg-C to 8 ± 2.6 mg-C) in bottles containing M. parvus in AAC (65.2 % ± 10.9 % carbon removal). No significant decrease was observed in bottles with AAC or Maastricht limestone only (P > 0.05).

![Figure 2.3. The total methane and carbon dioxide-carbon in the headspace of serum bottles containing M. parvus in Autoclaved Aerated Concrete (AAC) and Maastricht limestone incubated at high methane concentration. Error bars represent standard deviation (n=3).](image-url)
3.3 Influence of starting culture density and immersion time on MOB activity

After the activity test, additional experiments were performed to investigate the influence of several parameters to the activity of the immobilized MOB. Two parameters chosen to be varied were the starting culture density and the immersion time. *M. parvus* in Maastricht limestone was chosen for this experiment since they exhibited one of the highest activities at low methane concentration.

The methane removal potential of *M. parvus* at low methane concentration was dependent on the starting culture density and the immersion time (Figure 2.4). Significant methane removal was observed when the specimens were immersed for five hours or more (Figure 2.4a). Maximum methane removal was exhibited by *M. parvus* when the stones were immersed for 10 hours where the methane concentration in the headspace decreased from 35.9 ± 3.5 mg m\(^{-3}\) air to 8.6 ± 1.4 mg m\(^{-3}\) air after 100 hours of incubation (75.9 % ± 4.2 % methane removal). The highest methane removal rate was observed in the first 24 hours of incubation. After this period, 57.3 % ± 4.2 % of the methane has been removed. Lower methane removals were observed when *M. parvus* were immersed for 5 (21.2 % ± 3.7 % methane removal) and 20 hours (51.5 % ± 10.4 % methane removal). The methane removal by *M. parvus* was not significant when the stones were immersed for one hour (*P* > 0.05).

**Figure 2.4.** Influence of the (a) immersion time and (b) starting culture density on the methane removal of *M. parvus* in Maastricht limestone. Methane removal is depicted as the evolution of the methane headspace concentration in serum bottles at a specific time after the removal of the bacterial culture solution. Error bars represent standard deviation (n=3).

The methane removal potential of *M. parvus* was lower when smaller starting culture density was used (Figure 2.4b). A culture density of > 1 x 10\(^8\) cells ml\(^{-1}\) was needed to have a significant methane removal by *M. parvus* on Maastricht limestone. From the different starting culture densities tested, *M. parvus* at culture density of 8 x 10\(^8\) live cells ml\(^{-1}\) exhibited the highest activity after 100 hours of incubation. Here, methane concentration in the headspace decreased from 38.7 ± 3.7 mg m\(^{-3}\) air to 0.9 ± 0.6 mg m\(^{-3}\) air (97.7 % ± 1.4 % methane removal). The highest methane removal rate was observed in the first 20 hours of incubation where 92.8 % ± 3.4 % of the initial methane was removed. The lowest methane removal was exhibited by *M. parvus* when a culture density of 5 x 10\(^7\) live cells ml\(^{-1}\) was used (28.2 % ± 9.0 % methane removal).
3.4 MOB kinetic evaluations

To investigate the trend of the MOB activity on building material, the kinetic behavior of the immobilized MOB were studied. Evaluations were based on the Michaelis-Menten kinetic parameters obtained from the study. The kinetics of methane removal by the MOB were studied in Maastricht limestone because higher numbers of tested MOB cultures exhibited methane removal at both high and low methane concentrations when they were immobilized in this stone (Table 2.3).

Figure 2.5. Kinetic curve of different Methane Oxidizing Bacteria (MOB) in Maastricht limestone. Hyperbolic curve fitting (straight line = M. parvus; dotted line = M. alcaliphilum; dashed dotted line = M. trichosporium; dashed line = Mixed culture) was done to obtain the Michaelis-Menten parameters ($K_{m(app)}$ and $V_{max(app)}$).

The methane removal kinetics of immobilized MOB followed the hyperbolic model of Michaelis-Menten with a good fitting (Figure 2.5; $R^2 > 0.95$). From the Michaelis-Menten parameters obtained, *M. parvus* exhibited the lowest $K_{m(app)}$ value (0.4 % (v/v)) with the $K_{m(app)}$ of the mixed culture being the highest (14.9 % (v/v)) (Table 2.4). Furthermore, the mixed culture possessed the lowest $V_{max(app)}$ values (8.2 µg CH$_4$ (g building materials h)$^{-1}$) with the $V_{max(app)}$ of *M. alcaliphilum* being the highest (16 µg CH$_4$ (g building materials h)$^{-1}$). *M. parvus* also exhibited the highest $V_{max(app)}/K_{m(app)}$ value (27.7 µg CH$_4$ (g building materials h % (v/v))$^{-1}$) among all MOB cultures in Maastricht limestone. The kinetic of the mixed culture was obtained from a bacterial community predominantly composed of *Methylcystis*-related MOB (Type II, probes P_McyM309, O_Mcy255, P_Mcy270, and P_Mcy233) as revealed from the microarray analysis (Figure 2.6).

Table 2.4. Methane removal Michaelis-Menten kinetic parameters of different Methane Oxidizing Bacteria (MOB) in Maastricht limestone.

<table>
<thead>
<tr>
<th>Bacterial culture</th>
<th>$K_{m(app)}$ (% (v/v))</th>
<th>$V_{max(app)}$ (µg CH$_4$ (g building material h)$^{-1}$)</th>
<th>$V_{max(app)}/K_{m(app)}$ (µg CH$_4$ (g building material h % (v/v))$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. alcaliphilum</em></td>
<td>5.4</td>
<td>16.0</td>
<td>2.9</td>
</tr>
<tr>
<td><em>M. parvus</em></td>
<td>0.4</td>
<td>11.1</td>
<td>27.7</td>
</tr>
<tr>
<td><em>M. trichosporium</em></td>
<td>3.7</td>
<td>18.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>8.2</td>
<td>14.9</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Chapter 2

Figure 2.6. The Methane Oxidizing Bacteria (MOB) relative abundance of the mixed cultures used in this study was based on microarray analysis. Red key indicates the highest hybridization signals.

4. Discussion

For each MOB culture, higher methane removal was exhibited when MOB was immobilized on AAC at both high and low methane concentrations with *M. trichosporium* being the exception. Since in this experiment all building materials were immersed using the same value of starting culture density and immersion time, the high methane removal of MOB in AAC could be attributed to the higher macropores (pores with diameter > 5 µm) volume possessed by AAC compared to other building materials. AAC and Maastricht limestone exhibited significantly higher macropores volume (*P* <0.05) than other tested building materials. Thus, with bacteria size around 1 to 2 µm, building materials with high macropores volume could accommodate more bacteria than the ones with low macropores volume (Samonin & Elikova, 2004). Maximum accumulation of biomass occurs when the pore size of the support is one to five times the size of bacteria. AAC also exhibited a high volume of pores with diameter < 0.5 µm which gave rise to a higher surface area possessed by the material compared to other materials. However, MOB would not be able to penetrate and reside within these micropores. Although both porosity and surface area of the support are the two most important factors for a high microbial activity, in this study, macropores volume was the more important factor to have a high methane removal. Overall, with a higher amount of bacteria most likely incorporated in AAC, higher methane removal could therefore be expected when comparing the activity of the same cultures in different building materials.

For a given building material, different methane removal rates were observed for different MOB cultures and methane concentration. At high methane concentration, the methane removal rate by the MOB would approach a zero order kinetic and MOB would exhibit a methane removal rate approximating their *V*_*max*_ value. This was observed in the kinetic studies of different MOB culture in Maastricht limestone. MOB exhibiting the highest *V*_*max* (e.g., *M. trichosporium*) values exhibited the highest methane removal rate at high methane concentration. MOB exhibiting low *K*_*m(app)_ value indicates a higher affinity to methane. At low methane concentration, the methane removal rate of MOB would approach a first order kinetic where the *K*_*m(app)_ >> *S*, thus *M. parvus*, having the highest *V*_*max*/*K*_*m(app)_ value, exhibited the highest methane removal rate at low methane concentration.

The *K*_*m(app)_ value of *M. trichosporium* obtained in this study is comparable to the one reported by Lontoh and Semrau, 1998 (a study in liquid culture, which is around 0.64% (v/v), after multiplication with Bunsen coefficient) (Lontoh & Semrau, 1998). Although kinetic parameters of *M. parvus* and *M. alcaliphilum* have not been reported yet, the *K*_*m(app)_ values of both pure cultures are within the range of known MOB strains reported in the literature (*K*_*m(app)_ between 0.056% (v/v) to 4.6% (v/v)) (Conrad, 1996). Furthermore, the *K*_*m(app)_ value of the mixed culture can be considered as one of the highest values compared to the values of known MOB or upland soil (Gulledge et al., 2004). This value was comparable to the one of landfill biocover soil (*K*_*m(app)_ = 14 % (v/v)) (Chi et al., 2012), an environment with a relatively high atmospheric methane concentration which is comparable to the enrichment condition of the mixed culture prior to the tests.
There is a threshold immersion time and starting culture density for *M. parvus* to exhibit significant methane removal capacity in Maastricht limestone. *M. parvus* exhibited significant activity when the building materials were immersed for more than 1 hour or when a starting culture density of at least $2 \times 10^8$ live cells ml$^{-1}$ was used. The methane removal potential of MOB increased when higher starting culture density was used or when the stone was immersed longer. Both conditions increased the probability of bacteria to be incorporated inside the stone. With higher amount of bacteria adsorbed on the stone, MOB could exhibit higher methane removal rates.

The decrease of the total methane and carbon dioxide-C in the headspace of serum bottles containing Maastricht limestone inoculated with *M. parvus* could be attributed to biomass growth. Using the thermodynamic maintenance energy calculation and assuming a maintenance energy requirement of 5.94 kJ (C-mol biomass. h)$^{-1}$ at 28 °C (Tijhuis et al., 1993), the minimum methane concentration needed by the immobilized MOB for their metabolic maintenance is in the range of 2000 ppmv (*M. parvus*) to 12.2 % (v/v) (mixed culture). From substrate Herbert-Pirt relation, the total methane uptake would partly be utilized for maintenance and partly for growth. At high methane concentration (~20% (v/v)), the methane-C was utilized for both processes. The total methane and carbon dioxide-C in the headspace of serum bottles took into account the methane that has not been consumed by MOB and the carbon dioxide released from catabolic processes. Due to this partial carbon utilization for growth and maintenance, the total methane and carbon dioxide-C was decreasing over the incubation period.

The decrease of the total methane and carbon dioxide-C in the headspace of serum bottles containing AAC inoculated with *M. parvus* could be attributed to biomass growth and carbonation process. The main binding material of the AAC (*Ytong*) is Tobermorite-1,1 nm (Ca$_5$Si$_6$O$_{16}$(OH)$_2$.4H$_2$O; 20-40 % (wt/wt), a calcium silicate hydrate; [www.AAC.gr](http://www.AAC.gr)). Other major component in the material is quartz sand (i.e., SiO$_2$, 60 – 80 % (w/w); [www.AAC.gr](http://www.AAC.gr)). In the presence of water, Tobermorite reacts with carbon dioxide which leads to the formation of a silica-gel and calcium carbonate (Matsushita et al., 1999), according to reaction 2.1.

$$\text{Ca}_5\text{Si}_6\text{O}_{16}\text{(OH)}_2.4\text{H}_2\text{O} + 5\text{CO}_2 \rightarrow 5\text{CaCO}_3 + 6\text{SiO}_2\text{H}_2\text{O} + 4\text{H}_2\text{O} \quad (2.1)$$

Furthermore, tobermorite is in equilibrium with portlandite according to the following reaction (Kus & Carlsson, 2003):

$$\text{Ca}_5\text{Si}_6\text{O}_{16}\text{(OH)}_2.4\text{H}_2\text{O} \leftrightarrow 5\text{Ca(OH)}_2 + 6\text{SiO}_2 \quad (2.2)$$

Tobermorite is a stable component, however, additional carbonation reaction in this study might occur from the carbonation of calcium hydroxide:

$$\text{Ca(OH)}_2 + \text{CO}_2 \rightarrow 5\text{CaCO}_3 + \text{H}_2\text{O} \quad (2.3)$$

Two main process occur for the carbonation reaction of AAC: diffusion of carbon dioxide to the water phase and the reaction of carbon dioxide to form calcium carbonate. The whole process is typically controlled by carbon dioxide diffusion into the water phase (Hanecka et al., 1997). In this study, the water phase is the liquid layer surrounding AAC specimen. From different AAC models, it was found that the degree of carbonation for the AAC tested specimens had lower values than for the Portland cement based materials (Hanecka et al., 1997). This indicates that reaction 2.3 occurs at higher rate than reaction 2.2. The overall carbonation rate is typically high in the first 30 to 40 days of AAC specimens exposure. Maximum density increase around 50 kg m$^{-3}$ was reached after 24 months of the specimens exposure (Matsushita et al., 2000; Matsushita et al., 1999).
Overall, both biomass growth and carbonation reaction might occur over the incubation period of *M. parvus* in AAC at high methane concentration. Due to the additional carbonation reaction, a lower amount of carbon dioxide was released to the atmosphere. Therefore, a more pronounced decrease of the total methane and carbon dioxide-C was observed when *M. parvus* was immobilized on AAC compared to when immobilized on Maastricht limestone.

5. Conclusions

From this study, it was shown that MOB could remove methane when immobilized on building materials. *M. parvus* immobilized on AAC exhibited the highest MOR both at high and low concentrations due to the high porosity of the material. Additionally, AAC inoculated with MOB could act as a carbon sink for methane. This carbon capture and storage process is more pronounced when using AAC as the building material; besides for growth, the methane-derived carbon can also be converted to calcium carbonate via carbon dioxide reaction with tobermorite, the binding material.

For the biofilter studies (Chapter 3 and 4), mixed MOB culture used in this study were the culture selected for the biofilter inoculation. This was based on the fact that non-aseptic practice was preferred to keep the operating cost lower if the biofilter was to be applied to remove methane in places with high methane emission and atmospheric concentration < 1% (v/v). AAC was used as the carrier material for those studies. Studies of MICP by MOB (Chapter 5 and 6) focused on the use of both *M. parvus* and AAC as the model MOB and building material, respectively.

Acknowledgements

The project is funded by SIM-SHE SECEMIN project (SIM 2009-1) and the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005). Grateful thanks were given to Pieter Van den Abbeele and Stephen Andersen for critically reviewing the work in this chapter.
Supplementary Information

Table S1. pH of tested building materials. The following symbols were used for the different types of bricks: Safari Geel Bezand (Brick A), Rocher rood (Brick B), and Tenere (Brick C). AAC is Autoclaved Aerated Concrete.

<table>
<thead>
<tr>
<th>No</th>
<th>Building material</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AAC</td>
<td>9.1 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>Maastricht limestone</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>Euville limestone</td>
<td>9.1 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>Brick A</td>
<td>9.8 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>Brick B</td>
<td>8.8 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>Brick C</td>
<td>8.8 ± 0.1</td>
</tr>
</tbody>
</table>
CHAPTER 3
METHANE BIOFILTRATION USING AUTOCLAVED AERATED CONCRETE AS THE CARRIER MATERIAL

Abstract

The methane removal capacity of mixed Methane-Oxidizing Bacteria (MOB) culture in a biofilter setup using Autoclaved Aerated Concrete (AAC) as a highly porous carrier material was tested. Batch experiment was performed to optimize MOB immobilization on AAC specimens where optimum methane removal was obtained when calcium chloride was not added during bacterial inoculation step and 10 mm thick AAC specimens were used. The immobilized MOB could remove methane at low methane concentration (~1000 ppmv) in a biofilter setup for 127 days at an average removal efficiency of 28.7%. MOB also exhibited a higher abundance at the bottom of the filter, in proximity with the methane gas inlet where a high methane concentration was found. Overall, a more environmentally friendly methane biofilter performance can be obtained using AAC as the carrier material.

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Chapter 3

1. Introduction

Atmospheric methane plays a substantial role in global warming by contributing to 0.5 W m\(^{-2}\) of the 2.77 W m\(^{-2}\) of the total radiative forcing of long lived greenhouse gases (Chapter 1 section 1.1). Both natural (347 Tg year\(^{-1}\)) and anthropogenic processes (331 Tg year\(^{-1}\)) contribute almost equally to the total atmospheric methane emissions (Chapter 1 section 1.2). Among anthropogenic methane emissions sources, energy (e.g., fossil fuel combustion) and agricultural sectors (e.g., livestock, rice paddies) are the largest contributors. With increasing food and energy demand as a result of the human population growth, global anthropogenic methane emission is predicted to increase in the future. Therefore, mitigation strategies of the anthropogenic methane emission are essential for the regulation of the global methane budget.

Methane-Oxidizing Bacteria (MOB) are responsible for ~5 % of the global methane sink (Chapter 1 section 1.2). MOB are microorganisms capable of utilizing methane as their sole carbon and energy sources (Chapter 1 section 2.2). MOB possess Methane Monooxygenase (MMO) enzyme which enables them to oxidize methane to methanol. Via a series of oxidation reactions, methanol is subsequently converted to formaldehyde, a central component in the bacterial metabolism (Hanson & Hanson, 1996). Formaldehyde is the metabolic intermediate used to synthesize biomass and generate reducing equivalents. Due to its versatility and ease of applicability, MOB is central in the biotechnological applications to mitigate methane emission (Semrau et al., 2010).

Biofiltration is the typical biotechnological application to mitigate methane emission in several places such as the coal mines and livestock barns (Chapter 1 section 2.4). Methane biofiltration employs MOB embedded on a static carrier material to remove methane from waste gas flowing through the filter. Biofilter performance is influenced by several technical (e.g., reactor dimension) and operational parameters (e.g., inlet load). When designing a methane biofilter, the carrier material selection is essential for an optimum methane removal (Huang et al., 2011). Biofilter carrier material can be organic (e.g., compost) or inorganic (e.g., gravel stone). An advantage of using organic material is that the material provides additional nutrients for the bacteria (e.g., N and P sources) whereas inorganic material is more durable as it does not deteriorate with time (Akdeniz et al., 2011; Veillette et al., 2012a). Between the two types of carrier material, the use of inorganic carrier material ensures high elimination capacity and enhanced biofilter performance (Nikiema et al., 2005).

As seen in Chapter 2, one of the most important properties for MOB immobilization was porosity. Material with high porosity can accommodate a high number of bacteria by providing a vast adsorption site for the bacteria (Cohen, 2001; Samonin & Elikova, 2004) and Autoclaved Aerated Concrete (AAC) possesses this beneficial characteristic. AAC is a lightweight porous concrete consisting of calcium silicate hydrate typically used for wall, floor, and roof panels of residential and industrial buildings. AAC possesses a high porosity that can reach 80 % (v/v) as a result of gas entrapment by the aerating agent during manufacturing process (Narayanan & Ramamurthy, 2000). In Chapter 2 it was concluded that a high methane removal was exhibited at low (~100 ppmv) and high (~20 % (v/v)) methane concentrations by different MOB cultures when they were immobilized on AAC.

In this study, the performance of the immobilized MOB on AAC to remove methane in a biofilter setup was investigated. Firstly, batch tests to optimize MOB immobilization on AAC were performed. This was done by testing the influence of: (1) calcium chloride addition into the bacterial culture and (2) varying AAC specimens thickness on the methane removal capacity of the immobilized MOB. Calcium addition to bacterial culture is known to promote bioflocculation (Sobeck & Higgins, 2002) and this was anticipated to improve MOB immobilization on the AAC specimens. Secondly, a lab scale biofilter test
was conducted to investigate the capacity of the immobilized MOB on AAC to remove methane at low concentration (~1000 ppmv). The optimized conditions obtained from the batch test would be used as the basis for the biofilter design.

2. Materials and Methods

2.1. Methane Oxidizing Bacteria

MOB mixed culture was enriched from circum-neutral agricultural soil (pH ~7.9) originating from Ghent, Belgium (Chapter 2). The culture was predominantly composed of *Methylocystis spp.* as revealed by diagnostic microarray analysis targeting the pmoA gene of the MOB.

2.2. Autoclaved Aerated Concrete

AAC (*Ytong*, Belgium) was cut into triangular prism specimens for the batch optimization test (Figure 3.1a) or circular discs for the biofilter test (Figure 3.1b). The triangular specimens were 30 mm in radius with varying thickness (please refer to section 2.5 of this chapter for detail explanation of the experimental method). The circular discs were 10 mm thick with a diameter of 90 mm with four openings (15 mm x 10 mm). The openings were made for gas passage in the biofilter. The specimens were stored at 28°C prior to use.

![Image of AAC specimens](image)

**Figure 3.1.** Autoclaved Aerated Concrete (AAC) specimens used to immobilize Methane Oxidizing Bacteria (MOB) for the: (a) batch optimization test, and (b) biofilter test.

2.3. Biofilter configuration

Three identical biofilters were made from a hollow transparent polyethylene (PE) tube (ISPA plastic, The Netherlands) with a dimension of: 80 cm (length) x 9 cm (diameter). For each biofilter, 7 holes with 12 mm diameter were made at 9 cm apart along the biofilter length. The holes, which were used as gas sampling ports, were closed with butyl rubber stoppers and sealed with epoxy glue (Loctite, USA). Before being placed in the biofilter, the discs were inoculated with MOB using method described in section 2.5. The discs were subsequently placed inside the biofilter in a spiral trajectory in order to prevent gas flow obstruction caused by biofilm clogging (Figure 3.2a). The biofilter bed height was 45 cm and it was made out of stacks of AAC specimens starting at 15 cm from the bottom of the filter. A circular piece of plastic frame with numerous holes was attached to the biofilter wall at the base of the bottom disc in order to hold the discs and for gas inlet distribution. Both ends of the biofilter were subsequently closed with PVC flanges (ISPA plastic, The Netherlands) and tightened with 8 screws (M8 x 60 mm; Ijzewaar, Belgium) to make the biofilter gas tight. In this final configuration, five gas sampling ports were situated equally to different filter bed heights whereas the other two ports were located close to the gas inlet and outlet. The sampling ports were numbered sequentially from the bottom to the top part of the filter (i.e., 1<sup>st</sup> and 7<sup>th</sup> ports were the one adjacent to the gas inlet and outlet, respectively).
Figure 3.2. (a) Biofilter bed configuration when placed inside the biofilter and (b) the biofilter process configuration when all three biofilters (MBF-A, MBF-B, and MBF-C) were run in series. When MBF-A and MBF-B were run in series (see section 2.2.3), gas entered firstly in MBF-B. When all biofilters were run in series, gas entered firstly in MBF-C. Methane and compressed air are mixed and subsequently fed at the bottom of the biofilter at ~1000 ppmv methane concentration. Liquid containing essential nutrients is fed intermittently at the top of the filter using a sprinkler.

2.4 MOB enrichment

Prior to the experimental set-up, 200 ml of culture was sub-cultivated in Nitrate Mineral Salt (NMS) medium (Whittenbury et al., 1970) in a 1 L serum bottle (Schott Duran, USA). For this culture sub-cultivation, 10% (v/v) inoculum from a previous enrichment was added to fresh NMS medium before the bottle was sealed with a butyl rubber stopper and an aperture cap. Methane gas (99.5 % (v/v); Linde Gas, Belgium) was subsequently injected into the headspace until it reached ~20 % (v/v) headspace concentration. The bottle was incubated on a shaker (120 rpm) at 20 °C until the culture was enriched to ~2 X 10^8 cells ml^-1.

2.5 Batch optimization test

Forty grams of AAC specimens (porosity: 46 % ± 1.8 % and pores volume: 0.36 ± 0.04 ml g^-1; Chapter 2 section 3.1) were inserted into each 1 L serum bottles containing 200 ml of enriched MOB culture. The bottles were subsequently sealed with a butyl rubber stopper and an aperture cap. Methane gas was subsequently injected into the headspace until it reached ~20 % (v/v) headspace concentration. The bottles were incubated on a shaker (120 rpm) at 20 °C for 48 hours. Afterwards, the liquid was poured out of the bottles and the bottles were resealed before methane gas was injected into the headspace.

The methane removal capacity of the MOB was measured by analyzing the change in the methane concentration in the headspace of the bottles. Methane oxidation rate by the bacteria was calculated based on this headspace concentration change according to the method described in Chapter 2. Because oxygen is also a substrate for bacterial methane oxidation, the oxygen concentration in the headspace was maintained above 5 % (v/v) by injecting oxygen (99.5 % (v/v); Air Liquide, Belgium) into the bottles. Methane was replenished to maintain an ~20 % (v/v) starting headspace concentration.
For incubations with varying calcium chloride addition, different volume of 1 M calcium chloride was added to different bottles containing bacterial culture after the specimens were inserted to reach 30, 50, 70, and 90 mM final calcium chloride concentrations. Incubations of AAC specimens in MOB culture without calcium chloride addition served as reference incubations. In this test, 10 mm thick AAC specimens were used. A 10, 15, or 20 mm thick specimens were used for incubations with varying specimen thickness. For simplification, the following abbreviations were assigned to different incubations with the following calcium chloride addition: 0 mM (MC0, controls), 30 mM (MC30), 50 mM (MC50), 70 mM (MC70), and 90 mM (MC90). The following abbreviations were assigned for incubations with: 10 mm (MT10), 15 mm (MT15), 20 mm (MT20) thick specimens. Each treatment was performed in triplicate.

### 2.6 Biofilter test

The biofilter process configuration can be seen in Figure 3.2b. The biofilter was connected at its base to a gas line coming from a compressed air (Air Compact, Belgium) and methane gas (99.5 % (v/v); Air Liquide, Belgium) mixing point. Both gas flows were regulated using mass flow controllers connected to a control module (EX-FLOW; Bronkhorst, The Netherlands) ensuring that ~1000 ppmv methane/air concentration was fed into the biofilter. Prior to the biofilter test, the hollow filter was checked for leaks by feeding the gas into the filter for one week and the methane concentration in the filter during that period was monitored regularly. NMS medium stored in a 10 L tank was intermittently fed through the top of the filter countercurrent to the air flow through the biofilter. The liquid was fed every 6 hours for 1 minute (120 ml min⁻¹) by a pump (Cole-Parmer, USA) equipped with a timer (Chacon, Belgium). At the bottom of the filter, the liquid was collected and recirculated back to the tank. The liquid nutrient composition was checked regularly to ensure enough nutrient provision for the bacteria. When one of the nutrients was depleted, the liquid was replaced by fresh NMS medium. The biofilter was operated in a temperature controlled room (20° C). Summary of the biofilter operating and design parameters can be seen in Table 3.1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty Biofilter volume (m³)</td>
<td>0.0051</td>
</tr>
<tr>
<td>Biofilter bed volume (m³)</td>
<td>0.0026</td>
</tr>
<tr>
<td>Biofilter bed mass (kg)</td>
<td>1.02</td>
</tr>
<tr>
<td>Compressed air flow rate (m³ h⁻¹)</td>
<td>0.1998</td>
</tr>
<tr>
<td>Methane flow rate (m³ h⁻¹)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Inlet methane concentration (ppmv)</td>
<td>960</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>20</td>
</tr>
<tr>
<td>Empty Bed Residence Time (EBRT) (s)</td>
<td>47</td>
</tr>
<tr>
<td>Methane Inlet Load (IL) (g C-CH₄ d⁻¹ m⁻³)</td>
<td>944.7</td>
</tr>
<tr>
<td>Nutrient feeding rate (ml min⁻¹)</td>
<td>120</td>
</tr>
</tbody>
</table>

In the final configuration, three biofilters were run in series. Initially, one biofilter (MBF-A) was run for 36 days with the gas flow rate set at approximately half of the design capacity for the first 20 days. MBF-B was installed after 36 days and it was placed prior to MBF-A. Both biofilters were subsequently run in series for 37 days before the third biofilter (MBF-C) was installed. MBF-C was placed ahead of MBF-B and all biofilters were operated for 53 days before the experiment was stopped. The biofilters were organized in this arrangement to promote higher biomass growth in the newly installed biofilter by feeding it with methane at higher concentration. To observe the influence of liquid nutrient feeding on bacterial methane removal, liquid feeding was stopped from day 86 to 106.
Several parameters were calculated in this study. The Empty Bed Residence Time (EBRT) was calculated using the following equation:

\[
\text{EBRT (s)} = \frac{V_f}{Q}
\]  

(3.1)

where \(V_f\) is the filter bed volume (m\(^3\)) and \(Q\) is the inlet gas flow rate (m\(^3\) h\(^{-1}\)). The methane volumetric load is divided into Inlet Load (IL) and Outlet Load (OL) and they were calculated using the following equations:

\[
\text{IL (g m}^{-3}\text{ h}^{-1}) = \frac{(C_{\text{in}} \times Q)}{V_f}
\]  

(3.2)

\[
\text{OL (g m}^{-3}\text{ h}^{-1}) = \frac{(C_{\text{out}} \times Q)}{V_f}
\]  

(3.3)

where \(C_{\text{in}}\) and \(C_{\text{out}}\) were the inlet and outlet methane concentrations (g m\(^{-3}\)), respectively, measured from gas samples taken from the 1\(^{st}\) and 7\(^{th}\) sampling ports of MBF-A. \(C_{\text{in}}\) always corresponded to the bottom (1\(^{st}\)) sampling port of MBF-A, MBF-B, and MBF-C in the 1\(^{st}\) (day 1 to 36), 2\(^{nd}\) (day 37 to 72), and 3\(^{rd}\) (day 73 to 127) phase, respectively. To estimate the methane removal capacity of MOB, the Elimination Capacity (EC) was calculated using the following equation:

\[
\text{EC (g m}^{-3}\text{ h}^{-1}) = \left(\frac{Q}{V_f}\right) \times (C_{\text{in}} - C_{\text{out}})
\]  

(3.4)

The EC was calculated for each biofilter with the total EC being the sum of all. The methane Removal Efficiency (RE) of MOB was calculated using the following equation:

\[
\text{RE (%)} = \left(\frac{(C_{\text{in}} - C_{\text{out}})}{C_{\text{in}}}\right) \times 100\%
\]  

(3.5)

### 2.7 Gas composition analysis

For the batch optimization test, 1 ml of gas sample was taken from the headspace of each bottle using a gas tight syringe (Hamilton, Belgium) and directly analyzed. For the biofilter test, duplicate gas samples were taken from each sampling port using a gas tight syringe (Hamilton, Belgium) and transferred to 12 ml vacutainers (Becton Dickinson, Belgium) that had been vacuumed prior to the analyses. Oxygen, methane (for batch optimization test), and carbon dioxide were measured using a Compact Gas Chromatography (GC) (Global Analyser Solution, The Netherlands) equipped with a thermal conductivity detector, a Porabond pre-column, and a Molsieve SA column. Methane in the gas samples was analyzed using a Trace GC Ultra (Thermo Scientific, Belgium) equipped with a flame ionization detector. Gas pressure inside the biofilters and serum bottles was measured using a tensimeter (WIKA, Germany).

### 2.8 Nutrient composition analysis

Nitrate, nitrite, sulphate, and phosphate were monitored in the liquid of the nutrient tank. A 1 ml liquid sample was collected from the tank and diluted 10 times prior to analysis. The concentration of ions in the sample was analyzed using a 761 Compact Ion Chromatograph (Metrohm, Switzerland) equipped with a thermal conductivity detector using method described previously (van der Ha et al., 2011).

### 2.9 DNA extraction

At the end of the biofilters test, triplicate samples were collected from the surface of the specimen located beside the sampling port by scraping the surface of the specimen up to ~1 mm deep. There were thus five different types of specimen samples from each biofilter. These samples consisted of biomass mixed
with residual AAC specimens. After homogenization, an aliquot of the sample was then dried in an oven at 70°C for 24 hours. The samples were subsequently stored in a -20°C freezer until being used for DNA extraction.

Total DNA was extracted from the samples using a physical disruption method (Vilchez-Vargas et al., 2013). Upon lysis and disruption at 1,800 rpm for 3 min, phenol-chloroform-isooamyl ethanol (25:24:1) extractions were followed. DNA was precipitated and washed twice with cold ethanol and resuspended in 50 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]) (Hernandez-Sanabria et al., 2010). The quantity and quality of DNA were measured using an ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

### 2.10 qPCR analysis

Quantitative PCR (qPCR) assay was performed to investigate the MOB abundance on AAC specimens at different bed heights. The quantification of the *pmoA* gene was used as proxy for the total MOB community. The *pmoA* gene (gene encoding for a subunit of the particulate methane monoxygenase enzyme) is present in virtually all obligate methanotrophs and is congruent with the 16S rRNA gene phylogeny (Kolb et al., 2003), making the *pmoA* gene suitable for the detection of methanotrophs (Ho et al., 2011). qPCR targeting the *pmoA* gene was performed using the A189f/mmb661r primer combination. Briefly, each qPCR reaction (total volume 20 µl) consisted of 10 µl 2X SensiFAST SYBR (BIOLINE, the Netherlands), 3.5 µl of A189f forward primer (5 pmol µl⁻¹), 3.5 µl mmb661r reverse primer (5 pmol µl⁻¹), 1 µl Bovine Serum Albumin (5 mg ml⁻¹; Invitrogen, the Netherlands), and 2 µl diluted template DNA.

In a preliminary qPCR run, DNA template was diluted (10x, 50x, and 100x dilution) to determine the optimal target yield. Henceforth, DNA was diluted 100x to achieve the optimum *pmoA* gene copy numbers. The PCR program consisted of an initial denaturation step at 95°C for 3 min, followed by 45 cycles of 95°C for 10 s, 30°C for 10 s, and 72°C for 30 s. Fluorescence signal was obtained at 87°C (8 sec) after each cycle, and melt curve obtained from 70°C to 99°C (1°C temperature increase on each cycle). The qPCR was performed with a Rotor-Gene Q real-time PCR cycler (Qiagen, the Netherlands). Duplicate qPCR reactions were performed for each template DNA giving a total of six replicates per sampling point along the vertical sampling sites.

### 2.11 Statistical analysis

Except for the biofilter test (duplicate measurements), values are the mean of triplicate measurement values. Error bars represent standard deviations. one-way ANOVA test was carried out for means comparison, assuming significance of P < 0.05. Subsequent pairwise multiple comparisons tests (Holm–Sidak procedure) were performed to compare the differences between two mean values in the experiment (α = 0.05). Statistical analyses were carried out in SigmaPlot v12.0 (Systat Software Inc., USA). The mixed model procedure in SAS 9.4 was used to perform the analysis of the distribution of MOB abundance in a biofilter (MBF-A and MBF-B), according to the split plot design (SAS institute, Cary, NC, USA). This MOB distribution analysis was based on the significant differences of MOB abundance between each port in a specific biofilter at the end of the biofilter test.
3. Results and Discussion

3.1 Batch optimization test

The aim of the batch optimization test was to optimize MOB immobilization on AAC specimens for the biofilter test. This was done by investigating: (a) the influence of calcium chloride addition into the MOB culture or (b) AAC specimen thickness to the methane removal of immobilized MOB.

3.1.1 The influence of calcium chloride addition into the MOB culture to the methane removal of immobilized MOB

Calcium chloride was added into the bacterial culture to promote floc formation inside AAC specimens. This strategy was used previously to induce floc formation in wastewater (Moussa et al., 2006; Zita & Hermansson, 1994). Bivalent cations (e.g., calcium chloride) addition into wastewater increased the attractive energy (DLVO theory) between negatively charged bacteria by forming a “cation bridge”. This promoted floc formation and its subsequent settling (Zita & Hermansson, 1994).

In this study, we hypothesized that calcium chloride addition after specimen immersion in the culture would increase the probability of having MOB aggregate sizes exceeding the pore size of the specimen due to the floc formation after MOB entered the pores of the specimens. When separating the liquid, it was hoped that these aggregates remained in the specimens rather than being lost with the liquid. By retaining a higher number of bacteria inside the specimens, higher methane removal by the bacteria was anticipated. On the other hand, salt addition could also inhibit the bacterial methane uptake. Salt addition lowered the methane solubility in the medium and slows its transfer from the gas to the liquid phase (Schnell and King, 1996). Previous researches showed that salinity inhibited the methane removal capacity of mixed MOB culture (van der Ha et al., 2010). Thus, a suitable calcium chloride addition that would improve the activity of immobilized MOB without necessarily hampering the bacterial activity was investigated here. The following symbols represented different incubations with the following calcium chloride addition: 0 mM (MC0; controls), 30 mM (MC30), 50 mM (MC50), 70 mM (MC70), and 90 mM (MC90).

With the exception of MC90, methane was removed from the headspace of all bottles after each methane addition (Figure 3.3a.). MC90 was stopped after 32 days due to negligible methane consumption by the MOB. The highest initial methane removal rate was measured in MC30 (179.7 ± 3.9 µg C-CH₄ (g AAC d⁻¹)) and MC50 (179.8 ± 1.2 µg C-CH₄ (g AAC d⁻¹)). However, the difference was not significant to MC0 (P > 0.05). Similar rates (i.e., without significant difference (P > 0.05)) were also observed in MC0, MC30, and MC50 after subsequent methane injections. Based on this result, it can be seen that calcium chloride addition did not significantly improve the methane removal capacity of the immobilized MOB. This salt component might inhibit the capacity of the immobilized MOB to remove methane although higher number of bacteria could be present in the specimen due to the floc formation. The methane removal capacity of MOB was eventually lost upon 90 mM calcium chloride addition (Figure 3.3a.) as a likely result of plasmolysis caused by hyperosmotic shock on the cells. Plasmolysis inhibits molecular biosynthesis which results in impaired cellular activities (Csonka, 1989). Overall, calcium chloride would not be added during the inoculation step of AAC specimens prior to the biofilter run as significant improvement of methane removal by MOB was not observed.

3.1.2 The influence of AAC specimens thickness to the methane removal of immobilized MOB

In this experiment different AAC specimens with different thickness were tested as the carrier material for MOB. Thinner specimens possess higher surface area and higher methane removal rate may be
obtained using thinner specimens as a consequence of the higher contact area between bacteria and methane. Here we investigated the optimum specimen thickness that would give the maximum specific methane removal (i.e., normalized to the weight of the specimen) by the MOB. The following symbols were assigned for incubations with: 10 mm (MT10), 15 mm (MT15), 20 mm (MT20) thick specimens.

**Figure 3.3.** The methane removal in different incubations when: (a) different amount of calcium chloride was added into the culture or when (b) different thickness of Autoclaved Aerated Concrete (AAC) specimens were used. Methane gas was added when the amount in the headspace was depleted until it reached ~20% (v/v) headspace concentration. The following symbols were assigned to different incubations with the following calcium chloride addition: 0 mM (MC0, controls), 30 mM (MC30), 50 mM (MC50), 70 mM (MC70), and 90 mM (MC90). The following symbols were assigned for incubations with: 10 mm (MT10), 15 mm (MT15), 20 mm (MT20) thick specimens.

Differences in methane removal rate were not significant when specimens with different thickness were used up to 18 days of incubation (P > 0.05) (Figure 3.3b.). Afterwards, higher removal rate was examined in MT10 (304.2 ± 1.8 µg C-CH₄ (g AAC⁻ d⁻¹) than in MT15 (196.2 ± 26.6 µg C-CH₄ (g AAC⁻ d⁻¹) or MT20 (200.1 ± 11.7 µg C-CH₄ (g AAC⁻ d⁻¹)). The difference of methane removal rate by MOB on specimens with different thickness could be due to the higher biomass on specimens in MT10 than in other incubations. Higher surface area of specimens is obtained when thinner specimens are used. With higher surface availability for biomass on the specimens surface, higher biomass growth could occur in MT10 than in other incubations. As higher biomass was most likely obtained in MT10 incubations after 18 days of incubations, higher methane removal rate was observed. Based on this batch test, AAC specimens exhibiting higher surface area would be better suited to obtain higher MOB activity. Overall, 10 mm thick specimens would be used to construct the biofilter bed.
### 3.2 Biofilter test

The optimized conditions (i.e., no calcium chloride addition during bacterial inoculation step and using 10 mm thick specimen) obtained from the batch test was used as the basis of the biofilter design. The biofilter test was aimed to investigate the capacity of the immobilized MOB on AAC to remove methane at low concentration (~1000 ppmv) in a biofilter setup. The influence of the amount of biofilter operated in series to the overall methane Elimination Capacity (EC) and Removal Efficiency (RE) in the biofilter was also investigated. Further investigation was performed by analyzing the methane removal distribution along each filter height and the MOB abundance in each filter.

#### 3.2.1 Biofilter inoculation using mixed culture MOB

The methane biofilter in this study would be used to remediate methane in livestock barn (see Part 1 description; p. 37). Previous study showed that the typical atmospheric methane concentration in the livestock barn is ~100 ppmv (Jungbluth et al., 2001). MOB mixed culture was chosen as the inoculum for the biofilter test although the culture could not remove methane at low concentration (i.e., 100 ppmv) on AAC (Chapter 2; Table 2.3). Based on the kinetic study conducted in Chapter 2 (Table 2.4), *M. parvus* exhibited the lowest $K_{\text{m(app)}}$ value among other tested MOB cultures. This indicates that *M. parvus* is the most suitable culture for the biofilter inoculum if the biofilter is to be applied in the livestock barn. However, as explained in Chapter 2 (p. 53), using the obtained $K_{\text{m(app)}}$ (0.4 % (v/v); Table 2.4), a minimum methane concentration of 2000 ppmv is needed to maintain the viability of the bacteria. Hence, based on this kinetic analysis, the inoculation of AAC with *M. parvus* would not be feasible for the biofilter application in the livestock barn as the bacteria will not grow and eventually die.

From the microarray analysis (Figure 2.6), it was found that the mixed MOB culture used in this study was composed of *Methylocystis*-related MOB. Previous study showed that MOB capable to oxidizing methane at a high affinity are typically confined in *Methylosinus* and *Methylocystis* genera (Knief et al., 2003). The aim of using the MOB mixed culture in Chapter 2 as the inoculum of the biofilter was to enrich the high affinity MOB from this *Methylocystis*-based bacterial community. By obtaining mixed MOB predominantly composed of this high affinity MOB, higher activity of the bacteria would be expected at lower concentration (100 ppmv; Chapter 4). Moreover, this “newly composed” mixed MOB culture should have higher survivability at low concentration. Dunfield et al (1999) enrich a high affinity MOB mixed culture from soil at ~275 ppmv of methane for four years (Dunfield et al., 1999). In this study, 1000 ppmv was used to feed the biofilter.

After being enriched at high methane concentration (~20 % (v/v); Chapter 2) in a batch setup, MOB mixed culture could remove methane at significantly lower concentration (~1000 ppmv) in the biofilter for a relatively long period (~4 months) (Figure 3.4). As explained previously, this culture was initially comprised of *Methylocystis spp.* and *Methylocystis*-like *spp.* (Chapter 2). MOB from these groups was shown to oxidize methane at low atmospheric concentration (i.e., ppmv level) and grown under ~1000 ppmv methane concentration (Knief & Dunfield, 2005). Hence this might explain why the culture could remove methane at this low concentration for a prolonged period although they were always enriched in a much higher methane concentration.

#### 3.2.2 The influence of the number of biofilters in series to the overall EC in the biofilters

Using the principle of a steady state ideal plug flow reactor, higher overall EC could be achieved when a higher number of biofilters is operated in series. Assuming a first order kinetic reaction (see the basis of the assumption in Chapter 1, p. 24, the outlet methane concentration from the biofilter can be calculated according to the following equation (Levenspiel, 1972):
k in equation 3.6 is the constant of the first order kinetic reaction. From equation 3.6 it can be seen that by employing a higher number of reactors in a series, the overall reactor “length” is extended. This gives rise to a higher residence time of the substrate (i.e., methane) in the reactor. At higher residence time, lower methane outlet concentration (equation 3.6) and higher substrate conversion rate (i.e., higher EC and RE) should be obtained. To test this hypothesis, three biofilters (MBF-A, MBF-B, MBF-C) was operated by sequential addition in series. MBF-A was run individually for 37 days before MBF-B was installed prior to MBF-B. Both biofilters were run in series for 35 days before MBF-C was installed ahead of MBF-B. All three biofilters were run in series for 54 days before the experiment was stopped.

Methane was removed in the biofilter at varying EC and RE over the period of the test (Figure 3.4a and Figure 3.4b). Lowering RE was observed in MBF-A at the start of the 1st period before it increased after day 12. The RE decreased again as soon the IL was increased on day 16 before it concomitantly increased with the increasing of the IL (i.e., approximately at a set point doubled than the start of the operation). After MBF-B was installed, lower total EC (250.3 g-C CH₄ m⁻³ day⁻¹; day 44) was observed compared to when MBF-A was run individually (425.1 g-C CH₄ m⁻³ day⁻¹; day 36). Total EC increased from day 56 until MBF-C was installed on day 72 as the EC in MBF-A and MBF-B were increasing from day 56 and 64, respectively. The highest total EC (630 g-C CH₄ m⁻³ day⁻¹) and RE (65.54 %) during the 2nd period were reached on day 66.

A decrease in total EC was observed when MBF-C was added. The total EC decreased to 396 g-C CH₄ m⁻³ day⁻¹ (day 80) after all three biofilters were run in series. When liquid nutrient feeding was stopped on day 86, the total EC decreased further until it reached the lowest capacity (176.7 g-C CH₄ m⁻³ day⁻¹) on day 106. After liquid nutrient feeding was started again, the total EC remained approximately at the same level until the end of the test. Carbon dioxide concentration during the experiment always remained at atmospheric level (~300 ppmv; data not shown). The average RE (i.e., all biofilters included) of each phase of the experiment was 28.5 % (day 1 to 36), 31.5 % (day 37 to 72), and 26.1 % (day 72 to 127). Over the whole biofilter operation, the average RE was thus 28.7 %. When day 86 onwards was not included in the calculation to exclude the influence of the stoppage of the liquid nutrient feeding, the average RE from day 72 to 86 would be 34.6 %.

Higher RE in the biofilters wasn’t always obtained with the addition of the biofilter. Conversely, lower RE was obtained directly after day 36 and day 72 where MBF-B and MBF-C were added, respectively. This was likely due to the adaptation period of MOB in the biofilter to a new environmental condition (i.e., methane concentration) causing the lower activity of the bacteria. In this study, this occurred at the start of the biofilter operation and after MBF-B was added (day 37). The adaptation period for the MOB in MBF-A for the 1st and 2nd period lasted for approximately 12 days and 20 days, respectively. The methane removal capacity of MOB in MBF-A dropped again in the 1st period when the inlet load was doubled the set point after day 16 before it increased again after day 24. In the 2nd period, it took 28 days for the MOB in MBF-B to adapt to the biofilter environment after its start-up. Bacterial adaptation period, indicated by the lower RE, was observed as soon as environmental changes were applied to the biofilter system. Previous batch and biotrickling filter studies showed that MOB exhibited lower activity when one of the process conditions was altered (Knief & Dunfield, 2005). In that study, the methane removal capacity of MOB was lower when the bacteria were incubated at changing methane concentration. There, the MOB activity increased after a period of low activity. In this study, there are two identified MOB adaptation periods. They are the adaptation of MOB: (i) from the batch liquid enrichment at 20 % (v/v) methane concentration to the biofilter environment at 1000 ppmv methane concentration, and (ii) the changing of IL occurred on day 16 in MBF-A and after the installment of new
biofilters. After this adaptation period, higher methane removal capacity was always observed (Figure 3.4a). However, this increased activity was not observed in the 3rd period (day 73 onwards) and was likely the result of the absence of liquid feed from day 86 to day 106. Liquid feeding was crucial to the performance of the biofilter. This was further demonstrated when lower MOB activity was examined when liquid feeding was stopped and subsequently the activity increased when liquid feeding was started again (day 86 to 106). Liquid feeding in a biofilter is important to maintain the humidity inside the reactor, keep the reactor temperature constant, and provide essential nutrients to the bacteria (Cohen, 2001). Increasing EC in all biofilters were not observed and the EC remained approximately at the same level from day 106 until the end of the biofilter operation. Hence, it wasn’t conclusive whether MOB were adapting to the changing of the methane feeding concentration and the stoppage of the liquid feeding.

Figure 3.4. (a) Methane Inlet Load (IL), Outlet Load (OL), Removal Efficiency (RE), and (b) Elimination Capacity (EC) in the biofilters (MBF-A, MBF-B, and MBF-C) over 127 days of operation. Different area in (b) indicates the EC in each biofilter. MBF-A was run individually for 37 days before MBF-B was installed prior to MBF-A. Both biofilters were run in series for 35 days before MBF-C was installed ahead of MBF-B. All three biofilters were run in series for 54 days before the experiment was stopped. Liquid feeding was stopped on day 86 and started again on day 106.
The highest RE was obtained on day 66 and this was when two biofilters (MBF-A and MBF-B) were run (65.54%; day 66) and not three (day 72 onwards). It can be seen that, unlike a plug flow reactor, increasing the number of biofilter (thus increasing the reactor volume) did not translate to a higher RE in the overall system. Lower RE was always observed after MBF-C was added compared to the one on day 66. This was still observed even after liquid nutrient feeding was restarted from day 106 onwards.

3.2.3 The distribution of the methane removal in the biofilter along the biofilter height

The methane removal profile along the filter height varied over the period of the test (Figure 3.5). Initially, methane removal was equally distributed in MBF-A along the filter height (day 12). Afterwards, methane removal was relatively higher at the top (port 5 and 6) of the filter (days 27 and 36). When MBF-B was started, the methane removal profile in MBF-A reverted back to the linear trend examined on day 12 (day 44). The high total EC observed after day 44 occurred mainly at the bottom part of MBF-A (port 1 and 2; day 66). After MBF-C addition, the high activity shown at the bottom part of MBF-A was decreased and linear methane removal pattern was again observed along the filter height (day 78). As the three filters were continuously operated, higher methane removal was exhibited in the middle (port 3 and 4) and top (port 5 and 6) part of MBF-A (day 117) whereas a linear methane removal pattern was always shown in MBF-B and MBF-C.

The bacterial methane removal capacity was dependent on the availability of essential nutrients provision (e.g., P and Cu sources) to the bacteria (Hanson & Hanson, 1996). The increased activity observed at the top part of MBF-A after 36 days could be a consequence of the proximity of the filter bed to the liquid feed inlet (Figure 3.5). Hence, nutrients were more accessible to the bacteria at the top part of the filter. Higher methane removal observed at the lower bed of MBF-A after 64 days could be due to the bed proximity to the gas inlet exposing the bacteria to a higher methane concentration (Fig 5). From the kinetic study (Chapter 2), immobilized MOB on building materials always exhibited higher methane removal rate at higher methane concentration.

3.2.4 The distribution of the MOB abundance in the biofilter along the biofilter height

Among the three biofilters, significantly higher total MOB abundance was observed in MBF-B (9.5 x 10^8 ± 1.6 x 10^8 pmoA copies (g dw)^-1; P < 0.05) at the end of the biofilter operation (Figure 3.6.). The total MOB abundance was not significantly different in MBF-A and MBF-C although MBF-A was started 72 days prior to MBF-C (P < 0.05). From this analysis it can be seen that the highest total MOB abundance was not found in the MBF with the longest methane feeding period (i.e., MBF-A ; see Figure 3.4). Significantly higher total MOB abundance was instead found in MBF-B. However, the MOB abundance was higher in other sampling ports of MBF-A and, thus, MOB were uniformly distributed in MBF-A than MBF-B (P >0.05). Although this MOB abundance analyses were only performed at the end of the biofilter operation, this could give an indication why a higher EC was most of the time observed in MBF-A than MBF-B (Figure 3.4). The EC was higher in MBF-B than MBF-A on days 51, 56, 74, 78, 80, 88, and 106 (7 out of 23 measurements after MBF-B was installed). As the biofilters were run during a very short EBRT (0.78 min), having a more uniform MOB distribution along the filter length could increase the overall RE. This could be related to the fact that the feed gas was in contact with a higher number of MOB as it passed through the filter bed. Moreover, it is also not unreasonable to assume that a fraction of MOB population on MBF-A had been lysed and degraded at the end of the experiment making the total population similar to the growing biomass in MBF-C.
Figure 3.5. Methane removal profile along the biofilter bed. Top three figures depict the methane consumption profile in MBF-A for the first 36 days. The middle six figures depict the methane removal profile in MBF-A and B from day 37 to 72. The bottom nine figures depict the methane removal profile in MBF-A, B, and C from day 73 to 127. Methane always entered from port 1 with port 2 to 6 situated equally to different filter bed height. Sampling port 7 was placed close to the gas outlet.
For each MBFs, the highest MOB abundance was detected at the bottom part (filter height equal to port 1 and 2) of the filter whereas the lowest abundance was found at the middle region (filter height equal to port 3 and 4). The highest and lowest MOB abundance per gram of dry weight over the entire setup were detected on the filter bed in which height equal to port 2 (8.8 x 10^8 ± 3.5 x 10^8 pmoA copies) and 4 of MBF-B (8.9 x 10^4 ± 4.3 x 10^4 pmoA copies), respectively. This indicates that MOB grow best in areas exposed to higher methane concentration (i.e., MBF gas inlet). Although, as explained in section 3.2.1, higher activity, and thus higher probability of biomass growth, was observed at the top part of the MBF-A when run individually due to its proximity to the liquid feed inlet (Figure 3.5), the influence of the liquid inlet position was rendered insignificant towards having higher MOB abundance at the end of the biofilter operation. This could be due to the more uniform liquid distribution the longer the biofilters were run. Thus the bacteria had better access to the nutrient compared to the initial run.

A high RE on day 66 could be correlated to the high methane removal around the biofilter inlet (Figure 3.5). This high methane removal can be due to several reasons such as high total MOB abundance or a high abundance of the high affinity MOB. As explained previously, high affinity MOB can typically be found within the *Methylocystis* genera and MOB from this group were identified in the mixed culture inoculum used for the biofilter (Chapter 2). This correlation can be made using the qPCR analysis to investigate the total MOB abundance (Figure 3.6) and diagnostic microarray analysis to identify which MOB that have been enriched there (Chapter 2). However, the qPCR analysis was only performed at the end of the biofilter operation and diagnostic microarray analysis was not performed at all in this study. Hence, direct correlation of the high activity on day 66 to the high total MOB abundance and which MOB that were high in abundance couldn’t be done. The evolution of the MOB abundance in the biofilter was not investigated as the biofilter setup didn’t allow for invasive biomass sampling. This was due to the fact that the biofilter should be kept gas tight especially as bacterial activity was based on component removal in the gas phase (i.e., methane).
4. Conclusions

An optimum methane removal by immobilized MOB mixed culture was reached when 10 mm thick AAC specimens were used and calcium chloride was not added prior to the bacterial inoculation step. The immobilized MOB could remove methane at low methane concentration (~1000 ppmv) in a biofilter setup for 127 days at an average removal efficiency of 28.7%. Optimum RE (65.54 %; day 66) was obtained when two biofilters were operated in series. In all biofilters, MOB had preferential growth near the biofilter inlet where a high methane concentration along the filter length was found. The presented findings confirm the suitability of AAC as a methane biofilter carrier material and represent a novel strategy for constructing an environmentally friendly biofilters. Application of this biofilter for the removal of methane from in-situ livestock gas waste was subsequently investigated in Chapter 4.

Acknowledgements

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CHAPTER 4
METHANE BIOFILTRATION OF RUMINANTS GAS EFFLUENT USING AUTOCLAVED AERATED CONCRETE AS THE CARRIER MATERIAL

Abstract

The performance of Methane-Oxidizing Bacteria (MOB) immobilized on Autoclaved Aerated Concrete (AAC) in a biofilter setup to remove methane from ruminants effluent gas was investigated. Two dairy cows were housed in respiration chambers for two days where the exhaust gas from the chambers was used as the biofilter feed. MOB consumed methane at an average Removal Efficiency (RE) of 17.52% and Elimination Capacity (EC) of 67.3 g m\(^{-3}\) d\(^{-1}\). Several factors that might cause the lower RE and EC found in the lab scale study (Chapter 3; RE = 28.7%) are: (a) the lower methane concentration and (b) the presence of ammonia in the livestock effluent gas, (c) the higher gas flow rate into the biofilter, and (d) the lowering humidity level in the biofilter. By using AAC as carrier material, carbon dioxide in the effluent gas as well as the one produced by the methane oxidation by MOB were removed by the likely carbonation reaction with AAC. Thus, complete carbon sequestration from methane was obtained. Overall, our results showed that a more environmentally friendly methane biofilter process could be achieved when using ACC as the carrier material.

This chapter has been redrafted after:

1. Introduction

Methane emitted from livestock contributes up to ~40% of the global anthropogenic methane emission (Key & Tallard, 2012). Effective livestock methane emission mitigation strategies should focus on emission originating from the ruminants as it accounts for ~90% of the total livestock methane emission (USEPA, 2013). Current approaches include addition of feed supplements to reduce methane generation from rumen and management practices to improve meat/milk efficiency (Martin et al., 2010; Patra, 2012; USEPA, 2013). However, methane is constantly produced in the rumen and retained in the effluent gas.

Ruminants produce methane as a result of the microbial digestion of the food in the rumen and large intestines (Boadi et al., 2004). Protein, starch, and other polysaccharides are hydrolyzed and fermented partly to hydrogen, carbon dioxide, and acetic acid. These components are subsequently converted to methane by methanogens. Methane represents energy loss from the fermentation process and it is primarily emitted by eructation (Baker, 1997). Methane can also be emitted by flatulation or methanogenesis in the anaerobic part of manure, although they do not constitute a significant total emission. Carbon dioxide is produced from both respiration and eructation, while ammonia and nitrous oxide are emitted from the ammonification and nitrification/denitrification processes in the manure (Petersen & Sommer, 2011). These components were the main emission constituents of the ruminant effluent gas.

Considering the low methane concentration in the effluent gas, biotechnological applications are economically beneficial and environmentally friendly strategies to treat the gas (Lopez et al., 2013; Nikiema et al., 2005). Biofiltration, a typical biotechnological application for methane remediation, has been applied to mitigate ruminant methane emissions (Melse & Van der Werf, 2005; Pratt et al., 2012a). Methane-Oxidizing Bacteria (MOB) are the biocatalysts used to degrade methane in the biofilter (Chapter 1 section 2.4). By possessing the Methane Mono-Oxygenase (MMO) enzyme, MOB can oxidize methane and utilize it as the carbon and energy sources (Hanson & Hanson, 1996). In a methane biofilter setup, MOB are immobilized on a carrier material in a fixed bed system. Although a full scale biofilter application has not been established yet, several lab scale tests have been conducted to remove methane emission from livestock housing (Girard et al., 2012; Pratt et al., 2012a; Veillette et al., 2012a).

Different carrier materials have been tested to achieve an optimum methane removal by the MOB. Previously, we showed that Autoclaved Aerated Concrete (AAC) could be used to remove methane at low concentration (i.e., ~1000 ppmv) in a biofilter setup (Chapter 3). AAC is a lightweight building material exhibiting porosity up to 80% pore volume where more than 40% of the pore diameter is between 5 to 100 µm (Chapter 2). With bacteria diameter around 1 to 2 µm, this makes AAC a suitable carrier material to immobilize MOB in the biofilter setup as it can accommodate a high number of bacteria per gram of material (Samonin & Elikova, 2004). In this study, we used the MOB biofilter previously tested in the lab scale environment (Chapter 3) to remove methane from dairy cow effluent gas. We aimed to investigate whether MOB in the biofilter could remove methane emitted from livestock.

2. Materials and Methods

2.1 Methane biofilter

The methane biofilter A (MBF-A) from our previous test (Chapter 3) was used in this research. Although MBF-B exhibiting the highest MOB abundance among all biofilters, more mature biofilm was found in MBF-A as it was run the longest. Higher resistance towards disturbance was expected in a more mature
biofilm (Costerton et al., 1987) as we were testing the biofilter in a more challenging environment (e.g., lower methane concentration). The biofilter was filled with AAC specimens previously inoculated with an MOB mixed culture enriched from circum-neutral agricultural soil (pH ~7.9) originating from Ghent, Belgium (Chapter 2). In Chapter 3, methane gas at ~1000 ppmv concentration was used to feed the filter for 127 days with an average methane removal efficiency of 28.7% (the average over the whole biofilter operation). The present study was conducted one week after the lab scale test.

2.2 Biofiltration in ruminant respiration chamber

The biofilter test was performed in a ruminant respiration facility at the Institute for Agriculture and Fisheries Research (ILVO) in Melle, Belgium. The facility was dedicated to investigate greenhouse gas emission from livestock. In this facility, the dynamics of gasses emitted from ruminants under different conditions such as the feeding and milking period are investigated. The facility consists of six different chambers where the ruminants could be placed individually but only two chambers (i.e., one cow for each chamber) were used in this study (Figure 4.1). Each chamber dimension was 4 m (length) x 1.55 m (width) x 2.8 m (height) and they were made from polypropylene (50 mm thick; Paneltim, Belgium) mounted on an internal stainless steel frame (total effective volume: 12.3 m³). Detailed description of the facility construction has been described elsewhere (Campeneere & Peiren, 2014). During the test, the chambers operated at slightly below atmospheric pressure. Air flew from the front door (67 cm x 37 cm) of each chamber to the exhaust, equipped with a ventilator (diameter: 35 cm; Fancom, The Netherlands), that was located at the rear part of the chamber’s roof. The gas was subsequently released to the atmosphere via a roof panel fitted with an axial exhaust fan (Fancom, The Netherlands). This exhaust fan generated the air flow throughout the chamber.

![Figure 4.1](image.png)

**Figure 4.1.** Biofilter configuration in the exhaust system of the ruminant respiration chambers at ILVO Vlaanderen. A cow was housed in each chamber for two days test. The gas outlet from the chambers went into the exhaust system where the biofilter was. Gas flowed through the filter using a pump installed at the gas outlet. Two gas sampling lines were situated near the biofilter inlet and outlet where a volume of gas was sampled regularly for analysis.

The biofilter was placed horizontally between the roof opening of one of the chambers and the roof panel inside the exhaust system (Figure 4.1). The biofilter had four ports that were used as the effluent gas inlet/outlet (2 ports) and sampling gas lines (2 ports). Gas entered the biofilter through the bottom part and left from the top part of the filter (Figure 4.1). The gas outlet port was connected to a ME2C
pump (Vacuubrand, Germany) to create a gas flow inside the biofilter. The gas outlet was placed relatively far (i.e., approximately 3 meter) from the biofilter to prevent mixing with the inlet gas. The sampling and process gas lines were made using gas leak-proof PFA tubes (Cole-Palmer, USA).

Two dairy cows (from hereon they will be called C165 and C201) were the ruminants employed for the test and they were bred in ILVO. All biofilter connections were opened an hour before both cows entered the chambers. This was done so that the MOB in the biofilter could acclimatize to the environmental conditions around the exhaust system. The summary of the biofilter process and technical parameters can be seen in Table 4.1. The test was performed from the 20th (5 pm) to the 22nd of August 2014 (4 pm). The cows entered the chambers on the 20th of August at ~6 pm. During the measurement period, the feeding/milking of the cows were done at three separate periods. For C165, they were at 8.30 am (21st August), 17.40 pm (21st August), 8 am (22nd August) whereas for C201, they were at 8 am (21st August), 17.10 pm (21st August), 7.45 am (22nd August). Moreover, the cows’ feces and urine were gathered on the 21st of August. The summary of the cows’ feed composition and milk production can be seen in Table S1 and Table S2, respectively. After the test, both cows were guided out from the chambers and the biofilter was collected from the exhaust.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty biofilter volume (m³)</td>
<td>0.0051</td>
</tr>
<tr>
<td>Biofilter bed volume (m³)</td>
<td>0.0026</td>
</tr>
<tr>
<td>Biofilter bed mass (kg)</td>
<td>1.02</td>
</tr>
<tr>
<td>Flow rate biofilter (m³ h⁻¹)</td>
<td>1.2</td>
</tr>
<tr>
<td>Flow rate chambers’ exhaust fan (m³ h⁻¹)</td>
<td>400</td>
</tr>
<tr>
<td>Empty Bed Residence Time (EBRT) (s)</td>
<td>15.3</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>19.1</td>
</tr>
</tbody>
</table>

a average chamber temperature during the measurement period

2.3 Gas composition analysis

Gas measurements started as soon as the biofilter ports connections were opened (i.e., 20th August at 5 pm). Sampling lines from the biofilter were first connected to a Vacu-Guard filter (Whatman, UK), before reaching the analyzer. Additionally, two sampling ports were placed at the exhaust fan of each chamber to analyze the gas composition in the effluent gas from each cow. The gas composition in the samples were analyzed using photoacoustic multi-gas monitor Innova 1312 (LumaSense Technologies, Denmark) and it was used to measure methane, carbon dioxide, nitrous oxide, ammonia, and water concentrations. In the analyzer, the tubes were connected to an eight-channel multi sampler (CBISS, UK) with PFA tubing.

The parameters used for the biofilter evaluations were the removal efficiency (RE) and elimination capacity (EC). These parameters were calculated for methane, ammonia, and carbon dioxide. Additionally, the methane emission rate from the chambers was evaluated. The RE was calculated using the following equation:

\[
RE(\%) = \left(\frac{C_{in} - C_{out}}{C_{in}}\right) \times 100\% \quad (4.1)
\]

where \(C_{in}\) and \(C_{out}\) were the methane inlet (ppmv) and outlet concentrations (ppmv), respectively, from the biofilter. The EC was calculated using the following equation:

\[
EC \ (g \ m^{-3} \ h^{-1}) = \left(\frac{Q_{bf}}{V_{f}}\right) \times \left(C_{in} - C_{out}\right) \times 10^{-3} \times \frac{M}{V_{m}} \quad (4.2)
\]
where $V_f$ was the filter bed volume ($m^3$) and $Q_b$ was the biofilter inlet gas flow rate ($m^3 \cdot h^{-1}$). $V_m$ was the molar volume of methane at 19.1 °C and 0.89 atm (26.9 L mol$^{-1}$). $M$ was the methane molecular weight (16 g mol$^{-1}$). The methane emission rate from the chamber was calculated from the following equation:

$$\text{Methane emission (g d$^{-1}$)} = Q_f \times (C_{\text{chamber}} - C_{\text{background}}) \times 10^{-3} \times 24 \times M/V_m$$  (4.3)

where $C_{\text{chamber}}$ was the methane concentration analyzed from each chamber (ppmv), $Q_f$ was the exhaust fan flow rate ($m^3 \cdot h^{-1}$) and $C_{\text{background}}$ was the background methane concentration (ppmv).

3. Results and Discussion

3.1 Ruminants gas emission from the respiration chambers

Gas concentration profiles in the exhaust gas from both chambers during the measurement period can be seen in Figure 4.2. The average concentration of these gasses throughout the measurement period were 55.5 ppmv (C165) and 54.3 ppmv (C201) for methane, 1.4 ppmv (C165 and C201) for ammonia, and 0.3 ppmv (C165 and C201) for nitrous oxide. Higher average carbon dioxide concentration was analyzed in the gas emission from the C165 chamber (1068.3 ppmv) than the one of C201 (1032.6 ppmv). In average, C165 and C201 emitted 306.8 g methane d$^{-1}$ and 301.2 g methane d$^{-1}$, respectively.

![Figure 4.2](image)

Figure 4.2. Concentration of: (a) methane, (b) carbon dioxide, (c) ammonia, and (d) nitrous oxide in effluent gas from each chamber where cow C165 and C201 resided. Black lines indicates the change of day. Solid (C201) and dashed (C165) grey lines indicate cow feeding time and milking periods.

The dynamics of the methane emission in both chambers was mainly influenced by eructation. Higher methane concentration in the effluent gas was observed in between meals or in the evening (Figure 4.2) which coincided with the rumination period (Beauchemin et al., 1990). In agreement with previous
studies, lower eructation methane emission was analyzed before the morning feeding period (Bell et al., 2014; Garnsworthy et al., 2012). Increasing carbon dioxide emission is usually observed during eructation (Jungbluth et al., 2001). Higher carbon dioxide concentration during eructation could be attributed to the added emission from the ruminal bacterial fermentation (Bell et al., 2014). Ammonia and nitrous oxide emissions mostly likely originate from manure that was present in the chamber and not from the cow itself. The ammonia and nitrous oxide concentration observed in the current research was slightly lower compared to previous studies (Jungbluth et al., 2001; Sun et al., 2008). In conclusion, the effluent gas from the chambers were comparable to the one typically observed from a ruminant in a respiration chamber.

3.2 Biofilter performance

Methane was removed from the cows’ gas effluent in the biofilter throughout the test period with varying removal efficiency (Figure 4.3). The methane concentration in the biofilter inlet was relatively stable at an average concentration of 61.9 ppmv. Prior to the cows’ presence in the chamber, the methane concentration in the biofilter inlet was similar to the background concentration indicating that the additional methane entering the filter afterwards was originating from the cows. The average EC in the biofilter over the measurement period was 67.3 g m⁻³ d⁻¹. The average RE of methane (17.52 %) in the biofilter was lower than the one observed in our lab scale test (RE = 28.7 %; Chapter 3) which could be caused by several factors. Firstly, methane at much lower concentration was fed into the filter compared to the previous study (~1000 ppmv). The activity of the immobilized MOB on building material followed the hyperbolic Michaelis-Menten kinetic model and at lower methane concentration, smaller activity was exhibited by the bacteria (Chapter 2). Secondly, the biofilter operated with much lower residence time in this study as the gas flow rate was six times higher (Table 4.1). This decreased the contact time of methane with the bacteria and therefore the substrate conversion. Thirdly, the presence of ammonia may competitively inhibit the MMO. Ammonia, when dissolved in water is in equilibrium with ammonium. MMO is homologous to the Ammonium Mono-Oxigenase (AMO) enzyme possessed by Ammonia-Oxidizing Bacteria (Bedard & Knowles, 1989). Due to this enzyme catalytic site similarity, previous liquid culture studies showed that MOB were capable to oxidize ammonium. Ammonium oxidation by MOB was observed previously in cell-free extract Methylococcus capsulatus (Bath) containing sMMO (Green & Dalton, 1986). Ammonium is also known to inhibit methane oxidation by MOB in arable and forest soil (Gulledge & Schimel, 1998; Hutsch, 1998; King & Schnell, 1998). Application of ammonium fertilizer can therefore inhibit microbial processes involved in the methane uptake in agricultural sites e.g., rice paddy field (Bodelier et al., 2000). Moreover, according to the Michaelis-Menten kinetic model, a substrate competitive inhibition would lower the enzyme affinity to the substrate (1/Kᵢ). The Michaelis-Menten equation (eq. 1.1; p. 18) therefore becomes:

\[
V = \frac{V_{max(app)} \times C_{NH₄}}{(K_{m(app)} \left(1+\frac{1}{K_i}\right) + C_{CH₄})}
\]

with Kᵢ being the inhibitory component (i.e., ammonium) dissociation constant. At low substrate concentration, the kinetic model followed the first order kinetic (Prats & Forestier, 1988), hence, lower conversion rate at the same substrate concentration would be obtained when ammonium is present (eq. 4.1). This Kᵢ values varied among MOB strains, e.g., 10 mM (Methylomonas methanica; pH 7), 0.2 mM (Methylotrophus trichosporium OB3b; pH 8) (Oneill & Wilkinson, 1977). In soil, the addition of ammonium resulted in the increase of Kᵢ(app) and decrease in the Vₘₐₓ(app) Values (Gulledge & Schimel, 1998).

Finally, the MOB activity could be lowered due to the lower humidity level in the biofilter. This was based on the fact that there was higher water content in the filter gas outlet, presumably due to the water
evaporation from the biofilter (Figure 4.4). The biofilter humidity should be kept to maintain the bacterial activity in a biofilter setup (Cohen, 2001). The gas flowing through the biofilter often desiccates the carrier material by evaporating the moisture on the material.

![Figure 4.3. The methane (a) concentration at the biofilter inlet and outlet and (b) Removal Efficiency (RE) in the biofilter. Black lines indicate the change of day. Dashed line in (b) indicates the average RE in the biofilter. Background mixing ratio is the methane concentration outside the respiration chambers.](image)

Besides methane, the biofilter also influenced the dynamic of other gasses. Carbon dioxide and ammonia were also removed in the biofilter with average RE of 4.02% and 11.47%, respectively (Figure 4.4). Methane is converted by MOB to synthesize new biomass and carbon dioxide (Hanson & Hanson, 1996), however, at low concentration (0-100 ppmv), a complete methane conversion to carbon dioxide occurs (Knief & Dunfield, 2005). In this study, higher carbon dioxide emission was not observed in the biofilter outlet (Fig. 4.4). This could be attributed to the carbonation reaction of carbon dioxide with the binder material of AAC (i.e., tobermorite -1.1 nm) (Chapter 2). Furthermore, ammonia could be removed due to the stripping of the component when passing through the biofilter. The component was most likely dissolved in the water phase of the biofilter (see Figure 1.4). Moreover, with the possibility of ammonia binding and conversion in the MMO, lower ammonia concentration in the biofilter outlet was detected. Nitrous oxide was not removed from the effluent gas whereas higher water concentration was observed in the biofilter outlet.
4. Conclusions

In this study, the capacity of MOB in a biofilter setup using AAC to remove methane from ruminants gas effluent in a respiration chamber was investigated. MOB removed methane at an average RE and EC of 17.52 % and 67.3 g m$^{-3}$ d$^{-1}$, respectively. Compared to the lab scale test, several factors impacted the methane removal capacity of MOB, namely, higher gas flow rate into the biofilter, the lowering humidity level in the biofilter, the presence of ammonia and lower methane concentration in the effluent gas. The use of AAC as the filter bed present an added advantage compared to other materials by removing carbon dioxide produced from the bacterial metabolism and the ruminants by the likely carbonation reaction with tobermorite, AAC binder material.

Acknowledgements

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Supplementary Information

Table S1 Composition of the ration supplied to the animals in the experiment.

<table>
<thead>
<tr>
<th>Feed</th>
<th>Amount (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize silage</td>
<td>21.3</td>
</tr>
<tr>
<td>Haylage</td>
<td>11.7</td>
</tr>
<tr>
<td>Corn cob mixture</td>
<td>1.5</td>
</tr>
<tr>
<td>Sugar beet pulp</td>
<td>5</td>
</tr>
<tr>
<td>Balanced concentrate (F10-14)</td>
<td>0.8</td>
</tr>
<tr>
<td>Balanced concentrate (F09-08)</td>
<td>0.9</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>0.4 (C165)</td>
</tr>
<tr>
<td></td>
<td>0.5 (C201)</td>
</tr>
</tbody>
</table>

Table S2 The milk production of the tested dairy cows during the measurement period

<table>
<thead>
<tr>
<th>Dairy cow(^{b})</th>
<th>Date</th>
<th>Milking time</th>
<th>Milk production (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C165</td>
<td>20 August</td>
<td>5.40 pm</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>21 August</td>
<td>8.30 am</td>
<td>15.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.30 pm</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>22 August</td>
<td>8 am</td>
<td>14</td>
</tr>
<tr>
<td>C201</td>
<td>20 August</td>
<td>5.40 pm</td>
<td>N/A(^{a})</td>
</tr>
<tr>
<td></td>
<td>21 August</td>
<td>8 am</td>
<td>17.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.15 pm</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>22 August</td>
<td>8.45 am</td>
<td>18.4</td>
</tr>
</tbody>
</table>

\(^{a}\) N/A data not recorded
\(^{b}\) C165 and C201 gave birth on the 25\(^{th}\) of February and 21\(^{st}\) of June, respectively. The milk production of both cows increased for the first 90 days after they gave birth. Henceforth, the milk production of the cow decreased by approximately 9% per month.
Overview

Part 2 deals with the exploration of Methane Oxidizing Bacteria (MOB) application on building materials as an alternative biocatalyst for the material surface treatment. Part 2 is divided into two chapters. As biogenic building material surface treatment revolves around the use of Microbiologically Induced Carbonate Precipitation (MICP) (Chapter 1 section 3.2), Part 2 started with the exploration of the formate oxidation-driven MICP by MOB (Chapter 5). Subsequently, the concept was applied as an alternative process for the biogenic concrete surface protection and the effectiveness of this process was investigated (Chapter 6). The results obtained from studies in Chapter 5 and 6 are examined and the application suitability of formate-based MICP by MOB as the surface treatment of concrete is assessed in Chapter 7.
FORMATE OXIDATION DRIVEN CALCIUM CARBONATE PRECIPITATION BY *Methylocystis parvus* OBBP

**Abstract**

Application of Microbially Induced Carbonate Precipitation (MICP) using the urea-based approach in the construction industry poses several disadvantages such as ammonia release to the air and nitric acid production. An alternative MICP from calcium formate by *Methylocystis parvus* OBBP is presented in this study to overcome these disadvantages. To induce calcium carbonate precipitation, *M. parvus* was incubated at different calcium formate concentrations and starting culture densities. Up to 91.4 % ± 1.6 % of the initial calcium was precipitated in the methane amended cultures compared to 35.1 % ± 11.9 % when methane was not added. Because the bacteria could only utilize methane for growth, higher culture densities and therefore calcium removal was exhibited in the cultures when methane was added. A higher calcium carbonate precipitate yield was obtained when higher culture densities were used but not necessarily when more calcium formate was added. This was mainly due to salt inhibition of the bacterial activity at a high calcium formate concentration. A maximum of 0.67 ± 0.03 CaCO$_3$ Ca(CHOOH)$_2$ (g/g) calcium carbonate precipitate yield was obtained when $10^9$ cells mL$^{-1}$ and 5 g L$^{-1}$ of calcium formate were used. Compared to the current strategy employing biogenic urea degradation as the basis for MICP, the approach in this study presents significant improvements in terms of pollutant emission reduction if applied in the construction industry.

This chapter has been redrafted after:


DOI: 10.1128/AEM.01349-14
1. Introduction

Microbially Induced Carbonate Precipitation (MICP) is a well-known process and has been extensively investigated (Chapter 1 section 3.2.1). In short, MICP produces carbonate minerals, e.g., calcium carbonate, as a result of alterations in environmental conditions. In nature, examples of MICP include calcite formation in soils (Braissant et al., 2002), limestone caves (Cacchio et al., 2003), seas (Morita, 1980) and soda lakes (Thompson & Ferris, 1990). Four different key parameters that govern microbially induced calcium carbonate precipitation are the: (a) concentration of non-precipitated calcium, (b) concentration of the total inorganic carbon, (c) pH, and (d) availability of nucleation sites for calcium carbonate crystal formation (Hammes & Verstraete, 2002). Among the four parameters, bacterial activities mainly influence the total inorganic carbon concentration and the pH of the environment (De Muynck et al., 2010).

MICP is the basis for several biotechnological applications in the construction sector (Chapter 1 section 3.2). These include the use of calcium carbonate precipitate to protect concrete surface against the ingress of deleterious substances (e.g., chloride ions) (De Muynck et al., 2008a) or to heal cracks in aging concrete (Van Tittelboom et al., 2010; Wang et al., 2012b). Among the bacterial activities that can induce calcium carbonate precipitation, urea degradation by heterotrophic bacteria is typically used for applications on building materials. In biogenic urea degradation, urea is transformed to ammonia and carbonate ions to initiate precipitation (Stocks-Fischer et al., 1999). Bacillus spp. (e.g., B. sphaericus) is the most commonly applied urea degrader for MICP in the construction sector due to several advantages such as the high initial urea degradation rate by the strain and a highly negative $\zeta$ potential of the strain (Dick et al., 2006).

However, the use of urea degradation based MICP in the construction sector poses several drawbacks. Firstly, ammonia production can pollute the air. Secondly, with $pK_a$ of ammonium/ammonia around 9.25 at 25°C (Bates & Pinching, 1949), ammonium can be present inside the building material and nitrified by bacteria into nitric acid which in turn reacts with calcite from the building material to form calcium nitrate. Calcium nitrate is a highly soluble component and the dissolution of this component in the building material can contribute to the biodeterioration of the material (Piqué et al., 1992). Therefore, an alternative MICP for application in the construction sector needs to be investigated.

Methane-Oxidizing Bacteria (MOB) are a subset of methylotrophic bacteria capable of utilizing methane as their carbon and energy source (Chapter 1 section 2.1). As part of the dissimilatory methane oxidation pathway, MOB oxidize formate to CO$_2$ using the formate dehydrogenase enzyme. Methylocystis parvus OBBP, a type II MOB, has been previously investigated for biotechnological applications. M. parvus OBBP is known to synthesize Poly-3-Hydroxybutyrate (PHB), a biopolymer that is used as a raw material for bioplastics (Pieja et al., 2011b). The strain accumulates PHB intracellularly when it is provided with an excess of carbon source and in the absence of sufficient essential nutrients (e.g., nitrogen, phosphorus, etc) (Pieja et al., 2011a; Pieja et al., 2011b). For bioremediation purposes, the particulate Methane Monoxygenase enzyme expressed by Methylocystis spp. can also degrade several pollutants such as halogenated alkanes (Ho et al., 2012; Semrau et al., 2010).

In this study, M. parvus OBBP was investigated as an alternative biocatalyst to induce calcium carbonate precipitation from calcium formate. We hypothesize that formate utilization by M. parvus OBBP will lead to an increase of pH and carbonate production. With the availability of calcium ions from calcium formate and the potential use of M. parvus OBBP cell wall as the nucleation site, calcium carbonate precipitation is favored. This study is divided into two parts. First, a proof of principle of calcium carbonate precipitation by M. parvus OBBP from calcium formate was performed. Second, the influence
of the culture density and calcium formate concentration to the calcium carbonate precipitate yield was investigated.

2. Materials and Methods

2.1 Bacterial strain and culture condition

As *Methylocystis parvus* OBBP exhibited the highest methane removal rate when immobilized on different building materials (Chapter 2), this strain was utilized in this study. *Methylocystis parvus* OBBP was obtained from Colin Murrell (School of Environmental Science, University of East Anglia). *M. parvus* OBBP was grown in Nitrate Mineral Salt (NMS) medium (Whittenbury et al., 1970) in serum bottles (Schott-Duran, USA) under ~20 % (v/v) methane concentration in the headspace. The bottles were incubated on a shaker (120 rpm) at 28° C. For the precipitation experiments, twenty times lower phosphate buffer concentrations (i.e., 35.9 mg L\(^{-1}\) and 13.6 mg L\(^{-1}\) of Na\(_2\)HPO\(_4\).12H\(_2\)O and KH\(_2\)PO\(_4\), respectively) in the NMS medium were used. This was done to minimize the buffering capacity of the medium but for the medium to still sufficiently provide a phosphorus source for the bacterial growth. Experiments were performed using 125 mL PYREX\® serum bottles (Corning, USA). Bottles were acid washed by immersion in 1 M nitric acid (VWR, Belgium) for one day and left to dry to remove trace metals from the bottles’ surface. Incubations were performed aseptically by autoclaving the bottles at 120° C for 20 minutes before experiments and by preparing the set up under laminar flow.

2.2 Calcium carbonate precipitation by *Methylocystis parvus* OBBP

This experiment was performed to investigate MICP from calcium formate by *M. parvus* OBBP. *M. parvus* OBBP was grown until mid-logarithmic phase before the cells were collected by centrifugation at 10,000 X g for 10 minutes, washed twice with saline solution (8.5 g L\(^{-1}\) NaCl), and resuspended in NMS medium. Fifty µL of the culture was sampled to determine the total number of cells before they were added into the serum bottles. Sterile calcium formate and NMS medium were mixed in different bottles (working volume: 7 mL) to have final formate concentrations of: 0.04, 0.14, 0.44, 0.72, 1.1, 1.44, 1.83, 2.32, and 2.88 g L\(^{-1}\). A 1 mL of the culture was subsequently added to each bottle. Serum bottles containing bacterial culture and NMS medium without formate addition served as references. Two mL of liquid sample were taken afterward from each bottle, filtered using 0.22 µm filter (Milipore, USA), and stored at 4°C until further analysis. The bottles were then capped with butyl rubber stoppers (Rubber B.V., The Netherlands), sealed with crimp caps (Agilent Technologies, Belgium) and incubated on a shaker (120 rpm) at 28° C for four days, after which, samplings were performed. Approximately 2 mL of liquid were taken at the end of incubation period from each bottle for bacterial cell counting and liquid sample analysis. After cell count determination, the liquid samples were filtered using 0.22 µm filter (Milipore, USA) and stored at 4°C until further analysis.

The influence of methane addition on calcium carbonate precipitation by *M. parvus* OBBP was also investigated. Sealed serum bottles with bacterial inoculated mixture of calcium formate and NMS medium were injected with methane (99.5 % (v/v), Air Liquide, Belgium) to reach ~10 % (v/v) methane concentration in the headspace. Afterwards, the headspace gas composition was determined and the gas pressure was measured using a tensimeter (WIKA, Germany). This was repeated daily. Methane Oxidation Rate (MOR) by *M. parvus* OBBP was determined by linear regression following the methane depletion in the headspace over time according to the method described in Chapter 2. Liquid sampling procedure, as described previously, was done before and after the incubation.
For all type of incubations, reference incubations containing *M. parvus* OBBP in sodium formate and uninoculated calcium formate were performed. For both incubations, methane was added into the bottles. Incubations in sodium formate were done to investigate the evolution of measured parameters (e.g., bacterial growth) when calcium carbonate precipitation was absent. The uninoculated calcium formate incubations were performed to verify that the calcium carbonate precipitation was driven by the bacterial formate oxidation. Incubations were performed in triplicates. For simplification, the following abbreviations were assigned to the different treatments: bacterial incubations in methane and calcium formate (MCF), calcium formate (CF), methane and sodium formate (MSF). UMCF was assigned to the uninoculated incubations containing calcium formate under methane.

### 2.3 Optimization of calcium carbonate precipitate yield from calcium formate by *M. parvus* OBBP

This experiment was performed to determine the influence of calcium formate concentrations and bacterial cell densities to the calcium carbonate precipitation yield from calcium formate (g CaCO$_3$/g Ca(CHOOH)$_2$). Triplicate incubations from each of $10^6$, $10^7$, $10^8$, $10^9$ cells mL$^{-1}$ *M. parvus* OBBP and 0.5, 2.5, 5, 10 g L$^{-1}$ calcium formate were prepared. *M. parvus* OBBP was grown in NMS medium, and the cells were collected as described previously. The bacterial culture was resuspended in deionized water. For each treatment, 8 mL mixture of sterile calcium formate and the bacterial culture was made in the serum bottles. Before and after the incubation, 2 mL of liquid sample were taken, filtered using 0.22 µm filter (Milipore, USA), and stored at 4°C until further analysis. The serum bottles were then capped with butyl rubber stoppers (Rubber B.V., The Netherlands) and incubated for one day on a shaker (120 rpm) at 28° C. In this experiment, methane was not added into the bottles. When the process is applied on building wall, to minimize bacterial wash out due to environmental effects such as rain, then fast precipitation is preferred. Thus, a short incubation time was set in the experiment.

Additional 50 mL incubations of $10^9$ *M. parvus* OBBP cells mL$^{-1}$ without calcium formate addition and in 2.5, 5, and 10 g L$^{-1}$ calcium formate were made. The bigger liquid volume was used in order to obtain a sufficient biomass pellet from the culture after the experiment. The pellets were used to determine calcium carbonate crystals morphology, phase, and polymorphs. The biomass pellets were collected after the experiments as described previously.

### 2.4 Bacterial cell count

Bacterial cell count was performed using BD Accuri™ C6 flow cytometer (BD Biosciences, Belgium) according to the live/dead staining protocol as described previously (Van Nevel et al., 2013). For the analysis, each culture sample contains 500 µL mixture of: bacterial culture (5 µL or 50 µL, depending on the dilution factor), fluorescent dyes (5 µL; dye composition is described in (De Roy et al., 2012)), and sterile physiological solution (0.9% (v/v) of NaCl). The total number of propidium iodide and SYBR green tagged cells per mL of the analyzed sample was reported as the culture density.

### 2.5 Gas composition analysis

Methane and oxygen were measured using a Compact Gas Chromatograph (GC) (Global Analyser Solution, The Netherlands) equipped with a Thermal Conductivity Detector, a Porabond pre-column, and a Molsieve SA column using methods described in Chapter 2. A 1 mL of gas sample was taken from each serum bottle before being injected into the GC using a gas tight syringe (Hamilton, Belgium).
2.6 Liquid sample analysis

Liquid samples were analyzed for the: (a) formate concentration, (b) soluble calcium concentration, and (c) pH. The formate concentrations in samples were measured using DX-500 BioLC liquid chromatograph that was equipped with an AS1 column and an ED50 Conductivity Detector (Dionex, USA). The soluble calcium concentration was measured using AA-6300 Atomic Absorption Spectroscopy (Shimadzu, Japan). 100 µL and 200 µL of 65 % (v/v) nitric acid (VWR, Belgium) and 1 g L⁻¹ lanthanium standard solution (Chem-lab, Belgium), respectively, were added to each sample before analysis. The amount of calcium carbonate precipitated was calculated from the amount of the removed soluble calcium in the culture. The pH was measured using a C-532 pH electrode (Consort, Belgium).

2.7 X-ray diffraclometer (XRD)

Stored biomass pellets of 10⁹ cells ml⁻¹ M. parvus OBBP culture in 0, 2.5, 5, and 10 g L⁻¹ calcium formate were used for the identification of the calcium carbonate precipitated crystal phase by XRD analysis. XRD spectra for each sample was analyzed using a Thermo Scientific ARL X'TRA Powder Diffractometer equipped with a Peltier cooled detector. The X-ray diffractometer was operated at 40 kV and 30 mA with monochromated CuKα radiation. XRD data, over the range of 3 to 60° 2θ, were collected with a step size of 0.02° and a preset time of 1 sec at each step.

2.8 Scanning Electron Microscopy (SEM)

Stored biomass pellets of 10⁹ cells mL⁻¹ M. parvus OBBP culture in 5 g L⁻¹ calcium formate were used for the SEM analysis. The pellet was placed on an aluminum stub with a carbon conductive tab and dried at 60° C for approximately two hours to remove the water content in the pellet. In addition to the image analysis, elemental composition analysis of the samples was also carried out using an Energy-Dispersive X-ray Spectroscopy (EDS). SEM and EDS analysis were performed using a Phenom ProX desktop scanning electron microscope (Phenom-World B.V, Eindhoven, The Netherlands), with 10 kV and 15 kV accelerating voltages for image and EDS analysis, respectively. Before analysis, samples were sputtered with 2 nm Pt-Pd coating. Samples of non-biogenic calcium carbonate were also analyzed by EDS and used as reference.

2.9 Transmission Electron Microscopy (TEM)

Stored biomass pellets of 10⁹ cells mL⁻¹ M. parvus OBBP culture in 5 g L⁻¹ calcium formate were used for TEM analysis. The bacteria were fixed in 0.1 M cacodylate buffer containing 4% paraformaldehyde and 5% glutaraldehyde. TEM images were collected at 50 kV using method described previously (Hosseinkhani et al., 2012). Images were taken using Zeiss TEM 900 transmission electron microscope using (Carl Zeiss, Germany).

2.10 Statistical analysis

Besides pH measurements, except stated otherwise, values are the mean from triplicate measurements and error bars represent standard deviations. pH measurements were carried out once for each type of incubation. Statistical analyses were done in SigmaPlot v12.0 (Systat Software Inc, USA) to compare significant differences of values between different incubations by means of one way ANOVA test (p=0.05).
3. Results

3.1 Calcium carbonate precipitation by *M. parvus OBBP* from calcium formate

This experiment was performed to investigate MICP from calcium formate by *M. parvus* OBBP. This was done by incubating the MOB culture at different calcium formate concentrations. The influence of methane addition on calcium carbonate precipitation by *M. parvus* OBBP was also investigated. For simplification, the following symbols were assigned to the different treatments: bacterial incubations in methane and calcium formate (MCF), calcium formate (CF), methane and sodium formate (MSF). UMCF was assigned to the uninoculated incubations containing calcium formate under methane. Overview of different evaluated parameters from this experiment can be seen in Figure 5.1.

![Figure 5.1](image)

**Figure 5.1.** The (a) culture density, (b) methane oxidation rate, (c) calcium removal, and (d) formate removal exhibited in MCF (□), CF (○), MSF (▲), and UMCF (◇) incubations. The abbreviations represent different type of incubations: bacterial incubations in methane and calcium formate (MCF), calcium formate (CF), methane and sodium formate (MSF), UCFM was assigned to the uninoculated incubations containing calcium formate under methane. The dotted line in Figure 5.1a. indicates the initial *M. parvus* OBBP culture density (9 x 10^6 cells mL^-1). Values are the average of triplicate measurements. Error bars represent the standard deviation.
M. parvus could drive calcium carbonate precipitation when incubated in calcium formate and the degree of the precipitation varied according to the starting formate concentration used (Figure 5.1a to 5.1d). One to two log increase of M. parvus OBBP culture densities were observed in almost all methane amended cultures compared to their initial culture densities (Figure 5.1a). However, for all type of incubations, lower culture densities were exhibited by the bacteria at formate concentrations higher than 1.44 g L\(^{-1}\). The MOR exhibited by M. parvus OBBP also decreased at higher formate concentrations (Figure 5.1b). The bacteria showed higher MOR in MCF than MSF when the same amount of formate was added, except in 1.83 and 2.88 g L\(^{-1}\) of formate where the differences were not significant (\(P > 0.05\)). M. parvus OBBP exhibited higher calcium removal in MCF than CF at formate concentrations higher than 0.5 g L\(^{-1}\) (Figure 5.1c). In MCF, it was observed that the calcium removal by MOB exhibited a hyperbolic increase from 3.5 % ± 0.6 % to 91.4 % ± 1.6 % at 0.04 g L\(^{-1}\) and 2.88 g L\(^{-1}\) of formate, respectively. M. parvus OBBP could not remove more than 50 % of the initial calcium in CF at all formate concentrations tested. The bacteria removed formate completely at different formate concentrations in M1 whereas varying formate removals were exhibited by the bacteria in CF or MSF (Figure 5.1d.). Decreasing formate removal was exhibited by the bacteria in CF and MSF when formate concentration higher than 1.5 g L\(^{-1}\) was added. In CF, M. parvus OBBP exhibited only a maximum of 61.7 ± 9.1 % of formate removal and this was observed when 1.1 g L\(^{-1}\) of formate was added into the culture. The calcium and formate removal exhibited in all UMCF incubations were not significant. Furthermore, an increase of pH in all cultures was observed when formate was added into the M. parvus OBBP culture (Table 5.1). Higher pH increase was observed in MSF when formate more than 1.44 g L\(^{-1}\) was added. In MCF and CF, above 0.44 mg L\(^{-1}\) of formate, the pH changes in the cultures were not appreciable at increasing formate concentrations.

**Table 5.1** The initial pH and the pH differences in MCF, CF, and MSF cultures before and after the incubation. The abbreviations represent different type of incubations: bacterial incubations in methane and calcium formate (MCF), calcium formate (CF), methane and sodium formate (MSF).

<table>
<thead>
<tr>
<th>Initial formate concentration (g L(^{-1}))</th>
<th>MCF</th>
<th>CF</th>
<th>MSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH (t=0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>dpH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>pH (t=0)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>6.6</td>
<td>0</td>
<td>6.6</td>
</tr>
<tr>
<td>0.04</td>
<td>6.6</td>
<td>0.8</td>
<td>6.6</td>
</tr>
<tr>
<td>0.14</td>
<td>6.6</td>
<td>1</td>
<td>6.6</td>
</tr>
<tr>
<td>0.44</td>
<td>6.7</td>
<td>1.2</td>
<td>6.7</td>
</tr>
<tr>
<td>0.72</td>
<td>6.8</td>
<td>1.1</td>
<td>6.8</td>
</tr>
<tr>
<td>1.10</td>
<td>6.9</td>
<td>1.1</td>
<td>6.9</td>
</tr>
<tr>
<td>1.44</td>
<td>6.9</td>
<td>1.1</td>
<td>6.9</td>
</tr>
<tr>
<td>1.83</td>
<td>6.9</td>
<td>1.1</td>
<td>6.9</td>
</tr>
<tr>
<td>2.32</td>
<td>6.8</td>
<td>1.3</td>
<td>6.8</td>
</tr>
<tr>
<td>2.88</td>
<td>6.7</td>
<td>1.2</td>
<td>6.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>The initial incubation pH of M. parvus OBBP culture
<sup>b</sup>The pH increase in M. parvus OBBP culture before and after the incubation period
<sup>c</sup>ND, not determined

### 3.2 Influence of calcium formate concentration and bacterial cell density on the calcium carbonate precipitation yield

After proving that MICP could be driven by the formate oxidation by M. parvus, experiments were performed to investigate the optimum calcium carbonate precipitation yield (g CaCO\(_3\), g Ca(CHOOH)\(_2\)) from this process. This was done by testing different starting calcium formate concentrations (0.5, 2.5, 5, 10 g L\(^{-1}\)) and bacterial cell densities (10\(^6\), 10\(^7\), 10\(^8\), 10\(^9\) cells mL\(^{-1}\)). The results of this test can be seen in Table 5.2.
The calcium and formate removal of *M. parvus* OBBP was dependent on the calcium formate concentration and the culture density used (Table 5.2). At the same calcium formate concentration, higher calcium and formate removal were obtained in cultures when higher culture densities were used, with the exception being the bacterial calcium removal at 0.5 g L\(^{-1}\) of calcium formate. However, using the same value of culture density, higher calcium and formate removal were not necessarily exhibited by the bacteria when higher calcium formate concentrations were used. For example, in \(10^9\) cells m\(^{-1}\) culture, lower calcium and formate removal were observed in the cultures at 10 g L\(^{-1}\) of calcium formate (36.7 ± 7.1 (formate), 31.8 ± 5.6 (calcium)) than at 5 g L\(^{-1}\) of calcium formate (98.5 ± 0.1 (formate), 87.4 ± 3.8 (calcium)).

The maximum calcium carbonate precipitate yield obtained from different culture densities and calcium formate concentrations tested was 0.67 ± 0.03 CaCO\(_3\) Ca(CHOOH)\(_2\) (g/g) (Table 5.2). The yield was calculated from the amount of calcium carbonate precipitated (i.e., the removal of soluble calcium) over the amount of the calcium formate added. For each tested culture density, the maximum calcium carbonate precipitation yield could be obtained when 2.5 g L\(^{-1}\) of calcium formate was added. The maximum calcium carbonate precipitation yield, as described previously, was obtained from 5 g L\(^{-1}\) of calcium formate and \(10^9\) cells mL\(^{-1}\) cultures. However, there was no significant yield difference when either 2.5 g L\(^{-1}\) or 5 g L\(^{-1}\) of calcium formate was added (\(P > 0.05\)).

### 3.3 The morphologies, polymorphs, and cellular locations of the calcium carbonate crystals produced by *M. parvus* OBBP

Vaterite and calcite were the two main polymorphs identified in the calcium carbonate crystals from *M. parvus* OBBP cultures at varying formate concentrations (Figure 5.2). Several peaks of vaterite and calcite polymorphs were depicted in almost all XRD spectra from each type of culture. The highest vaterite peak was significantly shown in the culture at 10 g L\(^{-1}\) of calcium formate. The vaterite compositions in all incubations except at 5 g L\(^{-1}\) of formate were between 80 to 90 % (wt/wt) whereas the calcite composition at 5 g L\(^{-1}\) of formate was 58.5% (wt/wt) (calculated data). Aragonite was also detected in crystals from all type of incubations with a maximum of 6.6% (wt/wt) in culture at 10 g L\(^{-1}\) of formate (data not shown).

![Figure 5.2. XRD patterns from the biomass pellets of *M. parvus* OBBP cultures in 0 (without calcium formate addition), 2.5, 5, and 10 g L\(^{-1}\) of calcium formate. V and C indicate the XRD peaks that correlate with vaterite and calcite peaks, respectively.](image-url)
Table 5.2  Formate removal, calcium removal, and calcium carbonate precipitation yield exhibited in the cultures when *M. parvus* OBBP were incubated in varying culture densities and concentrations of calcium formate. Value appears in bold is the maximum calcium carbonate precipitation yield obtained from the test.

<table>
<thead>
<tr>
<th>Calcium formate (g L⁻¹)</th>
<th>10⁶</th>
<th>10⁷</th>
<th>10⁸</th>
<th>10⁹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formate removal (%)</td>
<td>5.8 ± 2.2</td>
<td>10.9 ± 1.9</td>
<td>0.08 ± 0.01</td>
<td>33.0 ± 1.4</td>
</tr>
<tr>
<td>Calcium removal (%)</td>
<td>2.5</td>
<td>0.7</td>
<td>8.5</td>
<td>0.07</td>
</tr>
<tr>
<td>CaCO₃ yield (g CaCO₃/ g Ca(CHOOH)₂⁻¹)</td>
<td>1.4 ± 0.8</td>
<td>9.0 ± 2.1</td>
<td>0.07 ± 0.02</td>
<td>2.5 ± 1.9</td>
</tr>
<tr>
<td>Formate removal (%)</td>
<td>10</td>
<td>0.7</td>
<td>7.5</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*Value is the average of duplicate measurements
The morphologies and likely cellular location of the bacterially induced calcium carbonate crystals were indicated in TEM and SEM images (Figure 5.3 and Figure 5.4). Calcium carbonate crystals seem to accumulate and adsorb on the surface of the bacterial cell wall (Figure 5.3). This observation was seen in almost all bacterial cells in the TEM images. Spherical crystals shape were observed in bacterial cultures when 5 or 10 g L$^{-1}$ calcium formate were added (Figure 5.4). Grouped spherulite crystals were observed in SEM image of the culture at 10 g L$^{-1}$ calcium formate but not at 5 g L$^{-1}$ calcium formate. The energy dispersive spectra of the spherulite was similar to the non-biogenic calcium carbonate whereas the mean peak of calcium (i.e., 3.69 keV) in the energy dispersive spectra at places without the spherical crystals were significantly lower.

**Figure 5.3.** (a-d) Transmission Electron Microscopy (TEM) images of *M. parvus OBBP* cultures in 5 g L$^{-1}$ of calcium formate. The arrows indicate the likely location of calcium carbonate crystals on the bacterial cells.

4. Discussion

4.1 Calcium carbonate precipitation by *M. parvus OBBP* from calcium formate

Significant bacterial growth was only observed in the methane amended cultures (Figure 4.1a). This was due to the fact that biomass could only be synthesized by the bacteria from methane but not formate. Methane oxidation by *M. parvus OBBP* generates formaldehyde, an intermediate for carbon assimilation via the serine cycle (Hanson & Hanson, 1996). Formate transformation to CO$_2$ generates NADH that can only be used as a reducing power in other metabolic processes, for example, hydroxypyruvate conversion to glycerate in the serine cycle (Asenjo & Suk, 1986). Moreover, formate addition could inhibit *M. parvus OBBP* growth as indicated by the lower MOR exhibited by the bacteria (Figure 4.1b). From the Herbert-Pirt equation, a low specific substrate utilization rate results in a low specific biomass growth (Tijhuis et al., 1993). In our study, the low MOR was likely caused by the low methane concentration in the liquid phase. From the hyperbolic substrate utilization kinetic (Knief & Dunfield, 2005), with methane as the sole carbon and energy source, MOB exhibited low MOR at a low methane concentration. The lower dissolved methane concentration upon formate addition was likely a consequence of the lower methane solubility; Salt addition lowers the water potential and thus lowers the methane diffusivity into the liquid phase (Schnell & King, 1996).
Figure 5.4. Scanning Electron Microscopy (SEM) images and Energy-Dispersive Spectroscopy (EDS) spectra of *M. parvus* OBBP culture in (a) 5 g L\(^{-1}\) and (b) 10 g L\(^{-1}\) of calcium formate. EDS analyses were done at predicted calcium carbonate crystal spots (i.e., spot 1 and 3; red line) and, at places without calcium carbonate crystals (i.e., spot 2 and 4; blue line). For reference, the EDS of non-biogenic calcium carbonate crystals are also shown in the graph (black line).
Without significant calcium and formate removal in M4, calcium carbonate precipitation occurred as a result of the bacterial formate oxidation (Figure 5.1c and Figure 5.1d). Formate conversion to CO₂ by \textit{M. parvus} OBBP led to an increase of the pH in the culture (Table 5.1). Previous studies have shown that bacterial or fungal utilization of low molecular organic compounds like formate (e.g., acetate) would lead to an increase in environmental pH (Braissant et al., 2003; Braissant et al., 2002; Martin et al., 2012). In a solution, formate and CO₂ are in equilibrium with formic acid and carbonic acid, respectively. The pH increase in the cultures occurred due to the bacterial conversion of formic acid to the weaker carbonic acid and this would shift the carbonate system towards carbonate ions production (equation 5.1; (Mucci, 1983)). Calcium carbonate was then formed from the reaction between calcium ions from calcium formate and carbonate ions from the formate conversion (equation 5.2).

\[
\text{CO}_2(g) \leftrightarrow \text{CO}_2(aq) + \text{H}_2\text{O} \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^- \leftrightarrow 2\text{H}^+ + \text{CO}_3^{2-} \quad (5.1)
\]

\[
\text{Ca}^{2+} + \text{CO}_3^{2-} \leftrightarrow \text{CaCO}_3 \quad (5.2)
\]

\[
\Omega = \frac{a(\text{Ca}^{2+})a(\text{CO}_3^{2-})}{K_{\text{so}}} \quad \text{with } K_{\text{so calcite,25°C}} = 3.8 \times 10^{-9} \text{ mol L}^{-1} \quad (5.3)
\]

Based on the thermodynamic approach, when the total ionic activity product from the calcium carbonate formation exceeds the calcium carbonate equilibrium constant (\(K_{\text{so}}\)) then the system is supersaturated (i.e., the saturation state (\(\Omega\)) > 1) and calcium carbonate precipitation is likely to occur (equation 5.3; (De Muynck et al., 2010a)).

\subsection*{4.2 Influence of calcium formate concentration and bacterial cell density on the calcium carbonate precipitation yield}

Higher calcium carbonate precipitate yields were obtained when higher \textit{M. parvus} OBBP culture densities were used but not necessarily at higher calcium formate concentrations (Table 5.2). At higher formate concentrations, the (i) precipitation time, (ii) salt stress, and (iii) crystallization surface area availability are limiting the precipitation rate. Longer incubation time was needed for the substrate (e.g., formate) conversion to induce precipitation at a high substrate concentration (Rivadeneyra et al., 2004). Higher formate and calcium removal exhibited by the bacteria are therefore anticipated at longer incubation time. A high calcium formate addition also increased the salt stress imposed on the cells thus limiting their activity (Ho et al., 2012). \textit{Methylocystis spp.} are generally known to be robust, and able to withstand different forms of stress (Ho & Frenzel, 2012; Ho et al., 2012; Ho et al., 2011), however, \textit{M. parvus} OBBP is not proven to be a halophilic MOB (Whittenbury et al., 1970). Hence, the addition of salt may eventually inhibit the activity of the bacteria. The inhibition effect of calcium formate to the precipitation yield was observed especially when 10 g L⁻¹ of calcium formate was added. Moreover, there was a limited surface area available for the bacteria to bind calcium ions for a given amount of biomass. Therefore further addition of calcium formate did not necessarily result in a higher calcium carbonate precipitate yield. Overall, as observed in another study (De Muynck et al., 2010b), there is an optimum process condition to obtain a maximum calcium carbonate precipitate yield. In our study, 5 g L⁻¹ of calcium formate and 10⁹ cells ml⁻¹ were the optimum calcium formate concentration and culture density, respectively, to obtain a maximum calcium carbonate precipitate yield.

\subsection*{4.3 The morphologies, polymorphs, and cellular locations of the calcium carbonate crystals produced by \textit{M. parvus} OBBP}

Proof of calcium carbonate precipitation by \textit{M. parvus} was further observed from XRD, TEM, and SEM analyses (Figure 5.2, Figure 5.3., Figure 5.4.). The three possible calcium carbonate crystal polymorphs
Formate oxidation driven calcium carbonate precipitation by *Methylocystis parvus* OBBP

(i.e., calcite, vaterite, aragonite) were obtained in *M. parvus* OBBP cultures in 5 g L\(^{-1}\) of calcium formate. Thermodynamically, vaterite and aragonite are metastable crystal phases whereas calcite is the more stable polymorph (Spanos & Koutsoukos, 1998). Vaterite, commonly formed at a high supersaturation, was suggested to be the precursor of calcite, which is formed at a low supersaturation (Jimenez-Lopez et al., 2008; Rodriguez-Navarro et al., 2003). In this study, vaterite seemed to be the main crystal phase at most incubation type. The type of calcium carbonate polymorphs is important for biotechnological applications in the construction industry. Calcite is the most preferred crystal phase due to its stability and its higher consolidating effect (Rodriguez-Navarro et al., 2003). However, vaterite is not a disadvantage as it could also be stabilized in the longer term (Rodriguez-Navarro et al., 2007).

From the EDS spectra analyses, it could be confirmed that the spherulite crystal in SEM images were composed mostly of calcium carbonate. The spherulite crystal observed from SEM analyses is known to be the final morphological stage of the biogenic calcium carbonate crystal development (Braissant et al., 2003; Rivadeneyra et al., 2004). However, in contrast to a previous study (Rivadeneyra et al., 2004), the grouped spherulite crystals were formed at a high salinity (i.e., 10 g L\(^{-1}\) of calcium formate) instead of at low salinity. The lower EDS peaks from calcium carbonate crystals spots compared to the pure calcium carbonate might indicate that the biogenic crystal was lower in purity. This could be due to the incorporation of an organic matrix, such as the cell debris, in the crystal. Moreover, although the exact role of bacterial cell in MICP is still debatable (De Muynck et al., 2010a), results from SEM and TEM analyses indicate that bacteria could act as the nucleation site for the crystals (Hammes & Verstraete, 2002). Previously, it was hypothesized that the bacterial cell wall might provide a template for the calcium carbonate crystal formation (Ferris et al., 1987). The cell wall, consisting of different functional groups (e.g., hydroxyl, carboxyl), binds calcium ions and further react with the carbonate ions to form calcium carbonate (Rodriguez-Navarro et al., 2003).

5. Conclusion

This study presents the first report of calcium carbonate precipitation from calcium formate, using *M. parvus* OBBP culture; furthermore, the optimum precipitate yields from different culture densities and calcium formate concentrations in grown *M. parvus* OBBP culture were also described. The results obtained in this study were used as the basis of the application of formate-based MICP by *M. parvus* OBBP for building materials (i.e., concrete) surface protection (Chapter 6).

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CHAPTER 6

BIOGENIC CONCRETE SURFACE PROTECTION BY

*Methylocystis parvus* OBBP

Abstract

The effectiveness of Microbiologically Induced Carbonate Precipitation (MICP) from the formate oxidation by *Methylocystis parvus* OBBP as an alternative concrete surface treatment was investigated. MICP was induced on Autoclaved Aerated Concrete (AAC) by immersing the material in $10^9$ *M. parvus* cells mL$^{-1}$ containing 5 g L$^{-1}$ of calcium formate. A 2 days immersion of the material gave the highest weight increase of the specimen which could be due to the calcium carbonate, biomass, and calcium formate deposition. This deposition mainly occurred on the wall of the pores on the surface of the specimen. Due to this surface deposition, a significantly lower water absorption was observed in the bacterially treated specimens compared to the non-treated ones (i.e., up to 2.92 ± 0.91 kg m$^{-2}$). A concomitant atmospheric methane removal (152.2 ± 40.1 µg of CH$_4$ m$^{-2}$ h$^{-1}$) was also observed in the bacterially treated specimens. Overall, compared to the currently employed urea hydrolysis process, formate-based MICP by *M. parvus* offers a more environmentally friendly approach for the biotechnological application to protect concrete surface as ammonia was not emitted and methane was removed from the air.

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*A sustainable biogenic concrete surface protection by Methylocystis parvus OBBP. Submitted to PLOS ONE journal.*
1. Introduction

Building materials (i.e., natural stones and concretes) are susceptible to physical, chemical, and biological weathering processes leading to the deterioration of the materials (Saiz-Jimenez, 1997). Building materials deterioration adversely affects the mechanical integrity of these materials, which in turn decreases their lifespan (Achal et al., 2011a; Labus & Bochen, 2012). Therefore, several conservation techniques have been applied to protect existing building materials (Cnudde et al., 2004; De Belie, 2010). These conservation techniques can focus on the application of water repellants or pore blockers. Both applications aim to minimize water impregnation, which triggers weathering, into the building material to prevent building material disintegration. As water repellent, inorganic/organic components have been applied previously to protect concrete surface. However, these treatments pose several shortcomings such as different thermal expansion coefficient of the treated layers and the need for constant maintenance (Brajer & Kalsbeek, 1999; Murray, 2013). Thus, pore blockers can be applied as an alternative treatment.

Microbiologically Induced Carbonate Precipitation (MICP) has been applied as an alternative pore blocker process to protect concrete surface (Chapter 1 section 3.2.2). MICP is the production of solid carbonate minerals (e.g., calcium carbonate) as a result of microbial activities. The resulting deposition of the minerals on concrete surface resulted in the significant decrease of capillary water uptake into the material (De Muynck et al., 2008a). Additionally, this biogenic treatment also improved the durability of concrete (Achal et al., 2011a; De Muynck et al., 2008b). These results have prompted considerable interest in further investigation of MICP-based applications to protect concrete surface (Achal et al., 2013; Pacheco-Torgal & Labrincha, 2013). MICP can be driven by several microbial metabolic processes, namely, oxidative deamination of amino acids, organic acid utilization, and the hydrolysis of urea (Chapter 1 section 3.2.1). Among these pathways, urea hydrolysis is the most investigated process as it offers several advantages such as the high rate of carbonate production by the bacteria (Hammes et al., 2003a; Hammes & Verstraete, 2002). However, several drawbacks when using this process include the emission of ammonia to the atmosphere and nitric acid production. Ammonia emission can contribute to environmental pollution and nitric acid presence in the building material can accelerate the deterioration of the material (De Muynck et al., 2010a). Hence, an alternative biogenic pathway should be employed for future biotechnological applications.

In Chapter 5, it was shown that calcium carbonate precipitation was induced from the formate oxidation by *Methylocystis parvus* OBBP. *Methylocystis spp.* are methane-oxidizing bacteria (MOB) belonging to the Alphaproteobacteria and possess the ability to utilize methane, a greenhouse gas, as both carbon and energy sources (Whittenbury et al., 1970). *M. parvus* have been utilized in several biotechnological applications e.g., bio-polymer production and pollutant remediation (Semrau et al., 2010). Formate oxidation by MOB generate reducing equivalents (i.e., NADH$_2$) needed for metabolic processes and carbonate ions that can be used to form calcium carbonate. Formate based MICP can offer several advantages over other pathways as it does not release by-products that can pollute the environment or that are detrimental to the material.

The objective of this study is to investigate the effectiveness of formate-driven MICP by *M. parvus* OBBP as an alternative process of building material surface treatment. We hypothesize that the resulting calcium carbonate precipitate on building material surface can protect the material by acting as pore blockers. In this research, Autoclaved Aerated Concretes (AAC) (*Ytong*, Belgium) was chosen as the model building material. AAC is a lightweight porous concrete consisting of calcium silicate hydrates.
formed by chemical reactions between calcareous and siliceous materials (Aroni et al., 1993). AAC possesses high compressive strength and is a good thermal insulator (Pytlik & Saxena, 1992). AAC is used mainly as wall, floor, and roof panels of residential and industrial buildings (Pytlik & Saxena, 1992). The material was selected because *M. parvus* exhibited a high activity when applied on AAC. In Chapter 2 we showed that a high methane removal rate was observed when *M. parvus* was immobilized on AAC compared to when immobilized on other building materials (i.e., bricks and limestones).

Several activities were carried out to reach the goal of this study. Firstly, MICP evaluations on AAC were carried out. Secondly, the influence of MICP on AAC characteristics was investigated. Thirdly, the effectiveness of the resulting biogenic calcium carbonate layer on AAC surface as pore blockers was evaluated. In this study, the capacity of the bacteria to concomitantly remove atmospheric methane and precipitate calcium carbonate after being inoculated on building material was also tested.

### 2. Materials and Methods

#### 2.1 Microorganism and growth conditions

*Methylocystis parvus* OBBP was obtained from Colin Murrell (School of Environmental Science, University of East Anglia) on Nitrate Mineral Salt (NMS) agar plate and it was the same strain used previously in the tests conducted in Chapter 5. Bacterial culture growth conditions and methods were similar to the ones in Chapter 5.

#### 2.2 Building materials

AAC blocks were cut into specimens with dimensions according to the type of the experiment as follows: (i) prisms of 2 cm x 2 cm x 4 cm (MICP on AAC and sonication test), (ii) prisms of 0.7 cm x 0.7 cm x 4 cm (methane removal by *M. parvus* on AAC), (iii) prisms of 3.5 cm x 2.5 cm x 1.5 cm (thin section analyses), (iv) cylinders of 1 cm in height and 0.6 cm in diameter (Scanning Electron Microscopy (SEM) and Microtomography analyses), and (v) cubes with 4 cm side (water absorption and drying behavior tests). Specimens were dried at 70°C and weighed daily until constant weight was achieved (i.e., the weight difference was less than 0.1% (w/w)).

#### 2.3 MICP on AAC

##### 2.3.1 MICP treatment procedure

The aim of the experiment was to have a proof of principle of formate-driven MICP by *M. parvus* OBBP on AAC. This was achieved by incubating AAC on *M. parvus* solution containing calcium formate. The experiment was performed aseptically by preparing the setup under laminar flow. *M. parvus* was grown in serum bottles to mid-logarithmic phase before the cells were collected by centrifugation at 11,000 x g for 20 minutes. The cells were subsequently washed two times with saline solution (8.5 g NaCl L⁻¹) and resuspended in 5 g L⁻¹ of calcium formate until a culture density of approximately 10⁹ cells mL⁻¹ was reached. In Chapter 5 it was shown that these conditions gave the maximum yield of calcium carbonate precipitate (g CaCO₃ Ca(COOH)₂⁻¹). Bacterial cell counts were done using 50 µL portion of the resuspended culture.

AAC specimens were placed into empty 150 mL plastic vessels (Novolab, Belgium) and fixed horizontally using double-sided tape. The specimens were UV-sterilized prior to the experiment. The resuspended bacterial culture was poured into the vessels until the specimens were completely immersed. The vessels were subsequently closed and incubated statically at 28°C. The influence of
immersion period on MICP on the specimens was investigated by immersing different specimens for 1, 2, 4, or 9 days. Before and after the immersion, 2 mL of liquid was sampled and filtered using a 0.22 μm pore size filter (Millipore, Belgium). The liquid samples were stored at 4°C until further analysis. After the immersion, 5 μL of liquid was taken from each vessel for bacterial cell counting before the liquid was poured out of the vessel. Specimens immersed in sterilized calcium formate and specimens immersed in calcium formate containing autoclaved bacteria served as the two controls. Experiments were done in quadruplicate.

2.4 MICP parameter evaluations

MICP evaluations on AAC were performed by assessing the following parameters: (a) AAC specimens weight increase, (b) soluble calcium and (c) formate removal in the liquid, and (d) pH increase.

2.4.1 Specimen weight increase

To investigate the influence of MICP on the specimens’ weight, after the liquid was poured out of the vessels, the specimens were removed and dried at 70°C in a ventilated oven. They were weighed daily until the weight differences were less than 0.1% (w/w). The weight increase was calculated as the difference between the weight of the specimen before and after the treatment.

2.4.2 Liquid sample analyses

The liquid samples were used to analyze: (i) calcium concentration, (ii) formate concentration, and (iii) pH. The calcium and formate concentrations were analyzed in the liquid samples using method described in Chapter 5. The pH was measured using a C-532 pH electrode (Consort, Belgium).

2.4.3 Bacterial cell counts

Bacterial cell counts were performed to obtain the culture densities before and after the treatment. The cell counts were performed in bacterial culture samples according to the live/dead staining method as described in Chapter 5. The total number of propidium iodide and SYBR green-tagged cells per mL of the analyzed sample was reported as the culture density.

2.5 The influence of MICP on the morphology of AAC

The influence of MICP on the morphology of AAC specimens was investigated by means of: (i) SEM, (ii) Thin Section, and (iii) X-ray micro-tomography analyses. Based on MICP parameters evaluation (see Results and Discussion section), 2 days specimen immersion in M. parvus culture was chosen as the optimum MICP method. The treatment procedure was performed as described previously and specimens were characterized before and after the treatment.

2.5.1 SEM analyses

For SEM analyses, the specimen was placed on an aluminum stub with carbon conductive tab before analysis. SEM was performed on the specimen using a Phenom ProX desktop scanning electron microscope with 5 kV accelerating voltages (Phenom-World BV, Eindhoven, The Netherlands).

2.5.2 Thin section analyses

Petrographic analyses of AAC specimens were outsourced to GEOS, an ISO 17025 accredited laboratory for concrete analyses, in Wellen, Belgium. Specimens were prepared and analyses were performed according to ASTM C 825.
2.5.3 Microtomography analyses

A sample of AAC was scanned using the X-ray microtomography (µCT) cone beam setup of the HECTOR scanner (Masschaele et al., 2013) at the Centre for X-ray Tomography of Ghent University (UGCT) (Masschaele et al., 2007). A total of 2401 projections was acquired from the specimen over an 360° angle with an exposure time of 1 s per projection. A thin aluminum filter (0.1 mm) was used to block low-energetic X-rays at the source to reduce beam hardening. In order to correct for inhomogeneities of the detector and the beam, 30 dark-field (no X-ray beam) and 40 flat-field (no sample) images were acquired. The X-ray tube provided a voltage of 90 kV with a power of 10 W. The source-detector and source-object distances were 1166 mm and 29.6 mm, respectively, resulting in a 5³ µm³ voxel size. The same acquisition parameters (e.g., number of projections, exposure time, filter, etc.) were used for the scans before and after the treatment. After the acquisition, the raw data were reconstructed using Octopus (Inside Matters bvba, Belgium; (Vlassenbroeck et al., 2007)). The same set of parameters for ring and spot removal, tilt and skew of the detector and beam hardening were adopted for both scans.

The two datasets, i.e. prior to and after the bacterial treatment, were loaded in the software DataViewer (SkyScan). The datasets were aligned manually and subsequently an automatic registration procedure was performed. This allowed to assess changes between the pre- and post-treated state of the sample by subtracting the volume of the pretreated state from the volume of the post-treated one. At locations where bacteria have precipitated carbonate, a change of the gray value was expected in the X-ray images. The differential volume obtained from the digital image subtraction represented the carbonate precipitation in the sample.

2.6 The effectiveness of MICP on AAC as an alternative surface treatment

The evaluations were performed on the specimens after 2 days of immersion in the bacterial culture using the method described previously. Non-treated specimens and specimens immersed in calcium formate containing autoclaved bacteria served as the two controls. All tests were performed in triplicates.

2.6.1 Capillary water absorption

The aim of the test was to investigate the effectiveness of MICP on the specimens against the transport of a deleterious substance (i.e., water) into the material. When a constant specimen weight was observed after the treatment, water absorption test was performed according to the EN 1925:1999 method described previously (De Muynck et al., 2011). The sorptivity coefficient was calculated using the following equation:

\[
\frac{Q}{A} = k \sqrt{t}
\]

where Q is the total absorbed water (cm³), A is the cross section of the side in contact with water (cm²), t is the time (s), and k is the sorptivity coefficient. k was calculated from the slope of the linear part of the curve (i.e., first five measurements) when Q/A is plotted over t⁰.⁵.

2.6.2 Drying behavior

The aim of the test was to investigate the difference in the drying behavior between treated and untreated specimens by evaluating the water evaporation rate from the water saturated specimens. The test was performed on specimens using the open air desorption test at 20 °C and 65% relative humidity as described by De Muynck et al (De Muynck et al., 2011). Water saturated specimens at the end of the
capillary water absorption test were used for the experiment. The drying behavior of the specimens was presented as the water weight loss over time.

### 2.6.3 Resistance to sonication

The aim of the test was to evaluate the adherence of the newly formed carbonate inside the specimens according to the method described by Rodríguez-Navarro et al (Rodriguez-Navarro et al., 2003). When the biogenic deposits was not adhered properly, the bacterially treated AAC specimens would exhibit similar weight loss to the non-treated specimens after sonication. Briefly, treated and untreated AAC specimens were subjected to 6 sonication cycles in a 37 kHz water bath (Elmasonic S 30/H; Elma GmbH, Germany) filled with demineralized water at 30 °C. In each cycle, the specimens were immersed for 5 minutes in the water bath and afterwards, the specimens were dried in an oven at 70°C and the weight was measured daily.

### 2.7 Methane removal by *M. parvus* on AAC

The aim of the test was to investigate the capacity of *M. parvus* to remove methane during / after calcium carbonate precipitation. After the immersion in bacterial culture, the specimens were removed from the culture and subsequently inserted to a perforated tube that was attached to a butyl rubber stopper. To have a gastight incubation of the specimens, the stoppers were subsequently placed in 250 mL serum bottles (Schott Duran, USA) (Figure 6.1). The bottles were screw capped and methane (95 % (v/v); Linde Gas, Belgium) was injected into the headspace until it reached ~160 ppmv methane headspace concentration. This concentration represents the methane atmospheric concentration in places known to emit methane (e.g., animal barns) (Jungbluth et al., 2001). The result of this test would then be used to assess whether the bacteria could remove methane when they were applied on AAC-based building material in those places.

![Figure 6.1. Schematic diagram of gastight incubation of bacterially inoculated specimen for the methane removal test.](image)

The methane removal capacity of *M. parvus* was examined by observing the evolution of the headspace methane concentration over time. This was achieved by analyzing a 1 mL gas sample taken from the headspace of the bottle using Trace Gas Chromatography Ultra (Thermo Fischer Scientific, USA) according to the method described in Chapter 2 at different incubation times. Specimens immersed in
sterilized calcium formate and specimens immersed in calcium formate containing autoclaved bacteria served as the two controls. The test was done in triplicate.

2.8 Statistical analysis

Values presented are the means of replicates of different treatment. Error bars represent the standard deviation. The comparison of mean values, assuming normal distribution, was done using the one-way ANOVA test (p =0.05) to evaluate the significant differences between the values. Subsequent pairwise multiple comparisons tests (Holm–Sidak procedure) were performed to compare the differences between two mean values in the experiment (a =0.05). Statistical analyses were carried out in SigmaPlot v12.0 (Systat Software Inc., USA).

3 Results and Discussion

3.1 Proof of principle of MICP on AAC

The aim of the experiment was to have a proof of principle of MICP on bacterially treated AAC and, if proven, to obtain the optimum MICP method. This was done by immersing the specimens in \(10^9\) *M. parvus* cells mL\(^{-1}\) containing 5 g L\(^{-1}\) of calcium formate at different immersion times. Specimens immersion in *M. parvus* culture resulted in a weight increase of AAC specimens (Figure 6.2a). Maximum weight increase (38 ± 19 mg) was obtained when the specimens were immersed for two days, however, the weight increase difference was not significant to other specimens (\(P >0.05\)). Weight decrease was observed in all control specimens and the effect was more pronounced the longer the specimens were immersed. Specimens immersed in calcium formate containing killed cells exhibited lower weight decrease compared to specimens immersed in only calcium formate.

Formate and calcium removal were only observed in the *M. parvus* culture (Figure 6.2b and Figure 6.2c). Significantly higher formate removal was observed when the specimens were immersed longer than two days (\(P <0.05\)). Calcium removal was observed in incubations containing *M. parvus* culture. Maximum calcium removal (0.24 ± 0.07 g L\(^{-1}\)) was observed when the specimens were immersed for two days, however, the difference was not significant to the removal in other incubations (\(P >0.05\)). Calcium release was observed in incubations with only calcium formate. Lower culture density was examined at the end of the immersion (Figure 6.2d). The culture density dropped to ~3 x 10\(^6\) cells mL\(^{-1}\) from starting culture density of 10\(^9\) cells mL\(^{-1}\). The final culture density did not vary significantly in all incubations (\(P >0.05\)).

Based on these observations, the specimen weight increase could be attributed to the calcium carbonate precipitation driven by the bacterial formate oxidation. *M. parvus* oxidizes formate to carbon dioxide as part of its catabolic activity (Hanson & Hanson, 1996). Carbon dioxide is in equilibrium with carbonic acid, bicarbonate and carbonate ions and the ratio of both ions is dependent on the pH of the culture. In Chapter 5, it was shown that formate oxidation by *M. parvus* led to a pH increase in the culture, resulting in carbonate ions production. In this study, carbonate ions reacted with calcium ions from calcium formate to produce calcium carbonate and when the system is oversaturated, calcium carbonate is precipitated. The specimen weight increase could also be attributed to the biomass deposition into the specimen. Based on the amount of absorbed liquid into the specimen (data not shown) and average mass of *M. parvus* (~5 x 10\(^{-13}\) gram cell\(^{-1}\)) (Pieja et al., 2011b), bacterial cell deposition could contribute up to ~50% of the specimens weight increase. Besides biomass and calcium carbonate deposition, the specimens weight increase could be contributed from the unconverted calcium formate. Biomass,
calcium formate, and calcium carbonate deposition were the most likely main weight increase contributors on the specimens.

**Figure 6.2.** Microbiologically Induced Carbonate Precipitation (MICP) parameter evaluations on Autoclaved Aerated Concrete (AAC) immersed in *M. parvus* culture containing calcium formate at different immersion time. The parameters are: (a) Specimen weight increase, (b) Calcium removal in the liquid culture, (c) Formate removal in the liquid culture, and (d) Bacterial culture density.

AAC specimens were dissolved during the immersion period. In the absence of calcium carbonate precipitation, specimens dissolution was observed from the calcium release in calcium formate incubations (Figure 6.2c) which resulted in the weight decrease of the specimens (Figure 6.2a). Due to
this material dissolution, the pH in the solution increased at the end of the immersion period (Figure 6.3). AAC, a material composed of 20-40 % (w/w) of calcium silicate hydrate (i.e., Tobermorite-1.1 nm; www.AAC.gr), can dissociate to calcium oxide and silicate oxide in solution. The pH was increased due to the calcium hydroxide formation as a result of calcium oxide reaction with water. Furthermore, calcium carbonate precipitation lowered the pH at the end of the incubation period by shifting the carbonate balance to the production of carbonate ions and protons. As a result, higher pH increase was observed in control incubations compared to the one with live cells (Figure 6.3). Overall, to obtain a maximum calcium carbonate deposition, two days immersion period was chosen for subsequent experiments (i.e., AAC characterization, surface treatment evaluations, and the methane removal test).

![Figure 6.3](image)

**Figure 6.3.** Initial (dark grey) and final (light grey) pH in the liquid of different types of incubations containing Autoclaved Aerated Concrete (AAC) for 1, 2, 4, and 9 days. The following symbols are defined as incubations containing: autoclaved *M. parvus* in calcium formate (AC MOB), calcium formate (CF), *M. parvus* in calcium formate (MOB). \( t_0 \) and \( dt \) indicate the initial pH and the pH difference at the end of the immersion period, respectively.

### 3.2 The influence of MICP on the morphology of AAC

The experiments were performed to investigate MICP influence on the specimen morphology using SEM, thin section, and microtomography analyses. SEM analyses showed that the bacterially treated specimen exhibited a dark layer on the pore’s surface (Figure 6.4). This layer was most likely the newly formed calcium carbonate layer. AAC specimen consisted of a matrix of rod-like aggregates (Figure 6.4e) and the likely calcium carbonate crystals layer filled the pores in between these aggregates (Figure 6.4f). As seen in Chapter 5, the biogenic calcium carbonate crystal had lower EDX spectra compared to pure calcium carbonate which indicated the impurity of the biogenic calcium carbonate crystal due to the inclusion of biomass in the crystal. It was shown in Chapter 5 (Table 5.2) as well that, at any tested starting calcium formate concentration and culture density, calcium formate was not fully converted to calcium carbonate. Hence, this suggests that the newly formed solid layer could be composed of calcium carbonate, calcium formate, and biomass. It was hypothesized in other study as well that the incorporation of organic matrix (i.e., biomass and salts) could attribute to the weight increase of the specimen (De Muynck et al., 2008b).
Indication of calcium carbonate deposits was also observed from thin section petrographs (Figure 6.5a and 6.5b). A 5% (w/w) higher calcite content in the sample aggregate relative to the specimen’s weight was obtained in the sample treated with MOB and calcium formate compared to the non-treated sample. This calcite fraction was formed around the quartz (i.e., sand) matrix of the specimen. From the microtomographic images, it can be seen that precipitated crystal volume was observed on the wall of the specimen’s pore (Figure 6.6c). Higher volume of crystals was identified at the specimen’s surface. These crystals were mostly formed around the bigger pores.

Based on the specimen characterization results, it can be concluded that the likely deposition of calcium carbonate, calcium formate, and biomass on AAC specimen had altered the morphology of the specimen. As seen from SEM analyses, the deposition mainly influenced the microstructure of the specimen. AAC
consists mainly of pores with diameters of 10-100 µm and 0.01-0.5 µm ((40% (v/v) and 55% (v/v) of the total pore volume, respectively) (Chapter 2) and *M. parvus* precipitated calcium carbonate with a crystal diameter of ~10 µm (Chapter 5). Hence the deposition could only alter the microcharacteristic of the specimen. Larger biogenic calcium carbonate crystals (20 – 100 µm diameter) were observed in other studies using *Bacillus* spp. and urea hydrolysis (De Muynck et al., 2011; De Muynck et al., 2010b). In those studies, higher substrate concentrations were used and, as a consequence, higher mineral production and larger crystals, were obtained. A 5 g L⁻¹ of calcium formate was the optimum concentration to give a maximum calcium carbonate precipitate when using the formate-driven MICP by *M. parvus* (Chapter 5).

![Figure 6.5. Thin section petrographs of Autoclaved Aerated Concrete (AAC) specimens. (a) and (b) depict sections of specimen before and after immersion in *M. parvus* culture, respectively. Red arrows in (b) indicate the likely biogenic calcium carbonate crystals.](image)

Higher solid deposition volume on the pore of specimen’s surface observed from microtomographic analyses indicated that precipitation mainly occurred on this part of the material. However, it should be noted that the method used in microtomography analyses was limited by the spatial resolution of the microtomographic datasets, i.e. 5 µm in this study. Hence, changes in pores with diameters less than 5 µm cannot be observed. In addition, small misalignments, partial volume effects and noise can result in apparent differences in the order of magnitude of the spatial resolution. Therefore, the crystal fraction on the inner part of the specimen could be attributed to these effects. Overall, biogenic calcium carbonate precipitation had altered specimen’s morphology by filling the specimen’s micropores.

3.3 The effectiveness of MICP on AAC as an alternative surface treatment

The experiments were performed to investigate the effectiveness of MICP on AAC as pore blockers. This was assessed by evaluating the decrease of water transport into AAC specimens after the bacterial treatment. Additionally, the drying behavior of the specimens after saturated with water and the cohesion of the newly formed carbonate to the specimen’s structure (sonication test) were also performed. From the capillary water absorption test, before the specimens reached water saturated state, up to 2.92 ± 0.91 kg m⁻² lower water absorption was observed in the bacterially treated specimens compared to the non-treated ones (Figure 6.7a). Lower water absorption was also observed in specimens treated with killed cells. However, the water absorption difference at a given measurement was not significant to the non-treated ones (P >0.05). A higher water sorptivity coefficient was therefore exhibited in non-treated specimens (107 ± 7 µm s⁻⁰.⁵) compared to the ones treated with killed (97 ± 7 µm s⁻⁰.⁵) or live bacteria (78 ± 6 µm s⁻⁰.⁵). All specimens reached a similar water saturated state after 48 hours.
Comparative drying behavior was shown by all water saturated specimens (Figure 6.7b). A higher water evaporation rate was exhibited by the bacterially treated specimens, however, the rate difference was not significant compared to control specimens ($P > 0.05$). All specimens showed high initial evaporation rate but the rate decreased afterwards. The water weight loss from the specimens reached a plateau at the end of the test where approximately 80% of the water content was evaporated. After 6 cycles of sonication, the highest and lowest total weight loss were exhibited by specimens treated with killed and
live bacteria, respectively (Figure 6.7c). The highest weight loss in each treatment was shown after 3 to 4 cycles of sonication. The specimens’ weight loss was not significant in the subsequent sonication cycle ($P > 0.05$).

Lower water absorption rate into bacterially treated specimens could be attributed to the increased resistance caused by the calcium carbonate and organic matrix deposition. The capillary water absorption is dependent on the pore volume and geometry of the material (De Muynck et al., 2008b; Dick et al., 2006). From SEM and microtomography analyses, the solid deposition had blocked the specimens’ micropores and this had likely slowed down the water intrusion into the specimens. As formate was not removed (Figure 6.2), lower water absorption rate in specimens treated with killed bacteria could be attributed to cell debris and calcium formate deposition on the specimens. Comparable drying behavior was shown in all specimens regardless of the treatment. From 3D microtomographic analyses (data not shown), it was observed that the large pores were well connected whereas only 1% of the total porosity was found to be closed pores. The drying behavior of the specimens treated with *M. parvus* could be due to the fact that the precipitated calcium carbonate occurred on micropores whereas water weight loss was mainly consisted of water evaporation in the macropores (i.e., pore diameter $\geq 100 \, \mu m$). Therefore, the drying behavior of the samples did not change significantly.

Sonication test is a standard test established by previous studies to investigate the consolidation efficacy of bacterial carbonate deposition on building materials (De Muynck et al., 2010b; Jimenez-Lopez et al., 2007; Rodriguez-Navarro et al., 2003). After 6 cycles of sonication tests, it was observed that MICP improved the cohesion of the material’s constituent as lower specimen weight loss was observed after the test. Consolidation effect of the newly formed carbonate was known to occur when MICP was applied on porous materials like AAC (De Muynck et al., 2011). However, the cohesive improvement effect was not significant compared to the non-treated specimens ($P > 0.05$). As observed by Jimenez et al (Jimenez-Lopez et al., 2007), higher weight loss was obtained in specimens treated with killed cells and this could be due to the easy removal of organic matrix (i.e., cell debris) deposition when subjected to the test. Overall, calcium carbonate deposition on AAC specimens increased the resistance of the material from the ingestion of water into the specimens.

### 3.4 Atmospheric methane removal by *M. parvus* on AAC

The experiment was aimed to investigate the methane removal capacity of *M. parvus* when concomitantly precipitating calcium carbonate. This was done by placing the specimens in incubations at an atmospheric methane concentration of $\sim 160 \, \text{ppmv}$ after bacterially treated. Atmospheric methane removal was observed in incubations filled with bacterially inoculated specimens, indicating *M. parvus* activity (Figure 6.8). A high methane removal rate ($152.2 \pm 40.1 \, \mu g \, \text{of CH}_4 \, m^{-2} \, h^{-1}$) was observed in the first 100 hours of incubation. When *M. parvus* was immobilized on AAC without calcium formate (i.e., without calcium carbonate biodeposition), the bacteria removed $5.5 \pm 0.9 \, \mu g \, \text{of CH}_4 \, m^{-2} \, h^{-1}$ (Chapter 2). A lower rate was obtained in that study because AAC was inoculated using lower culture density (i.e., $2 \times 10^8 \, \text{cells mL}^{-1}$) and tested at lower starting methane concentration (i.e., $\sim 100 \, \text{ppmv}$). Based on the hyperbolic Michaelis-Menten kinetic, a higher methane removal rate was exhibited by the bacteria at higher starting methane concentration (Chapter 2).
Figure 6.7. Evaluations of the effectiveness of Microbiologically Induced Carbonate Precipitation (MICP) using M. parvus in calcium formate as the surface treatment of Autoclaved Aerated Concrete (AAC) by means of. (a) Capillary water absorption. (b) Drying behavior. (c) Resistance to sonication tests. Different types of tested specimens are: non-treated specimens, specimens treated with killed M. parvus in calcium formate, and specimens treated with M. parvus in calcium formate.

Calcium carbonate precipitation could also be driven by the methane oxidation by M. parvus. Methane is assimilated as biomass, and/or fully oxidized to carbon dioxide (Hanson & Hanson, 1996). However, at ~160 ppmv methane concentration the amount of carbonate ions for calcium carbonate precipitation would stoichiometrically not be significant for the surface treatment of the material. However, this functionality has been covered by the amount of carbonate ions converted from formate. Atmospheric methane removal by the bacteria therefore represents an additional advantageous characteristic of the
process when it is applied on building material in places with methane atmospheric concentration \( \leq 1\% \) (v/v) such as the cow stable.

**Figure 6.8.** Evolutions of methane in the headspace of incubators containing different Autoclaved Aerated Concrete (AAC) specimens treated with calcium formate, killed *M. parvus* in calcium formate, and *M. parvus* in calcium formate.

### 4 Conclusions

Calcium carbonate was precipitated on the surface of AAC from the formate oxidation by *M. parvus*. From different immersion times, 2 days of immersion resulted in the highest weight increase of the specimen. The deposition mainly occurred on the wall of the pores on the surface of the specimen. As a result, significant lower water absorption was observed in the bacterially treated specimens compared to the non-treated ones. A concomitant atmospheric methane removal was also observed in the bacterially treated specimens.

### Acknowledgements

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CHAPTER 7
GENERAL DISCUSSION AND RESEARCH OUTLOOK
1. General discussion

1.1 Revisiting the research gaps

This thesis is divided into two parts and it centers around the application (housing) of MOB on building materials.

1.1.1 Research gap 1: Methane biofiltration

Greenhouse gas like methane is an important factor in the warming of the climate. As explained in Chapter 1 section 1.3, methane emission is set to increase in the forthcoming future and this will mainly be driven by the population growth and energy demand. Biofiltration is the typical biotechnological application to remediate methane emission at low concentration. This technology has been applied to remediate methane emission from landfill, animal house, and manure storage. Optimization of a biofilter performance has been done by varying operating parameters such as liquid feeding rate and frequency. Equally important is the biofilter design itself, and carrier material selection forms one of the crucial design steps. Some types of building materials possess some of the important parameters needed for a good carrier material (e.g., high porosity, good thermal insulation, etc). Although some biofilters tested some of the raw materials for building materials production (e.g., stone, perlite, tobermorite; Table 1.5), no studies have tested “ready-made” building materials as the biofilter carrier material. Some foreseen advantages of using “ready-made” building materials compared to their raw material are the absence of competition between building material manufacturer, the possibility of recycling used building material, and the possibility of using existing building wall in places where high methane concentration (i.e., ≤ 1 % (v/v)) is found (e.g., animal barns) as the niche for MOB. The use of building material in a biofiltration system hasn’t also been tested to remove methane emission from livestock. Therefore, in this part of this thesis, an exploration of the use of building materials as the carrier for methane biofiltration and its application to remove methane emission from livestock were done.

1.1.2 Research gap 2: Biogenic building materials surface protection

Following up the setup given in Part 1 where MOB were immobilized on building materials, another potential application was foreseen in this thesis. Microbial processes have been used for the past two decades in the construction industry (Chapter 1 section 3.2). Main applications are the use of bacteria for building materials surface protection. The capacity of bacteria to drive calcium carbonate precipitation is central in these applications. The resulting calcium carbonate precipitate is then used as the protective layer on building material’s surface. Due to several advantages possessed when using the bacterial urea hydrolysis by Bacillus spp. (e.g., the high carbonate production by the bacteria, the capacity of the bacteria to produce spores), this process is the typically used one for the previously mentioned applications. However, a drawback of using this system is the ammonia production that can harm the environment. Nitric acid can also be potentially produced when nitrified by bacteria which may harm the supposedly protected material. Alternatively, calcium carbonate precipitation can be driven by organic acid utilization by bacteria. MOB are able to oxidize formate to carbon dioxide which could drive calcium carbonate production. According to our knowledge, the application of this alternative pathway using MOB hasn’t been explored previously. Some advantages foreseen when using the formate-oxidation driven pathway by MOB to protect building material surface compared to the urea hydrolysis is the absence of ammonia production and the possibility to concomitantly remove methane from the air while protecting the material’s surface. Using the setup of “housing MOB on building materials”, in this part of the thesis, the capacity of MOB to drive calcium carbonate precipitation and its usage for the protection of building material surface was explored.
1.2 Research outcome

1.2.1 Research outcome 1: Methane bioremediation of livestock gas effluent by biofiltration using mixed culture MOB on AAC

In Chapter 2, screening of different MOB cultures and building materials were done by means of testing the activity at both high (~20% (v/v)) and low (~100 ppmv) methane concentrations. *M. parvus* on AAC was found to exhibit a high methane removal rate at both levels of tested methane concentrations compared to other tested MOB-building materials combination. This could be attributed to the high affinity nature of *M. parvus* on AAC and the high porosity nature of the building material. Moreover, the use of AAC as the carrier material also gave an advantage as the material could sequester the resulting carbon dioxide from MOB metabolism by reacting with tobermorite, the binder component of the material. Thus AAC was selected as the building material for the biofilter tests. In Chapter 3, a lab scale methane biofilter test using mixed culture MOB on AAC was performed for four months. For the biofilter tests (Chapter 3 and 4), mixed culture MOB was utilized as it was practically more laborious and economically non-beneficial to operate the biofilter in aseptic manner. Additionally, the possibility to enrich MOB with higher affinity to methane was foreseen. It was calculated in Chapter 2 that even when using *M. parvus* on AAC, the bacteria could not sustain themselves when applied in cow stable where the methane atmospheric concentration is ~100 ppmv. The decision was taken due to the fact that MOB from the *Methylocystis* genera were detected from the MOB mixed culture analysis (Chapter 2). *Methylocystis* spp. are known to exhibit high affinity to methane among other MOB. From the batch tests aimed to optimize MOB immobilization on AAC, it was found that calcium chloride would not be added during the inoculation step prior to the biofilter test and 10 mm thick AAC specimens would be used for the biofilter test. Furthermore, it was found that optimum methane removal was reached when two biofilters were operated in series. MOB also developed a preferential growth near the methane gas inlet where a high methane concentration was found. After four months of operation in the lab, the biofilter was tested to remove methane from livestock effluent gas (Chapter 4). In this field test, lower methane removal efficiency in the biofilter was obtained due to the lower methane concentration found in the biofilter inlet gas, the presence of ammonia in the effluent gas, the higher biofilter inlet gas flow, and the lowering humidity level in the biofilter. Nevertheless, a relatively efficient methane removal from the cow gas effluent was achieved.

1.2.2 Research outcome 2: Formate-driven MICP by *M. parvus* for the surface protection of AAC

In Chapter 5, the capacity of *M. parvus* to drive MICP from the dissimilatory formate oxidation was investigated. It was found that *M. parvus* could precipitate calcium carbonate when incubated in calcium formate with or without the addition of methane. It was also found that a maximum of $0.67 \pm 0.03 \text{ g CaCO}_3 \text{Ca(OH)(CH}_2\text{O)}_2 \text{ (g/g)}$ calcium carbonate precipitation yield could be obtained when $10^9$ *M. parvus* cells ml$^{-1}$ and 5 g L$^{-1}$ of calcium formate were used. Moreover, vaterite and calcite were the main calcium carbonate polymorphs formed with vaterite being the majority. This process was then used as the basis for the biogenic building material surface protection (Chapter 6). Based on the results obtained in Chapter 2, AAC was used as the tested material. In a proof of concept experiment, it was found that 2 days immersion of the material gave the highest weight increase of the specimen and this was most likely due to the calcium carbonate, biomass, and calcium formate deposition. This deposition mainly occurred on the wall of the pores on the surface of the AAC specimen. Due to this surface deposition, a significantly lower water absorption was observed in the bacterially treated AAC specimens compared to the non-treated ones. A concomitant atmospheric methane removal was also observed in the bacterially treated AAC specimens.
1.3 Positioning the research outcome in the related biotechnology field

1.3.1 Methane biofiltration

The screening of MOB-building materials for the methane biofilter in Chapter 3 was based on the methane removal kinetic analysis (Chapter 2). As explained in Chapter 1 section 2.3, this kinetic analysis was done by evaluating the Michaelis-Menten parameters from the hyperbolic model of the kinetic equation. Based on existing literatures, the kinetic analysis performed in Chapter 2 was the first to be performed using different MOB cultures on building materials. The studies found in the literature were all conducted in liquid culture. Here, a comparison of Michaelis-Menten parameter is made to existing kinetic studies (Table 7.1). It should be kept in mind that besides being determined in the liquid culture, the experimental conditions in which the kinetic parameters were assessed in other studies were different to the ones in Chapter 2. Hence, different kinetic parameters can be obtained even when the same pure MOB strain was used. For example, the $K_{m(app)}$ of *Methylocapsa acidiphilia* B2 determined by Knief and Dunfield (2005) is 3.4 μM whereas the one obtained by Dedys et al (2001) is 2 μM. $K_{m(app)}$ is a function of reaction rate constants of the individual enzymatic reaction (Prats & Forestier, 1988). These constants will vary when, for example, different temperature, compared to the other study, is used.

**Table 7.1. Comparison of Michaelis-Menten kinetic properties ($V_{max(app)}$ and $K_{m(app)}$) obtained in this thesis to the ones found in the literature (adaptation of Table 1.4 and Table 2.4)**

<table>
<thead>
<tr>
<th>MOB culture</th>
<th>$V_{max(app)}$ (10$^6$ nmol CH$_4$ cell$^{-1}$ h$^{-1}$)</th>
<th>$K_{m(app)}$ (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. alcaliphilum</em></td>
<td>13,800</td>
<td>77.1</td>
<td>This thesis (Chapter 2)</td>
</tr>
<tr>
<td><em>M. parvus</em> OB3P</td>
<td>9,600</td>
<td>5.7</td>
<td>This thesis (Chapter 2)</td>
</tr>
<tr>
<td><em>M. trichosporium</em> OB3b</td>
<td>15,700</td>
<td>52.8</td>
<td>This thesis (Chapter 2)</td>
</tr>
<tr>
<td>Mixed culture MOB</td>
<td></td>
<td>116.4</td>
<td>This thesis (Chapter 2)</td>
</tr>
<tr>
<td>Methyloccus sp LR1$^a$</td>
<td>18.7 – 27.8</td>
<td>2.2 – 12.6</td>
<td>(Dunfield &amp; Conrad, 2000)</td>
</tr>
<tr>
<td>Methylobacter sp. BG8$^b$</td>
<td>133 ± 45–648 ± 195</td>
<td>916 ± 235 ± 5,024 ± 1,234$^i$</td>
<td>(Benstead et al., 1998)</td>
</tr>
<tr>
<td>Methyloccus sp. capsulatus (Bath)</td>
<td>3710</td>
<td>23</td>
<td>(Carlson et al., 1991)</td>
</tr>
<tr>
<td>Methyloccus sp.</td>
<td></td>
<td>44</td>
<td>(Gulledge et al., 2004)</td>
</tr>
<tr>
<td>Methylosinus trichosporium OB3b$^a$</td>
<td>24.0 ± 1.5</td>
<td>1.0 ± 0.3</td>
<td>(Calhoun &amp; King, 1998)</td>
</tr>
<tr>
<td>Methylococca acidiphilia B2$^a$</td>
<td>100 ± 10 – 167 ± 25</td>
<td>1.00 ± 1.00 – 2.03 ± 0.45</td>
<td>(Dedys et al., 2001)</td>
</tr>
<tr>
<td>Methylosmicribium album NCIMB 11123$^a$</td>
<td>310 ± 50</td>
<td>4.7</td>
<td>(Knief &amp; Dunfield, 2005)</td>
</tr>
<tr>
<td>Methylobacter sp.</td>
<td></td>
<td>680 ± 20</td>
<td>(Knief &amp; Dunfield, 2005)</td>
</tr>
<tr>
<td>Methyloccus sp. BF1$^a$</td>
<td>1,280 ± 110</td>
<td>5.6</td>
<td>(Knief &amp; Dunfield, 2005)</td>
</tr>
<tr>
<td>Methyloccus sp. L6$^a$</td>
<td>340 ± 20</td>
<td>4.3</td>
<td>(Knief &amp; Dunfield, 2005)</td>
</tr>
<tr>
<td>Methyloccus sp. DWT$^a$</td>
<td>280 ± 20</td>
<td>4.3</td>
<td>(Knief &amp; Dunfield, 2005)</td>
</tr>
<tr>
<td>Methyloccus sp. SC2</td>
<td>110 ± 10 – 2,410 ± 140</td>
<td>0.11 – 2.2</td>
<td>(pmoA 2)</td>
</tr>
<tr>
<td></td>
<td>1,860 ± 60 – 2,000 ± 110</td>
<td>9.2-9.3</td>
<td>(pmoA 1)</td>
</tr>
</tbody>
</table>

Several assumptions and data were made and used, respectively, in the recalculation of the kinetic parameters in Table 2.4. Firstly, the Michaelis-Menten parameter conversion from our study was based on 1.75 ppmv methane concentration being equal to a concentration of 2.5 nM (Knief and Dunfield, 2005) or in another way by calculating the parameter on 1 bar basis and assuming a Henry constant of 1.5 x 10$^3$ atm M$^{-1}$ (http://www.henrys-law.org/henry.pdf). Thirdly, the amount of MOB absorbed was 0.36 ml per gram of building materials (porosity of Maastricht limestone in Table 2.1 assuming saturated with MOB culture after the immersion) with the culture density of 2 x 10$^9$ cells ml$^{-1}$ (Chapter 2).

$^a$ Unstarved cells
$^b$ Kinetic parameter ranges are taken from both batch and chemostat (methanol limited) tests
$^c$ Assumption of the weight of one cell is 1 x 10$^{-12}$ g
$^d$ Kinetic parameter ranges are taken from 1 week starved cell and 24 hours reincubated cell under 2 % (v/v) methane/air atmosphere after 1 week starvation period
$^e$ $V_{max}$ was recalculated according to the following formula; $V_{max} = a_x \times K_{max} \times \frac{1}{1 + 0.4 L^{1/0.03395 \times 10^{-18}}}; 0.1 L = $ liquid volume; 0.4 L = gas headspace volume; 0.03395 = Ostwald constant at 25º C
$^f$ Kinetic parameters are taken from trial 1
$^g$ Concentration in the liquid phase

Several analysis can be made when looking at the kinetic data in Table 7.1. Firstly, kinetic data comparison is made on the same MOB strain used in our study and the one in the literatures (*M.*
trichosporium OB3b). Approximately six times and 600 times larger $K_{m(app)}$ and $V_{max(app)}$, respectively, exhibited by M. trichosporium OB3b were obtained in Chapter 2 in comparison with the one obtained by Calhoun and King (1998). Hence, when incorporating these kinetic parameter into the Michaelis-Menten equation (equation 1.1), at the same methane concentration, higher methane removal rate will be exhibited by the bacteria using the building material setup used in Chapter 2. This can be attributed to several factors. Firstly, the diffusion resistance found in the liquid system is higher in comparison with the “wet building material” condition in Chapter 2 and this gave rise to the higher methane consumption rate by MOB on building materials. This was due to the fact that the liquid layer in this “wet building material” was thinner than the one of the liquid system. Secondly, high methane removal rate could be obtained in the “wet building material” due to the higher surface area of the “wet building material” in comparison with the liquid culture. The kinetic study conducted in Chapter 2 was conducted in the “wet building material” system due to the similarity of conditions found in the biofilter studies (Chapter 3 and 4).

The performances of the lab scale methane biofilter and the biofilter in the respiration chamber in Chapter 3 and 4, respectively, were compared with the biofilters from other studies (Table 7.2). Previous studies showed that high methane removal efficiency were obtained in biofilter studies using all types of carrier materials (Table 7.2). Besides the study conducted by Streese and Stegmann (2003), all methane biofilters using soil or a mixture thereof exhibited removal efficiencies above 50 %. Sly et al. (1993) obtained 98 % removal efficiency when using glass tubes as the carrier material whereas Josiane and Michelle (2009) obtained 90% removal efficiency with gravel stone. The majority of the biofilters from previous studies exhibited higher removal efficiencies than the one in Chapter 3 and 4. This can be attributed to several factors. Firstly, the biofilters in Chapter 3 and Chapter 4 operated at the lowest EBRTs (0.78 minutes (Chapter 3) and 0.25 minutes (Chapter 4)) compared to other biofilters (Table 7.2). When operating at a high EBRT, there is more contact time between methane and MOB and this could give rise to a high methane conversion. Conversely, low removal efficiency can be obtained at lower EBRT. For example, Pratt et al (2012) obtained complete methane removal at an EBRT of 90 minutes when their biofilter operated at a similar inlet methane concentration (0.1 % (v/v)) to our lab scale biofilter (Chapter 3). This is also the case when comparing the removal efficiency our lab scale biofilter with the one of Gebert and Grongroft (2006). It is not unreasonable to assume that when biofilters in Chapter 3 and 4 were operated at a much higher EBRT, higher methane removal efficiency would be obtained. Secondly, the mixed MOB culture used to inoculate our biofilter was known to exhibit a low activity at a low methane concentration (Chapter 2). This was indicated by the low specific affinity (i.e., $V_{max(app)} / K_{m(app)}$) of the mixed MOB culture (Table 2.4). As explained in Chapter 1 section 2.3, at methane concentration lower than the $K_{m(app)}$, the Michaelis-Menten kinetics equation will approach a first order kinetics (equation 1.2). This means a high activity will be exhibited by MOB culture having a high $V_{max(app)} / K_{m(app)}$. This first order kinetics approach was applicable for the kinetic of the mixed MOB culture in the biofilters in Chapter 3 and 4. It was foreseen in Chapter 2 that based on the kinetic analysis, none of the tested culture could sustain themselves at ~100 ppmv, the magnitude of the methane concentration where the biofilter would be applied. As explained in Chapter 3, one of the aims of using mixed MOB culture was to enrich MOB with higher affinity. We hypothesized that, during the lab scale biofilter, this high affinity MOB was not enriched as low RE was always obtained. As no kinetic and MOB composition analyses were performed from the biomass sample after the biofilter test, firm conclusion could not be drawn.
Table 7.2 Comparison of biofilter performance in Chapter 3 and 4 to the previous reports (adaptation of Table 1.5)

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Carrier material</th>
<th>C_{max} (% v/v)</th>
<th>Filter bed (m)</th>
<th>Q (m³ h⁻¹)</th>
<th>IL (g m⁻³ h⁻¹)</th>
<th>EC (g m⁻³ h⁻¹)</th>
<th>RE (%)</th>
<th>EBRT (min)</th>
<th>PCO₂ (g m⁻³ h⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed culture</td>
<td>AAC</td>
<td>0.1</td>
<td>0.09 (d) : 0.45 (h)</td>
<td>0.2</td>
<td>944.7</td>
<td>271.1</td>
<td>28.7</td>
<td>0.78</td>
<td>not produced</td>
<td>This thesis (Chapter 3)</td>
</tr>
<tr>
<td>Mixed culture</td>
<td>AAC</td>
<td>-0.005</td>
<td>0.09 (d) : 0.45 (h)</td>
<td>1.2</td>
<td>384.1</td>
<td>76.3</td>
<td>17.5</td>
<td>0.25</td>
<td>-28.5</td>
<td>This thesis (Chapter 4)</td>
</tr>
<tr>
<td>Soil mixture</td>
<td>Soil mixture</td>
<td>2.5</td>
<td>15.1</td>
<td>0.15</td>
<td>165</td>
<td>64</td>
<td>39</td>
<td>6</td>
<td>50</td>
<td>(Streefe &amp; Stegmann, 2003)</td>
</tr>
<tr>
<td>Activated sludge</td>
<td>Compost / perlite</td>
<td>0.85</td>
<td>0.48 (d) : 0.86 (h)</td>
<td>0.75</td>
<td>25</td>
<td>8</td>
<td>32</td>
<td>13.5</td>
<td>NRb</td>
<td>(Melše &amp; Van der Werf, 2005)</td>
</tr>
<tr>
<td>Landfill soil</td>
<td>Landfill soil</td>
<td>0.1</td>
<td>0.14 (d) : 0.3 (h)</td>
<td>0.003</td>
<td>24</td>
<td>100</td>
<td>90</td>
<td>20</td>
<td>20</td>
<td>(Pratt et al., 2012b)</td>
</tr>
<tr>
<td>NRb</td>
<td>Gravel stone</td>
<td>0.13</td>
<td>0.15 (d) : 1 (h)</td>
<td>0.33</td>
<td>18.6</td>
<td>8</td>
<td>43</td>
<td>8.8</td>
<td>50</td>
<td>(Nikiema &amp; Heitz, 2010)</td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Stones</td>
<td>0.7</td>
<td>0.15 (d) : 1 (h)</td>
<td>0.25</td>
<td>61.8</td>
<td>33</td>
<td>53</td>
<td>4.2</td>
<td>50</td>
<td>(Ramirez et al., 2012)</td>
</tr>
<tr>
<td>Landfill soil</td>
<td>Hyuga stone and activated carbon</td>
<td>1.5</td>
<td>0.08 (d) : 1 (h)</td>
<td>0.015</td>
<td>30.4</td>
<td>18</td>
<td>59</td>
<td>20</td>
<td>NRb</td>
<td>(Kim et al., 2013)</td>
</tr>
<tr>
<td>Pine bark</td>
<td>Pine bark and perlite</td>
<td>0.5</td>
<td>0.17 (d) : 0.3 (h)</td>
<td>0.04</td>
<td>400.6</td>
<td>280.4</td>
<td>70</td>
<td>30</td>
<td>NRb</td>
<td>(du Plessis et al., 2003)</td>
</tr>
<tr>
<td>Compost</td>
<td>Compost</td>
<td>6.6 – 10.8</td>
<td>0.5 (d) : 1.5 &amp; 2 (h)</td>
<td>0.5 – 2.8</td>
<td>5 – 20b</td>
<td>27.5</td>
<td>95</td>
<td>102 – 450</td>
<td>-58.1, p</td>
<td>(Habrichs &amp; Widmann, 2006)</td>
</tr>
<tr>
<td>Type II MOB mixed culture</td>
<td>Mixture of gravel, clay, sand, and soil</td>
<td>0.1</td>
<td>6 m³ and 9 m³</td>
<td>0.3 – 15</td>
<td>46.4 – 80b</td>
<td>80f</td>
<td>100f</td>
<td>24 – 1200</td>
<td>NRb</td>
<td>(Gebert &amp; Gronroft, 2006)</td>
</tr>
<tr>
<td>Municipal solid waste</td>
<td>Municipal solid waste</td>
<td>0.1</td>
<td>0.3 (d) : 0.5 (h)</td>
<td>0.005 – 0.02</td>
<td>2.5 – 6.5b</td>
<td>5.6</td>
<td>85</td>
<td>240 – 840</td>
<td>-2.75, f, q</td>
<td>(Einola et al., 2008)</td>
</tr>
<tr>
<td>Landfill soil and earthworm cast</td>
<td>Landfill soil and earthworm cast</td>
<td>5 – 25</td>
<td>0.14 (d)</td>
<td>0.006 – 0.22</td>
<td>31 – 560b</td>
<td>280f</td>
<td>100f</td>
<td>42 – 72</td>
<td>-10.1, f</td>
<td>(Park et al., 2009)</td>
</tr>
<tr>
<td>Soil</td>
<td>Gravel or compost</td>
<td>0.7</td>
<td>0.15 (d) : 1.35 (h)</td>
<td>0.25</td>
<td>71.2 / 65.8</td>
<td>29.2 / 12.5</td>
<td>41 / 19</td>
<td>4.3</td>
<td>65</td>
<td>(Nikiema et al., 2005)</td>
</tr>
<tr>
<td>NRb</td>
<td>Gravel</td>
<td>0.3</td>
<td>NRb</td>
<td>0.18</td>
<td>16.2</td>
<td>54</td>
<td>6</td>
<td>11.5 – 30</td>
<td>NRb</td>
<td>(Veellite et al., 2012b)</td>
</tr>
<tr>
<td>Compost</td>
<td>Compost</td>
<td>NRb</td>
<td>NRb</td>
<td>235f</td>
<td>188</td>
<td>80</td>
<td>75f</td>
<td>20</td>
<td>7f</td>
<td>(Huner &amp; Lechner, 1999)</td>
</tr>
<tr>
<td>M. fodenunum</td>
<td>Glass tubes</td>
<td>0.25 – 1</td>
<td>NRb</td>
<td>750f</td>
<td>735</td>
<td>98</td>
<td>20</td>
<td>10</td>
<td>(Sly et al., 1993)</td>
<td></td>
</tr>
<tr>
<td>Peat, landfill and agricultural soils</td>
<td>Peat, landfill and agricultural soils</td>
<td>1.73</td>
<td>0.15 (d) : 1 (h)</td>
<td>0.009</td>
<td>320f</td>
<td>160</td>
<td>50f</td>
<td>117</td>
<td>10f</td>
<td>(Stein &amp; Hettiaratchi, 2001)</td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Gravel stone</td>
<td>0.13 – 1</td>
<td>0.15 (d) : 1 (h)</td>
<td>0.25</td>
<td>12 – 95</td>
<td>49.5</td>
<td>90</td>
<td>70</td>
<td>Up to 4.2</td>
<td>(Josiane &amp; Michele, 2009)</td>
</tr>
<tr>
<td>NRb</td>
<td>NRb</td>
<td>0.13 – 1</td>
<td>0.15 (d) : 1 (h)</td>
<td>95</td>
<td>36</td>
<td>38</td>
<td>Up to 4.2</td>
<td>90</td>
<td>(Nikiema et al., 2009)</td>
<td></td>
</tr>
<tr>
<td>NRb</td>
<td>NRb</td>
<td>0.7</td>
<td>0.15 (d) : 1 (h)</td>
<td>25</td>
<td>67</td>
<td>58</td>
<td>4.2</td>
<td>NRb</td>
<td>(Menard et al., 2010)</td>
<td></td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Stone</td>
<td>0.08 – 1</td>
<td>0.15 (d) : 1 (h)</td>
<td>0.25</td>
<td>75</td>
<td>44.7</td>
<td>59.6</td>
<td>5.7</td>
<td>77.5</td>
<td>(Nikiema et al., 2010)</td>
</tr>
<tr>
<td>NRb</td>
<td>Inorganic material</td>
<td>0.3</td>
<td>0.15 (d) : 1 (h)</td>
<td>0.18</td>
<td>20</td>
<td>13</td>
<td>65</td>
<td>6</td>
<td>22.59</td>
<td>(Veellite et al., 2011)</td>
</tr>
<tr>
<td>NRb</td>
<td>Gravel stone</td>
<td>3.3</td>
<td>0.15 (d) : 1 (h)</td>
<td>0.25</td>
<td>46.7</td>
<td>18.8</td>
<td>60</td>
<td>4.2</td>
<td>NRb</td>
<td>(Girard et al., 2012)</td>
</tr>
<tr>
<td>Soil</td>
<td>Soil / perlite</td>
<td>4.4</td>
<td>0.35 (d) : 1 (h)</td>
<td>NRb</td>
<td>30</td>
<td>16</td>
<td>53</td>
<td>&gt; 180</td>
<td>6.4</td>
<td>(Pratt et al., 2012b)</td>
</tr>
<tr>
<td>NRb</td>
<td>NRb</td>
<td>4.8</td>
<td>0.15 (d) : 1 (h)</td>
<td>71.2</td>
<td>39.9</td>
<td>42</td>
<td>4.25</td>
<td>75</td>
<td>(Ramirez et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Activated sludge</td>
<td>Polyurethane foam</td>
<td>15.3 ± 0.5</td>
<td>0.08 (d) : 1 (h)</td>
<td>1.1</td>
<td>~ 229</td>
<td>25</td>
<td>11</td>
<td>4</td>
<td>60</td>
<td>(Estrada et al., 2014)</td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Perlite</td>
<td>~ 5</td>
<td>0.08 (d) : 1 (h)</td>
<td>0.015</td>
<td>106.5</td>
<td>65.1</td>
<td>43 – 88</td>
<td>180</td>
<td>0.56 ± 0.07 f</td>
<td>(Kim et al., 2014b)</td>
</tr>
<tr>
<td>Previous biofilter</td>
<td>Tobermorite</td>
<td>~ 5</td>
<td>0.08 (d) : 1 (h)</td>
<td>0.015</td>
<td>96.6</td>
<td>31.9</td>
<td>33 – 42</td>
<td>20</td>
<td>0.39 ± 0.14 f</td>
<td>(Kim et al., 2014a)</td>
</tr>
<tr>
<td>NRb</td>
<td>Inorganic material</td>
<td>0.2 – 0.9</td>
<td>18.1d</td>
<td>0.25</td>
<td>18.5 – 83.3</td>
<td>39.4</td>
<td>47.2</td>
<td>4.3</td>
<td>2.26</td>
<td>(Menard et al., 2014)</td>
</tr>
<tr>
<td>M. methanica</td>
<td>Glass rings</td>
<td>0.0 – 0.12</td>
<td>0.08 (d) : 0.7 (h)</td>
<td>0.012</td>
<td>0.35</td>
<td>0.32</td>
<td>90.4</td>
<td>17.6</td>
<td>2.5</td>
<td>(Apel et al., 1991)</td>
</tr>
</tbody>
</table>

1. Soil mixture is an equal volume of yard waste compost, peat, and squeezed spruce wood fiber
2. NR, not reported
3. Filter bed volume composition = compost : perlite (60 : 40 % (v/v))
4. Dimension unknown
5. Average value
6. Liquid feeding simulation period included
7. Liquid feeding simulation period not included
8. Symbols: Q (flow rate), IL (Inlet Load), EC (Elimination Capacity), RE (Removal Efficiency), EBRT (Empty Bed Residence Time), PCO₂ (carbon dioxide production)
9. CO₂ consumption by methanogens
10. Two biofilters with gravel or compost as carrier material. Biofilter with gravel material was the one inoculated with soil. Data reported first in the table is the one with gravel material
It is also further assumed that the type of inoculum might have an influence on the biofilter performance. High removal efficiencies were obtained in biofilters inoculated with environmental samples from niches known to emit methane such as landfill (Table 7.2). Kim et al (2013) and Park et al (2009) obtained 59% and 100% removal efficiency, respectively, when using inoculum from landfill. Furthermore, if a biofilter was designed to remove methane at a low concentration (e.g., ~0.1 % (v/v)), then an inoculum coming from an environment which inhabits MOB known to exhibit a high methane specific affinity (e.g., soil) could be used as an inoculum. This was why soil was used as the starting environmental sample for the MOB enrichment in Chapter 2.

Although having lower performance compared to the previously reported biofilters, there are several advantages when using the AAC-based biofilter. As observed in the kinetic (Chapter 2) and biofilter studies (Chapter 3 and 4), carbon dioxide was not emitted when AAC was used as the carrier materials. In fact, carbon dioxide was removed when the biofilter was tested in the cows respiration chambers (Chapter 4) at an elimination capacity of 28.5 g m$^{-3}$ h$^{-1}$ (Table 7.2). Therefore, a complete methane-derived carbon sequestration was achieved making AAC a more environmentally friendly carrier material. The use of AAC also gives an advantage over organic carrier materials as it provides less compaction problem (i.e., pressure did not fluctuate significantly; data not shown).

As explained in Chapter 1 section 2.4, the use of inorganic material such as AAC as the carrier material, may present some inconveniences. For instance, unlike organic material (e.g., compost), AAC cannot provide additional nutrients for the bacteria. Hence, external nutrient supply should be provided into the filter. This was further demonstrated in Chapter 3 when lowered methane removal capacity of MOB was observed when the liquid nutrient feeding was stopped. Moreover, bacterial inoculation step was needed when using AAC as the carrier materials. This, together with the liquid nutrient feeding can give rise to higher biofilter operational costs.

Overall, the use of AAC as the carrier material for a methane biofilter presents several advantages and disadvantages and these considerations should be taken into account when designing a methane biofilter. When looking at the performance of the biofilter when removing methane in ruminant gas effluent, it can be seen that, due to the various factors (e.g., lower methane concentration, higher gas feed flow rate), lower removal efficiency was found in the biofilter. Nevertheless, as previously mentioned, this technology offers an advantage of carbon dioxide sequestration and this advantage is not found in the biofilters of other studies (Table 7.2).

1.3.2 Biogenic building material surface protection

In this thesis, the application of MOB-based MICP on building material was focused on the material surface treatment and not for self-healing concrete due to the incapacity of MOB to form endospore. MOB are only capable to form cyst and exospore as their resting stages (Table 1.3). As explained in Chapter 1 section 3.2.3, bacteria need to be added in the endospores form because of the harsh environment met during concrete production (e.g., high temperature and pH). Therefore, only bacteria capable to do so (e.g., Bacillus spp.) that have been investigated so far in the area of self-healing concrete (Jonkers et al., 2010; Van Tittelboom et al., 2010).

In Chapter 1 section 3.2.1, it was explained that there were several microbial pathways known to drive calcium carbonate precipitation. For building material surface protection, the pathways used are the oxidative deamination of amino acid, organic acid utilization, and urea hydrolysis (De Muynck et al., 2010a). In Chapter 5, MICP driven from the formate oxidation by M. parvus OBBP was studied. The aim was to provide a more environmentally friendly biogenic process than the urea hydrolysis for building material surface treatment. In order to know the biotechnological potential of the formate-
driven MICP by \textit{M. parvus}, the performance (i.e., biomineralization rate) of this alternative pathways is compared to the urea hydrolysis ones (Table 7.3).

Typically, the specific rate of calcium carbonate production using the microbial urea hydrolysis is expressed either per cell, Colony Forming Unit (CFU), or OD basis. As the study conducted in Chapter 5 was performed using pure culture strain, the biomineralization rate can be expressed on per cell basis. On per cell basis, the optimum biomineralization rate obtained in Chapter 5 (i.e., at 5 g L\textsuperscript{-1} calcium formate and 10\textsuperscript{9} cells ml\textsuperscript{-1}) was 36 times lower than the maximum urea based biomineralization rate (\textit{B. sphaericus}; Table 7.3). Other strain, \textit{B. pasteurii} ATCC 6453 has also been applied in other areas of environmental biotechnology as the biocatalyst to remediate contaminated soils (Mahnanty et al., 2013a; Mahanty et al., 2013b). Based on existing literatures, urea based biomineralization studies were typically performed using \textit{Bacillus spp.} because they are known to possess the urease gene and exhibit a high urea degradation rate (Dick et al., 2006; Hammes et al., 2003a). Accordingly, the construction industry mostly utilized these strains for the MICP based biotechnological applications (e.g., concrete surface treatment and self-healing concrete) (De Belie, 2010; De Muynck et al., 2010a; Jonkers et al., 2010).

The type of calcium carbonate polymorphs is important for biotechnological applications in the construction industry. In Chapter 5, it was found that calcite, vaterite, and aragonite are the types of calcium carbonate polymorph formed by the formate-based MICP by \textit{M. parvus}. Previous studies showed that for urea-based MICP, the typical crystal polymorphs obtained is calcite (De Muynck et al., 2008a; De Muynck et al., 2011). Vaterite was obtained when oxidative deamination of amino acid was used (Jimenez-Lopez et al., 2008; Rodriguez-Navarro et al., 2003). In Chapter 5, the calcite composition when using 5 g L\textsuperscript{-1} of formate was 58.5\% (wt/wt) with the rest being dominated by vaterite. For building material surface protection, calcite is the most preferred crystal phase due to its stability and higher consolidating effect (Rodriguez-Navarro et al., 2003). However, vaterite is not a disadvantage as it could also be stabilized in the longer term (Rodriguez-Navarro et al., 2007).

When looking at Table 7.3, it can be seen that the urea-based MICP employs urea and calcium source as the substrates. The typical calcium source used is calcium chloride. However, for application on concrete, other component than calcium chloride is preferred as chloride ions can cause the corrosion of the reinforced steel in concrete. The presence of chloride ions in the crystal deposits can also result to the distortion of the crystal lattice. Alternatively, other calcium salt (e.g., calcium acetate) has been used (Jimenez-Lopez et al., 2008). One advantage of using \textit{Bacillus spp.} is that the strain can still be active to degrade urea at a high concentration of urea and calcium salt. De Munyck et al found that an optimum concentration of urea and calcium chloride to have an optimum biodeposition are 20 g L\textsuperscript{-1} and 50 g L\textsuperscript{-1}, respectively (De Muynck et al., 2010b). This has resulted in a high amount of calcium carbonate precipitated on the limestone surface in that study. In Chapter 5, the use of calcium formate concentration higher than 5 g L\textsuperscript{-1} resulted in the lower precipitation yield. This could be attributed to the salt stress imposed to the bacteria which resulted in the impaired \textit{M. parvus} activity. Therefore, for the surface protection test (Chapter 6), 5 g L\textsuperscript{-1} was the starting calcium concentration used.
Table 7.3 Comparison of calcium carbonate biomineralization rate obtained in Chapter 5 with the urea-based approach.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Biomineralization ratea</th>
<th>CaCO$_3$ crystal polymorphs</th>
<th>Biominalization substrates</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. parvus OBBP</strong></td>
<td>6.0 ± 0.4b</td>
<td>Calcite, Vaterite, Aragonite</td>
<td>Ca(CHOOH)$_2$ (5 g L$^{-1}$)</td>
<td>This thesis (Chapter 5)</td>
</tr>
<tr>
<td>B. sphaericus</td>
<td>218</td>
<td></td>
<td>Urea (60 g L$^{-1}$) and CaCl$_2$ (40 g L$^{-1}$)</td>
<td>(Wang (2013))</td>
</tr>
<tr>
<td>B. pasteurii ATCC 6453</td>
<td>16</td>
<td>Calcite</td>
<td>Urea (3 g L$^{-1}$) and CaCl$_2$ (2.8 g L$^{-1}$)</td>
<td>(Mahanty et al., 2013a; Mahanty et al., 2013b; Stocks-Fischer et al., 1999)</td>
</tr>
<tr>
<td>S. pasteurii ATCC 11859e</td>
<td>7.2</td>
<td>Calcite and Vaterite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (2.8 mg L$^{-1}$)</td>
<td>(Bachmeier et al., 2002; Chou et al., 2011; Dupraz et al., 2009a; Dupraz et al., 2009b; Mitchell &amp; Ferris, 2006; Warren et al., 2001)</td>
</tr>
<tr>
<td>B. megaterium ATCC 10788</td>
<td>0.16d</td>
<td>Calcite and Vaterite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (2.8 g L$^{-1}$)</td>
<td>(Achal &amp; Pan, 2011)</td>
</tr>
<tr>
<td>B. megaterium AP6</td>
<td>0.83e</td>
<td>Calcite</td>
<td>Urea (3 g L$^{-1}$) and CaCl$_2$ (2 g L$^{-1}$)</td>
<td>(Bachmeier et al., 2002)</td>
</tr>
<tr>
<td>E. coli HB101g</td>
<td>13</td>
<td>Calcite and Vaterite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (18.8 g L$^{-1}$)</td>
<td>(Ghashghaei &amp; Emtiazi, 2013)</td>
</tr>
<tr>
<td>E. ludwigii</td>
<td>33</td>
<td>Calcite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (28.3 g L$^{-1}$)</td>
<td>(Nemati et al., 2005)</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>4.3</td>
<td>Calcite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (28.3 g L$^{-1}$)</td>
<td>(Park et al., 2010)</td>
</tr>
<tr>
<td>S. soli KNUC401</td>
<td>23.8</td>
<td>Calcite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (28.3 g L$^{-1}$)</td>
<td>(Park et al., 2010)</td>
</tr>
<tr>
<td>B. massiliensis KNUC402</td>
<td>13.1</td>
<td>Calcite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (3.7 g L$^{-1}$)</td>
<td>(Park et al., 2010)</td>
</tr>
<tr>
<td>A. crystallopoietes KNUC403</td>
<td>13.9</td>
<td>Calcite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (3.7 g L$^{-1}$)</td>
<td>(Park et al., 2010)</td>
</tr>
<tr>
<td>L. fusiformis KNUC404</td>
<td>15.2</td>
<td>Calcite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (3.7 g L$^{-1}$)</td>
<td>(Park et al., 2010)</td>
</tr>
<tr>
<td>S. ginsegisoli CR5</td>
<td>5.4</td>
<td>Calcite, Vaterite, Aragonite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (2.8 mg L$^{-1}$)</td>
<td>(Achal et al., 2012a)</td>
</tr>
<tr>
<td>K. flava CR1</td>
<td>2.7</td>
<td>Calcite and Aragonite</td>
<td>Urea (20 g L$^{-1}$) and CaCl$_2$ (3.7 g L$^{-1}$)</td>
<td>(Achal et al., 2011b; Achal et al., 2012b)</td>
</tr>
</tbody>
</table>

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*a*Biomineralization rate is expressed as the soluble calcium removal rate in the bacterial culture.

*Biomineralization rate value is taken from the cultures that exhibited the highest calcium carbonate precipitation yield (5 g L$^{-1}$ calcium formate and 10$^9$ cells ml$^{-1}$). Value is the average of triplicate measurements. Error value represents the standard deviation.

*b*If several literatures are given for a specific strain then the substrate composition and the biomineralization rate were taken from or calculated based on the first reference provided.

*Biomineralization rate was calculated assuming the mass of one bacterium is 1 x 10$^{-12}$ g.

*Previously known as *Bacillus pasteurii* ATCC 11859.

*35 % (v/v) of CO$_2$ was added in the headspace of the incubator.

*Plasmid pBU11 has been constructed with the entire sequence of the urease gene cluster taken from *S. pasteurii* ATCC 11859.
Due to the lower calcium formate starting concentration used in Chapter 6, the influence of the resulting precipitate on the building material to the building material characteristics was smaller compared to other treatments. Firstly, a much lower AAC specimens weight increase was obtained in this thesis (Chapter 6). A maximum weight increase of 38 ± 19 mg was obtained using two days AAC specimens immersion in 10\(^{9}\) M. parvus cells ml\(^{-1}\) containing 5 g L\(^{-1}\) of calcium formate. As a comparison, a maximum of ~500 mg of limestone weight increase was obtained by De Muynck et al (2010) using 20 g L\(^{-1}\) of urea and 50 g L\(^{-1}\) of calcium chloride (De Muynck et al., 2010b). Zamarreno et al (2009) obtained a maximum ~100 mg of limestone weight increase by F4 isolate using 50 g L\(^{-1}\) of calcium acetate (Zamarreno et al., 2009). For application on concrete, De Munyck et al (2008) obtained a maximum 77.1 ± 3.8 mg and 81.8 ± 1.4 mg of specimens weight increase after a one time biodeposition treatment using 10 g L\(^{-1}\) urea and 26 g L\(^{-1}\) calcium acetate, respectively (De Muynck et al., 2008b). Secondly, due to the lower amount of deposition, the resulting precipitate obtained in our study (Chapter 6) was limited to the deposition of calcium carbonate on the wall of the pores on the surface of the specimen. From SEM analyses, due to the small size of crystals produced (i.e., crystal diameter of ~10 µm), these crystals mainly filled the pores in between the aggregates of AAC specimens. As mentioned in Chapter 6, Larger crystal size (20 – 100 µm diameter) were observed in other studies using Bacillus spp. and urea hydrolysis (De Muynck et al., 2011; De Muynck et al., 2010b). Accordingly, smaller sizes of crystals (diameter of 2-4 µm) was obtained by Jimenez Lopez et al (2008) when smaller concentration of substrate (1 g L\(^{-1}\) of calcium acetate) was used (Jimenez-Lopez et al., 2008).

Although using a relatively small substrate concentration, the resulting calcium carbonate deposition on AAC specimens (Chapter 6) could still effectively lower the water intrusion into the material. In Chapter 6, three different evaluations were performed to assess the effectiveness of the formate-based MICP by M. parvus, namely, capillary water absorption, drying behavior, and sonication tests. Previous studies showed that surface treatment as pore blockers resulted in effectively lowering the water intrusion rate (Jimenez-Lopez et al., 2007; Rodriguez-Navarro et al., 2003). Accordingly, when higher substrate concentration were used, the rate would be further decreased (De Muynck et al., 2010b). Varying degree of the lowering water absorption rate was obtained when other bacterial strains or metabolic processes were used (Dick et al., 2006; Le Metayer-Levrel et al., 1999). Furthermore, based on the drying behavior test, water was evaporated with ease from AAC specimens after being saturated with water (Chapter 6). This gives further advantage when using this process. Previous research showed that when higher amount of calcium carbonate deposits was obtained, the material’s porosity was significantly reduced and water was evaporated at lower rate from the material (De Muynck et al., 2011). This indicated that the material had a higher water holding capacity after bacterial treatment and this increased the probability of material dissolution. From the sonication test, every other reports have indicated that the biogenic carbonate mineral layer exhibited some consolidating effect on the subjected building material and this was also the case when using the formate-based MICP by M. parvus (Chapter 6). However, as observed in Chapter 6, the cohesive effect of the deposition was not significant (P > 0.05). It should also be mentioned that, some other evaluation techniques (e.g., gas permeability test) were not conducted when evaluating the surface treatment by M. parvus in Chapter 6.

Finally, comparisons are also made with the non-urea based treatment. These are the comparison with the two other aerobic microbial pathways known to drive calcium carbonate precipitation: Oxidative Deamination of Amino Acid (ODAA) and Organic Acid Utilization (OAU). Using OAU, other organic acid precursors (in the calcium salt form) that have been used are acetate and lactate among others (Jonkers et al., 2010; Jroundi et al., 2010). Some studies used a combination of ODAA and OAU as the driving process for MICP. Using Micrococcus sp. and B. subtilis, a 60 % water absorption reduction rate was observed when the bacteria were applied on a limestone surface compared to the non-treated
samples (Tiano et al., 1999). In that study, Tiano et al. used B4 medium containing 0.25 g L\(^{-1}\) and 0.4 g L\(^{-1}\) of calcium acetate and yeast extract, respectively. In Chapter 6, only up to 30\% (2.92 ± 0.91 kg m\(^{-2}\)) lower water absorption was observed compared to the non-treated samples. As explained previously, in Chapter 6, the consolidation effect of the solid deposition was not significant as observed from the sonication test (6 cycles; 5 min per cycle). Using 2 \(\times\) \(10^9\) cells ml\(^{-1}\) of \(M.\) \textit{xanthus}, Rodriguez-Navarro et al., (2003) found that the new carbonate crystals are strongly attached to the substratum after five cycles of sonication test (5 min per cycle) (Rodriguez-Navarro et al., 2003). These were mostly due to epitaxial growth on preexisting calcite grains. In that test 1 g L\(^{-1}\) of both calcium acetate and Bacto casitone (i.e., protein source) were used as the biomineralization precursors. Using the same strain and precursors, Jimenez-Lopez et al., (2008), could obtain a weight loss of treated stones of less than 0.30\%, whereas untreated stones weight lost was up to 0.85\% of the initial weight (Jimenez-Lopez et al., 2008).

Using calcium acetate (5 g L\(^{-1}\)) as the only biomineralization substrate and \textit{Pseudomonas} (isolates D2 and F2) and \textit{Acinetobacter} (isolate B14) as the bacterial strains, Zamarreno et al., (2009) up to 49\% of limestone pore reduction was observed (Zamarreno et al., 2009). In Chapter 6, although porosity analysis was not performed after the bacterial treatment, it was observed that the solid biodeposition only influenced the micropores of the AAC specimen. Overall, from the previously mentioned studies, better performance was observed even when non-urea bacterial treatments were applied in comparison with the formate-based approach using \(M.\) \textit{parvus} in Chapter 6.

2. Research outlook

Based on results obtained from different studies in this thesis, several suggestions are made for future research.

2.1 Research outlook for the use of building material for methane bioremediation

Based on the results obtained in part I of the thesis, it was observed that methane was removed at lower efficiency when AAC was used as the carrier material compared to when other carrier materials were used (as seen in previous studies). Optimization of the process was not performed to obtain the maximum methane removal. Detail studies were also not performed to explain some phenomena observed in Chapter 2, 3, and 4. No proof of carbonation reaction was given to explain the lower total methane- and carbon dioxide-C in the headspace of incubators containing bacterially inoculated AAC (Figure 2.3). The composition of mixed culture MOB on AAC specimens and the subsequent kinetic at the end of the biofilter test was also not checked. Hence, the enrichment of MOB with higher affinity after the lab scale biofilter test was not confirmed. In Chapter 4, the suitability of the biofilter for long term application could not be confirmed as the test was only performed for two days. Potential problems foreseen if the experiment was run longer was the inactivity of the bacteria due to the several factors such as the lowering humidity in the biofilter and the accumulation of ammonia in the biofilm. Overall, to address these shortcomings, several suggestions are made in this thesis.

Future research should investigate the carbonation of AAC from the methane oxidation by MOB. A strategy is to incubate MOB on AAC in a closed incubation fed with \(^{13}\)C-based methane. If the methane available for the MOB is only sufficient for the maintenance of the bacteria, then methane is fully converted to carbon dioxide (Knief & Dunfield, 2005). If carbonation occurs, then AAC specimen would contain higher \(^{13}\)C mass after a period of incubation. This would give further indication of the carbonation reaction.

If the biofilter will be constructed in a “classical biofilter” configuration (Chapter 3 and 4), then the following points are suggested. The influence of different EBRT (i.e., varying gas flow rate) on the RE in the biofilter should be investigated to determine the optimal gas flow rate to obtain maximum RE.
For the bacterial inoculation of AAC, then a mixture of the mixed MOB culture and \textit{M. parvus} is suggested to be the inoculum source as \textit{M. parvus} exhibited the highest methane removal rate on AAC (Chapter 2). Moreover, if the same mixed culture inoculum as the one used in Chapter 3 is used, then the evolution of MOB abundance and their impact on the methane removal by MOB should also be further studied. This should be done with a new reactor design that allows invasive sampling without affecting process performance. Using this new setup, the composition of the mixed MOB culture should also be checked at the end of the biofilter operation. Different composition might be present at the end of the biofilter operation than the one observed in the kinetic study in Chapter 2. Kinetic study of the mixed MOB culture residing on AAC should also be conducted after the lab scale biofilter test. These two tests should be done to investigate whether the MOB mixed culture after the biofilter test show higher affinity than the one in Chapter 2. When applied in the stable, the biofilter system should incorporate a humidification column prior to the biofilter. Essential nutrients can also be added intermittently via a separate liquid line similar to the one set in Chapter 3. Effluent gas from a certain niche (e.g., respiration chamber) is fed firstly to this humidification column to carry water vapor into the biofilter. This is done to keep the humidity on the carrier material and to strip ammonia from the feed gas. A test investigating the sensitivity of MOB in the biofilter to ammonia in the gas should also be performed. This can be done by feeding methane at varying ammonia concentrations into the biofilter. This is important to determine the maximum ammonia concentration giving the maximum RE in the biofilter. Such studies have been performed using other inorganic based carrier materials (Veillette et al., 2012b; Veillette et al., 2011). A higher number of biofilter (for example by adding MBF-B such as in Chapter 3) should be installed when operating the biofilter in the livestock barn in order to improve the RE in the filter system. This should be done especially when the filter operates at a low RE such as the one in Chapter 4. From results obtained in Chapter 3, the operation of two biofilters (MBF-A and MBF-B) were the optimum setup to obtain maximum RE (see Figure 3.4). Additionally, the biofilter should be tested in a real animal barn where several challenges could be found than the respiration chamber test (Chapter 4) e.g., a more extreme diurnal temperature variation is found. This factor also influences the MOB activity (Hanson & Hanson, 1996) and it would be interesting how this would impact the biofilter performance. It is also suggested to use a mixture of organic and inorganic material (e.g., AAC and soil) as the carrier material for application purposes in order to reap the advantages offered by both types of materials (i.e., nutrient provision from the carrier material and carbon dioxide sequestration) when operating the biofilter.

Another suggestion is to construct the biofilter using existing building material like the “green façade” system explained in Chapter 1 section 3.1 and seen in Figure 1.5. Potential applications are building structure in niches with a high methane atmospheric concentration (i.e., ≤ 1 % (v/v)). From the tested building materials in Chapter 2, bricks and AAC are the two potential materials for this application as they are the mostly utilized materials in animal barns. AAC has been increasingly utilized for animal barns construction materials in West Flanders. A drawback of using the same type of brick is that, due to the low porosity of the material, low number of MOB might be incorporated into the material. It was tested previously in Chapter 2 (data not shown) that MOB did not exhibit a high methane removal rate at low concentration (~100 ppmv; similar to the one in animal barns) when the bacteria were immobilized on bricks.

To construct a “green façade” type of biofilter (see Chapter 1) then the following steps need to be done: building material colonization and biomass system maintenance. Building material colonization can be done via external facilitation or natural colonization. Externally, MOB can be enriched in separate facility and incorporated into the existing building material. For existing building material, incorporation of biomass cannot use immersion method used in Chapter 2 and 3. Several suggested methods are by
spraying or brushing the bacterial culture on the building material. After this incorporation, MOB biofilm will expectantly grow on the surface after a period of time. A crucial factor of this application is to ensure the viability of the MOB. A test was done previously to investigate the viability of *M. parvus* and *M. trichosporium* on Maastricht limestone at low methane concentration (~100 ppmv) after the initial immobilization using method described in Chapter 2 (Figure 7.1).

It can be seen that the activity of both *M. parvus* and *M. trichosporium* was lost after approximately two months of activity test using the methane pulse feed batch test. Knief and Dunfield (2005) investigated that, based on the Herbert-Pirt equation which relate the limiting substrate (i.e., methane) specific consumption rate to the bacterial specific growth and maintenance, *M. trichosporium* needed a minimum of ~100 ppmv for the cell maintenance (Knief & Dunfield, 2005). As can be seen from Figure 7.1, although fed with methane at approximately the same level, the capacity of the bacteria to consume methane was eventually lost after two months. This could be attributed to the cell death or inactivity due to the lack of other nutrient supply. Therefore, if a methane biofilter would be built using the “green façade” concept, then other essential nutrients (e.g., nitrogen and phosphorus sources) should be supplied to the bacteria frequently. Hence, as a second step after MOB colonization of the building material, to ensure a stable MOB biofilm growth, nutrient solutions can then be applied frequently by brushing or spraying. Future studies should then investigate the influence of the frequency of the nutrient application to the activity/viability of the MOB. This should further relate to the operational cost of the system and the feasibility of the process.

![Figure 7.1](image.png)

**Figure 7.1** Period of methane removal by *M. parvus* (dotted line) and *M. trichosporium* (straight line) immobilized on Maastricht limestone (*n*=2) at low methane concentration (~100 ppmv).

Based on the results obtained in Chapter 2, for the biofilter with “green façade” concept, then *M. parvus* is the best MOB strain candidate for the initial colonization of the building material. As the system is open to the environment, the biofilter will eventually consist of an ecosystem of different organism. Therefore, it would be interesting to see whether *M. parvus* would still be present after a period of its initial application. It should also be further investigated whether the biofilter can be self-sustainable after operated for a certain period and a “mature” (MOB and other organisms) ecosystem is established. As explained in Chapter 1 section 3.1, the presence of other organism (e.g., higher plant) can provide metabolite intermediate (e.g., acetate) from the plant exudates. If this methane biofilter is self-sustainable, then application of nutrient solution on the building material is not necessary anymore. Moreover, if algae is present in this ecosystem, then the MOB-algae system can provide an additional
functionality to the biofilter. The CO$_2$ produced by MOB can be used by algae for photosynthesis (Methalgae concept; see PhD thesis David van Der Ha (2013)).

Building material colonization can also occur naturally. In Chapter 1 section 3.1, it was explained that properties of building material (e.g., pH, porosity) need to be adjusted to have an optimum colonization (see PhD thesis Sandra Manso (2014)). In addition to the building material surface characteristics adjustment, future studies can also investigate a method to “attract” MOB colonization on the material’s surface. A suggestion is to apply silicone oil which might increase methane solubility on the wet building material surface. Additional trait of this “methane biofilter wall” is, as is the case with “green façade” and “living wall” (Chapter 1 section 3.1), to provide aesthetic.

2.2 Research outlook for the use of formate based MICP by *M. parvus* for building material surface protection

Based on the results obtained in part II of the thesis, it was observed that the biomineralization rate of *M. parvus* was still inferior than several strains used for the urea-based approach (e.g., *B. pasteurii*). Moreover, the optimum calcium formate concentration to obtain a maximum calcium carbonate precipitation yield was 5 g L$^{-1}$. Using the urea-based approach, up to 20 g L$^{-1}$ of urea and 50 g L$^{-1}$ of calcium chloride could be used (De Muynck et al., 2010b). This implies that higher amount of calcium carbonate would be obtained using the urea-based approach and potentially longer protection effect for the building material. Faster precipitation rate could also be obtained using *B. sphaericus* and *B. pasteurii*. Although the formate-based approach using *M. parvus* is more environmentally friendly, the performance of the process on building material is still not optimum compared to the urea-based approach. Moreover, as seen in the results in Chapter 6, the resulting solid deposition did not exhibit significant consolidation effect to the building material as opposed to the effect of the urea-based approach.

For future study, an optimization of the biomineralization rate of the MICP based on calcium formate utilization by *M. parvus* OBBP should be conducted. This can be done by testing other available MOB strains available (e.g., *M. trichosporium*). For the application on building materials, several suggestions are made. Firstly, the use of mixed culture MOB as the biological agent should be explored. The use of mixed culture MOB could lower the operational cost related to the enrichment of the bacteria where an aseptic procedure should be applied. If mixed culture MOB is the chosen bacterial culture, then culture enrichment exhibiting higher affinity to methane than the ones in Chapters 2, 3, and 4 should be utilized. This is done so that higher methane removal activity and longer period of activity would be obtained if the culture is used as the catalyst for the building material surface protection. Secondly, future studies should look into the application of formate-based MICP on natural stones to test the effectiveness of the process as the surface treatment for this type of material. Higher compatibility of the newly formed carbonate solid compared to the results obtained in this study (i.e., from sonication test) could be achieved if the process is applied on materials composed mostly from calcium carbonate such as natural stones. Finally, field test application (e.g., in cow stables) should be conducted to investigate the effectiveness of the process to existing building materials.

Overall, biogenic building material surface protection using the formate-oxidation by *M. parvus* presents a novel MICP approach to protect building material surface. By employing *M. parvus* to oxidize formate, ammonia and nitric acid were not produced in the process. The formate-based approach also proved to be effective as a surface treatment by significantly reducing the water penetration rate into the material. Additionally, methane was removed from the air by the bacteria making the process exhibits less
emission compared to others. This alternative pathway gives a more environmentally friendly solution to the urea hydrolysis one.

2.3 Research outlook for a combined study: concomitant methane bioremediation and building material surface protection by MOB on building materials

In this thesis, the concept of methane bioremediation by MOB using building material as the carrier material and the biogenic building material surface protection by MOB are introduced. It was explained here that there are several limitations if the results obtained in this thesis are to be applied. These limitations mean that a combined concept of building material surface protection and methane bioremediation by MOB on building material will not be sustainable. For example, carbonation reaction contributes to the deterioration of building material if AAC is used. This has a contradictory effect the surface protection by the calcium carbonate precipitation. Moreover, it was foreseen that bacteria will not be viable after driving calcium carbonate precipitation. The formation of solid deposition around the bacteria cell wall will increase the mass transfer resistance of nutrients/metabolites into and out of the cell. Hence, application of MOB on building material for a concomitant methane bioremediation and building material surface protection will not be a sustainable application.

An alternative here is to use the concept of MOB on building material to protect the material surface with atmospheric methane removal being an additional trait of the application. The concept can be applied in places where elevated methane concentration is found e.g., the animal barn. The concept is as follows: MOB and calcium formate are applied by spraying / brushing on the building wall of an animal barn. MOB converts formate to carbonate and subsequently carbonate react with calcium to form calcium carbonate. Additionally, the bacteria also oxidize methane from the air to carbonate (Figure 7.2). From the measurement campaign in the respiration chamber (Chapter 4), the average methane concentration measured from the gas effluent in respiration chamber was around 55 ppmv. In an animal barn, especially in the open barn one, the methane atmospheric concentration is lower due to the mixture with the circulating air. As explained in Chapter 6, at low methane concentration (i.e., <50 ppmv), the amount of carbonate contributed from the methane oxidation for the building material surface protection would not be significant, however, a major fraction of calcium carbonate precipitate would be obtained from the formate oxidation. It can be seen from Figure 7.1 that a sustainable methane oxidation by MOB on building material could not be obtained by a one-time bacterial application on building material as MOB could not sustain themselves at this low concentration and the bacterial need for additional essential nutrients. As seen in the MOB metabolic pathway (Figure 1.3), reducing equivalent (i.e., NADH$_2$) is generated from the formate oxidation. Theoretically, due to this additional reducing equivalent production, MOB could prolong their viability for more than two months when compared with application without calcium formate (Figure 7.1). Overall, in this type of application, MOB (and calcium formate) application on building material is aimed for the building material surface protection with an additional trait of a period of methane removal from the air.
Figure 7.2 A concept for future study of MOB on building material: building material surface protection and additional trait of atmospheric methane removal by Methane Oxidizing Bacteria (MOB). Illustration by Tim Lacoere.
3. Conclusion

Two novel biotechnological applications of MOB are presented in this thesis: the use of building materials for methane bioremediation (Part 1) and the use of MOB as an alternative biocatalysts for biogenic building material surface protection (Part 2).

Part 1

Several building materials and MOB were screened for the application of the methane bioremediation (Chapter 2). The screening process was based on the kinetic of methane removal by the immobilized MOB at both high (~20 % (v/v)) and low (~100 ppmv) methane concentrations. *M. parvus* on AAC exhibited the highest methane removal at both concentrations due to the high porosity of the material and high affinity nature of the bacteria at low concentration. In Chapter 3, mixed culture MOB used in Chapter 2 and AAC were used in a lab scale biofilter setup at ~1000 ppmv for approximately four months with an average of 28.7 % removal efficiency. This removal efficiency was reached using three biofilters set in series where one biofilter (MBF-A) was initially started and the other biofilters were added consecutively. The biofilter used in Chapter 4 (MBF-A) was tested to treat gas effluent from livestock in a respiration chamber setup situated in ILVO Vlaanderen. Lower methane removal efficiency was observed in MBF-A (17.5 %) compared to the efficiency in lab scale test due to the lower methane concentration found in the gas effluent and the higher gas flow rate into the biofilter among others.

Part 2

A novel MICP pathway using MOB as the biocatalyst was tested in this part of the research. *M. parvus*, selected as it exhibited the highest methane removal in Chapter 2, was used as the MOB strain. Calcium carbonate precipitation was induced from the formate oxidation by *M. parvus* when the bacteria were incubated in calcium formate (Chapter 6). A maximum of $0.67 \pm 0.03 \text{ CaCO}_3 \text{ Ca(CHOOH)}_2$ (g/g) calcium carbonate precipitate yield was obtained when $10^9$ cells mL$^{-1}$ and 5 g L$^{-1}$ of calcium formate were used. This process was subsequently used to protect AAC surface (Chapter 7). Calcium carbonate was precipitated mainly on the wall of the surface of AAC specimens. Due to this precipitation, lower water absorption rate (i.e., up to $2.92 \pm 0.91 \text{ kg m}^{-2}$) into the specimens was observed. The precipitated calcium carbonate was also able to consolidate the building material matrix.
ABSTRACT

Methane is the most important organic greenhouse gas emitted to the atmosphere for its contribution to the global warming. The gas has a strong infrared absorbance (i.e., 25 times more efficiently than carbon dioxide) which makes it a more effective greenhouse gas than carbon dioxide although having a shorter lifetime in the atmosphere (~9 years). Driven by the anthropogenic emission due to the increase of global population and energy demand, methane emission is set to increase in the future. Several anthropogenic methane emission mitigation has been applied in various sectors (agriculture, energy, and waste). The use of a biochemical reactor can be an alternative to remediate methane emission at low concentration (< 1 % (v/v)) as it environmentally friendly and economically more beneficial. In the biochemical oxidizer, Methane Oxidizing Bacteria (MOB) are used as the biocatalyst. MOB are part of methylotrophic bacteria, a group of bacteria capable of utilizing one carbon compounds as their carbon and energy sources. For gaseous waste having low solubility like methane, the typical bioreactor used to treat methane gas waste is biofilter where MOB are immobilized on a carrier material.

When designing a biofilter, carrier material selection is arguably the most crucial step. The preferred carrier materials possess a high porosity and surface area to provide space for the bacteria to grow and to increase the contact area between the bacteria and methane, respectively. Based on these criteria, building materials have the potential to be a good carrier material for a methane biofilter. Using this concept of “housing” MOB on building material, another biotechnological application of the bacteria was explored. Microbiologically Induced Carbonate Precipitation (MICP) is the production of carbonate mineral driven by environmental condition (e.g., pH) alteration as a result of microbial activity. In the construction industry, the typically applied urea hydrolysis based MICP poses several disadvantages such as ammonia release to the air and nitric acid production. In this thesis, the capacity of MOB to induce calcium carbonate precipitation as the basis for a more environmentally friendly biogenic building material surface protection was also explored. Therefore the thesis is divided into two parts: Part 1 deals with the exploration of building material utilization as the carrier material for methane biofiltration (Chapter 2 to 4) whereas Part 2 deals with the exploration of MOB application on building materials as an alternative biocatalyst for the material surface treatment (Chapter 5 and 6).

In Chapter 2 a screening of different building material and MOB culture was done to select the combination of both which allow the bacteria to exhibit the highest methane removal capacity. Experiments were performed with different MOB inoculated on building materials at high (~20 % (v/v)) and low (~100 ppmv) methane concentrations. Methylocystis parvus in Autoclaved Aerated Concrete (AAC) exhibited the highest methane removal rate at high (28.5 ± 3.8 µg CH₄ g⁻¹ building material h⁻¹) and low (1.7 ± 0.4 µg CH₄ g⁻¹ building material h⁻¹) methane concentration. Due to the higher volume of pores with diameter > 5 µm compared to other materials tested, AAC was able to adsorb more bacteria which might explain for the higher methane removal observed. The total methane and carbon dioxide-carbon in the headspace was decreased for 65.2 ± 10.9 % when M. parvus in AAC was incubated for 100 hours. AAC was therefore selected for the carrier material for the subsequent methane bioremediation studies (Chapter 3 and 4) and M. parvus was selected as the MOB strains for MICP studies (Chapter 5 and 6).

In Chapter 3, the methane removal capacity of mixed MOB culture in a biofilter setup using AAC as a highly porous carrier material was tested. Although it was found that M. parvus exhibited the highest methane removal capacity on AAC (Chapter 2), mixed MOB culture was the selected culture for the biofilter inoculation in this study. This was based on the fact that non-asceptic practice was preferred to keep the operating cost lower if the biofilter was to be applied to remove methane in places with high methane emission (atmospheric concentration < 1 % (v/v)). Batch experiment was performed to
optimize MOB immobilization on the AAC specimens where optimum methane removal was obtained when calcium chloride was not added during bacterial inoculation step and 10 mm thick AAC specimens were used. The immobilized MOB could remove methane at low methane concentration (~1000 ppmv) in a biofilter setup for 127 days at an average Removal Efficiency (RE) of 28.7%. MOB also exhibited a higher abundance at the bottom of the filter, in proximity with the methane gas inlet where a high methane concentration was found. It was concluded here that a reasonably efficient and a more environmentally friendly methane biofilter performance can be obtained using AAC as the carrier material. Hence, the setup was used in a field test application (Chapter 4).

The performance of MOB immobilized on AAC to remove methane from ruminants effluent gas was investigated in Chapter 4. A biofilter employed in Chapter 3 was used as the biofilter in this study. Two dairy cows were housed in respiration chambers for two days where the exhaust gas from the chambers was used as the biofilter feed. MOB consumed methane at an average RE of 17.52 % and elimination capacity (EC) of 67.3 g m⁻³ d⁻¹. Several factors that might cause the lower RE and EC compared to the lab scale study (RE = 28.7 %) in Chapter 3 are: (a) the lower methane concentration and (b) the presence of ammonia in the livestock effluent gas, (c) the higher gas flow rate into the biofilter, and (d) the lowering humidity level in the biofilter. By using AAC as carrier material, carbon dioxide in the effluent gas as well as the one from the methane oxidation by MOB were removed by the carbonation reaction with AAC. Thus, complete carbon sequestration from methane was obtained. Overall, in part 1 of this thesis (Chapter 2 to 4) it was concluded that a more environmentally friendly methane biofilter than the ones previously tested could be achieved when using ACC as the carrier material.

An alternative MICP from calcium formate by Methylocystis parvus OBBP is presented in Chapter 5. To induce calcium carbonate precipitation, M. parvus was incubated at different calcium formate concentrations and starting culture densities. Up to 91.4 % ± 1.6 % of the initial calcium was precipitated in the methane amended cultures compared to 35.1 % ± 11.9 % when methane was not added. Because the bacteria could only utilize methane for growth, higher culture densities and therefore calcium removals were exhibited in the cultures when methane was added. A higher calcium carbonate precipitate yield was obtained when higher culture densities were used but not necessarily when more calcium formate was added. This was mainly due to salt inhibition of the bacterial activity at a high calcium formate concentration. A maximum of 0.67 ± 0.03 CaCO₃ Ca(CHOOH)₂⁻¹ (g/g) calcium carbonate precipitate yield was obtained when 10⁹ cells mL⁻¹ and 5 g L⁻¹ of calcium formate were used. Compared to the current strategy employing biogenic urea degradation as the basis for MICP, the approach in this study presents significant improvements in terms of pollutant emission reduction if applied in the construction industry. The process was subsequently applied on building material as an alternative surface treatment (Chapter 6).

The effectiveness of MICP from the formate oxidation by Methylocystis parvus as an alternative concrete surface treatment was investigated in Chapter 6. MICP was induced on AAC by immersing the material in 10⁹ M. parvus cells mL⁻¹ containing 5 g L⁻¹ of calcium formate. A 2 days immersion of the material gave the highest weight increase of the specimen due to the calcium carbonate deposition. The deposition mainly occurred on the wall of the pores on the surface of the specimen. Due to this surface deposition, a significantly lower water absorption was observed in the bacterially treated specimens compared to the non-treated ones (i.e., up to 2.92 ± 0.91 kg m⁻²). A concomitant atmospheric methane removal (152.2 ± 40.1 µg of CH₄ m⁻² h⁻¹) was also observed in the bacterially treated specimens. Overall, in part 2 of this thesis (Chapter 5 and 6) it was concluded that compared to the currently employed biogenic processes, the formate-based MICP by M. parvus offers a more environmentally friendly approach for the biotechnological application to protect concrete surface.
The results obtained from part 1 and 2 in this thesis were subsequently positioned in their related biotechnology field and the outlook for the respective researches was presented in Chapter 7. The AAC-based methane biofilter had lower methane removal efficiency compared to the previously reported biofilters, although the other biofilters operated with higher Empty Bed Residence Time (EBRT) which might increase the overall methane conversion in the biofilter. The AAC-based methane biofilter, however, offers an advantage of carbon dioxide sequestration and this advantage is not found in the other biofilters. For the formate-oxidation based MICP by *M. parvus*, it was found that, on per cell basis, the optimum biomineralization rate obtained in Chapter 5 (i.e., at 5 g L\(^{-1}\) calcium formate and 10\(^9\) cells ml\(^{-1}\)) was still approximately three times lower than the maximum urea based biomineralization rate by *B. pasteurii* ATCC 6453. Moreover, unlike the urea-based MICP where a high urea / calcium source concentration could be employed, the influence of the formate-oxidation based MICP on the building material characteristics was smaller. Nevertheless, the resulting calcium carbonate deposition could effectively lowered water intrusion into the material. Based on the results obtained, several suggestions were made. To construct the biofilter like the “green façade” concept, MOB should be applied by brushing / spraying on existing building material with consecutive applications of nutrient applications to sustain the MOB growth on the building structure. For the MOB-based MICP, the application on natural stones to test the effectiveness of the process as the surface treatment for this type of material should be performed. Future studies should also look into the use of mixed culture MOB as it may lower the cost of this type of application.


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EDUCATION

2011 – 2015  PhD in Applied Biological Science, Laboratory of Microbial Ecology and Technology (LABMET), Faculty of Bioscience Engineering, Ghent University
PhD thesis: Housing Methane-Oxidizing Bacteria on Building Materials: towards a Sustainable Air Bioremediation and Building Materials Surface Protection

2006 – 2008  MsC in Biochemical Engineering, Department of Biotechnology, Delft University of Technology
• MsC thesis: Integration of Discreet and Continuum Biofilm Modeling (Environmental Biotechnology Group)
• Conceptual process design: Sustainable Fermentation Production of Fumaric Acid

2000 – 2005  BsC in Chemical Engineering, Faculty of Industrial Technology, Bandung Institute of Technology
• BsC thesis: Examination of Glutaraldehyde Effectiveness as Carbon Steel Corrosion Inhibitor in Seawater Containing Sulphate Reducing Bacteria
• Preliminary plant design: Biotechnological Production of Poly-β-Hydroxybutyrate from Glucose
• Industrial Internship: Standard Operating Procedure for Ammonia pre-Heater, Granulator, and Pipe Reactor in Fertilizer Process Production in PT. Petrokimia Gresik (national fertilizer Company), Indonesia

PROFFESIONAL EXPERIENCE


2008  Assistant Researcher (Industrial internship), DSM Anti-Infectives, Delft, The Netherlands
Topic: Recovery of Fumaric Acid from low pH Fermentation Broth (obtained a patent from the European Patent Office; Patent No: 09158953.1 – 121).

2005 – 2006  Process Engineer, Laboratory of Electrochemical Conversion, Bandung Institute of Technology
ACADEMIC EXPERIENCE

January 2015   Visiting scientist in Fennel Laboratory, Department of Environmental Science, Rutgers University (FWO fellowship)
2011 – 2014   Scientific tutor for five master student for their thesis work
2011 – 2014   Coordinator of the Introduction of Environmental Microbiology practical course
2003 – 2004   Assistant Researcher, Laboratory of Electrochemical Conversion, Bandung Institute of Technology

EXTRACURRICULAR ACTIVITIES

2011 – 2013   Member of the Indonesian Student Association in Ghent, Belgium
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PUBLICATIONS

- Ganendra et al. Methane Biofiltration using Autoclaved Aerated Concrete as Carrier Material. Manuscript submitted
- Ganendra et al. Methane Biofiltration from Gas Waste Originating from Ruminants in Respiration Chamber using Autoclaved Aerated Concrete as Carrier Material. Manuscript submitted
- Ganendra et al. A Sustainable Biogenic Concrete Surface Protection by *Methylocystis parvus OBBP*. Manuscript submitted

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- Strategic Initiative Material in Flanders. User Meeting. Antwerpen, Belgium, 2011 (poster presentation)
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- 18th National Symposium on Applied Biological Sciences. Faculty of Bioscience Engineering, Ghent University, Belgium, 2013 (oral presentation)
- International Conference on Self-Healing Materials. Ghent University, Belgium, 2013 (oral presentation)
- 34th Annual Cement and Concrete Science Conference. The University of Sheffield, England, 2014 (poster presentation)
- Knowledge of Growth: Flanders Bio Annual Life Sciences Convention. Flanders Bio, Ghent, Belgium (oral and poster presentation)
- 16th EMBL PhD symposium. European Molecular Biology Laboratory, Heidelberg, Germany
- Strategic Initiative Material User Meeting. Antwerpen, Belgium, 2014 (oral and poster presentation)
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“The fear of the Lord is the beginning of knowledge”

Proverbs 1:7