Voor alle Don Quichot(a)s
Vechtend tegen windmolens

Opgedragen aan tante Magda
We hebben elkaars afscheid gemist…

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David van der Ha

METHANOTROPHIC MICROBIOMES AS DRIVERS FOR ENVIRONMENTAL BIOTECHNOLOGY

Thesis submitted in fulfilment of the requirements for the degree of Doctor (PhD) of Applied Biological Sciences

Titel van het doctoraat in het Nederlands:

Methanotrofe microbiomen als drijvende kracht voor milieubiotechnologische toepassingen

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Notation index

AME-D	Aerobic methane oxidation coupled to denitrification
AMO	Ammonia monooxygenase
AMS	Ammonium mineral salts medium
ANME	Anaerobic methane oxidizing *Archaea*
AOB	Ammonia oxidizing bacteria
AOM	Anaerobic oxidation of methane
ATP	Adenosine triphosphate
BOD	Biological oxygen demand
CDW	Cell dry weight
CFC	Chlorofluorocarbons
Co	Community organization
COD	Chemical oxygen demand
CSTR	Continuously stirred tank reactor
dAMS	Diluted ammonium mineral salts medium
DGGE	Denaturating gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNA-SIP	DNA-based stable istotope probing
dNMS	Diluted nitrate mineral salts medium
dNTP	Deoxyribonucleotide triphosphate
DO	Dissolved oxygen
EPS	Extracellular polymeric substances
ETC	Electron transfer chain
GC	Gas chromatograph
GHG	Greenhouse gas
GWP	Global warming potential
HAO	Hydroxylamine cytochrome c oxidoreductase
HRAP	High rate algal pond
HRT	Hydraulic retention time

IC	Ion chromatograph
IPCC	The intergovernmental panel on climate change
MAC	Coculture of methane oxidizing bacteria and algae
MMO	Methane monooxygenase
MOB	Methane oxidizing (proteobacterial) bacteria
MOC	Methane oxidizing community
MOR	Methane oxidation rate
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NMS	Nitrate Mineral Salts medium
OD	Optical density
PAR	Photosynthetically active range
PCA	Principal component analysis
PCR	Polymerase chain reaction
PHB	Poly-hydroxy-butyrate
PHBV	Poly-hydroxy-butyrate/valerate (mixture)
PFP	Primary facultative pond
pMMO	Particulate methane monooxygenase
ppb	Parts per billion
ppm	Parts per million
PSI/II	Photosystem I/II
PUFA	Polyunsaturated fatty acid
R	Richness
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
RuBisCo	Ribulose-1,5-biphosphate carboxylase oxygenase
RuBP	Ribulose biphosphate
RuMP	Ribulose monophosphate
sCOD	Soluble chemical oxygen demand
sMMO	Soluble methane monooxygenase
SBR	Sequence batch reactor
SCFA	Short chain fatty acids

SCP	Single cell protein
SIP	Stable isotope probing
SRT	Sludge retention time
TC	Total carbon
TCE	Trichloroethylene
TIC	Total inorganic carbon
TOC	Total organic carbon
TSA	Tryptic soy agar
TSS	Total suspended solids
VSS	Volatile suspended solids
WSP	Wastewater stabilization pond
WWTP	Wastewater treatment plant

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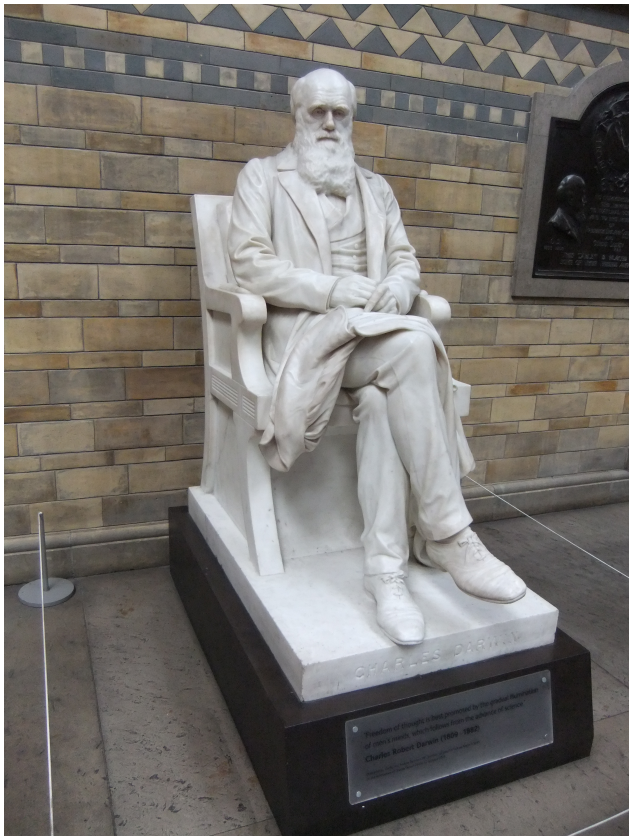
CHAPTER I

INTRODUCTION



Charles Darwin, Natural History Museum, London

hero [ˈhɪərəʊ]

1. a man/woman distinguished by exceptional courage, nobility, fortitude, etc.

2. a man/woman who is idealized for possessing superior qualities in any field

3. the principal character in a novel, play, etc.

4. he/she who can shake up my world, just for a second

Chapter I. Introduction

I.1 Global warming

I.1.1 The greenhouse effect

Life on Earth as we know it is only possible due to the influx of solar energy. Without an atmosphere, half of the incoming solar radiation would reflect on the earth surface and subsequently be send back to outer space. However, thanks to the specific atmospheric architecture, Earth's surface is covered by a heat blanket (Kandel and Viollier, 2005); in the troposphere, molecules of so-called greenhouse gases (GHG) are present, with the ability to absorb reflected heat, i.e. the long wave radiation within the thermal infrared range. As a result, this heat is reradiated to the lower troposphere, causing - in addition to cloud formation - a temperature increase at the surface of the earth (Casper, 2009). Without this radiative forcing effect, the surface of the earth would be on average 33°C colder and inhabitable for most life forms (Le Treut et al., 2007). Water vapor is the most abundant and contributing GHG in the atmosphere, responsible for about 50% of the greenhouse effect, in addition to 25% attributed to clouds (Schmidt et al., 2010). However, a majority of scientists believes that human activity does not significantly affect water vapor concentrations, and therefore it is not perceived as a GHG *sensu strictu*; an assertion that will also be used in this dissertation. Still, increasing water vapor concentrations can result in positive feedback effects. For example, when the temperature rises, more ice will melt, leading to more water vapor and a faster temperature increase (Held and Soden, 2000).

I.1.2 Global warming

The main idea behind the global warming concept is that the rise in temperature since the late 19^{th} century is mainly caused by human activity, as since the industrial revolution enormous amounts of anthropogenic GHG have been emitted to the atmosphere (Crowley, 2000). The concept of global warming gained momentum in the seventies as correlations were observed between the amount of emitted anthropogenic GHG and the observed increase of temperature (Broecker, 1975). Quickly, CO_2 was identified as one of the major

culprits, with 375 billion tons of CO_2-C emitted since 1750, the year typically chosen as reference before the industrial revolution (Ballantyne et al., 2012). During this period, the increase of the global temperature was significantly steeper than during the whole prior millennium (Ballantyne et al., 2012, Broecker, 1975). Over the years, more advanced models have been developed which were able to assign a large part of this increased heating to anthropogenic GHG. Since the ban on chlorofluorocarbons (CFC), the largest contributors to the global warming effect are, in order of magnitude, carbon dioxide (CO_2), methane (CH_4), dinitrogenoxide (N_2O) and ozone (O_3) (Forster et al., 2007, Milich, 1999). The atmospheric concentrations of these GHG indeed increased over the last decennia, as models show that the CO_2 concentration in the atmosphere increased between 1750 and 2011 from 280 to 391 ppm (Figure I-1). The same cumulative trend is observed for N_2O (270 to 324 ppb) and CH_4 (715 to 1813 ppb) (Ballantyne et al., 2012). Estimations of CH_4 concentrations indicate that during the last 8000 centuries, CH_4 concentrations have never been as high as today (Loulergue et al., 2008, Spahni et al., 2005).

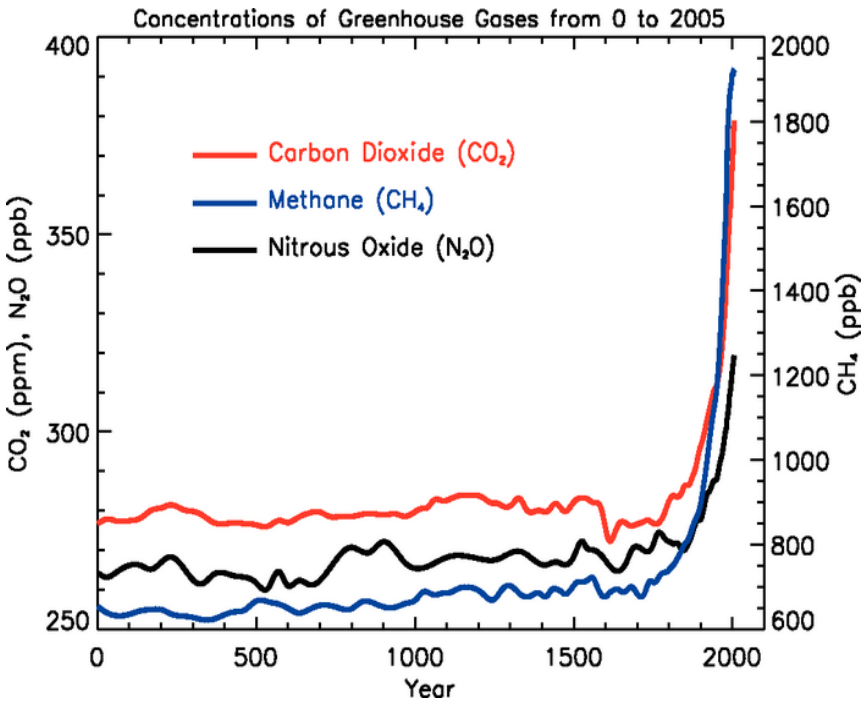


Figure I-1. Estimated trends of the atmospheric concentrations for the three most influencing greenhouse gases indicate that until the 17th century, GHG concentrations were stable. From the start of the industrial revolution on, a steep increase occurred (Forster et al., 2007).

I.1.3 Global Warming Potential

Each GHG has a different global warming effect, which depends on its radiative forcing and the residence time in the atmosphere. To compare the effectiveness of greenhouse gases, the global warming potential (GWP) index was developed, whereby the GWP of a specific greenhouse gas is compared with CO_2 and expressed as CO_2-equivalents (Shine et al., 2005). The applied GWP values are regularly modified and even contested, as the enhancing factors, feedback effects, lifetime and indirect effects are all model based estimations. The Intergovernmental Panel on Climate Change (IPCC) is generally accepted as reference institute. In its last report, the GWP was considered to be 25 for CH_4, 298 for N_2O and 10,900 for CFC-12 on a timescale of 100 years (Boucher et al., 2009, Forster et al., 2007). This index is weight based and expresses that prevention or mitigation of 1 ton CH_4 has the same effect as mitigating 25 ton of CO_2. In Table I-1, some characteristics are depicted for the three main GHG.

Table I-1. Comparison of some global warming related parameters for the three main GHG: the contribution to relative forcing of long-lived greenhouse gases, atmospheric lifetime, global warming potential (100 years), global atmospheric concentration in 2011, relative abundance compared to the beginning of the industrial revolution and the relative increase of the atmospheric concentration in 2011 (Ballantyne et al., 2012, Forster et al., 2007, WMO, 2012).

	CO_2	**CH_4**	**N_2O**
Contribution to radiative forcing	64%	18%	6%
Atmospheric lifetime	30-95 y	12±3 y	114±18 y
Global warming potential	1	25	298
Global atmospheric concentration	390.9±0.1 ppm	1813±2 ppb	342.2±0.1 ppb
Abundance relative to 1750	140%	259%	120%
Relative increase during 2011	0.51%	0.28%	0.31%

I.1.4 Effects and awareness

Recent models predict a further increase of the global temperature of at least 0.1°C per upcoming decennia if the emission patterns of GHG do not change (Betts et al., 2011, Roeckner et al., 2011, Sanderson et al., 2011). This could lead to numerous unwanted effects and a higher risk for calamities: melting of ice caps and glaciers, more intense periods of drought, shifts of climate belts, insecure food production in fragile areas, etc. (IPCC, 2007a). Moreover, indirect effects could lead to an accelerating pattern. The melting of polar ice could cause extra heat absorption, while tons of sequestered CH_4 could seep out of melting permafrost (Isaksen et al., 2011).

Although not all climatologists are yet convinced that the current global warming is caused by anthropogenic sources, politicians and scientists in most parts of the world are taking initiative to lower the anthropogenic GHG emissions. An important milestone was the Kyoto protocol, which was signed on December 11th of 1997 by 184 countries (UNFCCC, 1998). The involved countries committed to lower their greenhouse gas emission by the year 2012 with on average 5% compared to 1990. This agreement also led to the creation of carbon markets since 2007, whereby countries with a surplus of emission rights can sell these at market price to countries who were not able to sufficiently reduce their emissions (Kossoy and Ambrosi, 2010). All these initiatives should limit further global warming to less than 2°C in 2100, compared to pre-industrial values. However, without more efforts from important emitting countries like the USA and China, this goal will not be easily reached.

I.2 The carbon cycle

I.2.1 Sources and sinks of CO_2

Carbon is the central element in all organic compounds, and therefore the backbone of the earth's life forms. Plants and autotrophic bacteria are categorized as primary producers as they are able to produce organic compounds by reducing inorganic CO_2, thereby creating an important sink of CO_2. Most other life forms depend strongly on these

primary producers: Animals and humans include plant material in their diet, as they are not able to produce some essential compounds themselves. Also heterotrophic bacteria depend on these compounds for their carbon and energy needs (Malhi et al., 2002).

Respiration and photosynthesis are among the most important biochemical pathways whereby carbon-containing compounds are transformed. Respiration is the controlled oxidation of energy-rich organic compounds such as sugars, fats and proteins by means of enzymes (Madigan et al., 2012). All aerobic organisms – plants, animals, bacteria and archaea – are CO_2 sources as they all apply this transformation during their catabolic activities. The following reaction represents respiration, whereby glucose ($C_6H_{12}O_6$) is used as model organic compound (Thauer et al., 1977):

$C_6H_{12}O_6 + 6\ O_2 \Rightarrow 6\ CO_2 + 6\ H_2O$ $\qquad\qquad$ $\Delta\ G° = -478\ kJ\ mol^{-1}\ CO_2$

Photosynthesis is the reverse reaction, whereby light energy is used for CO_2 fixation, resulting into the production of energy-rich carbon compounds. Overall, this is a transformation of inorganic carbon into organic carbon and therefore trees, grasses and algae, all performing photosynthesis, form an import carbon sink. Forests for example are responsible for about 60 Gton CO_2-C y^{-1} sequestration worldwide (Malhi et al., 2002), while grasslands have, despite the respiratory processes taking place, a net sequestration rate of 104±73 g CO_2-C m^{-2} y^{-1}, taking care of about 40% of global CO_2 sequestration worldwide (Soussana et al., 2007). Other important CO_2 sinks have an abiotic nature. While oceans are CO_2 sources due to respiratory activity and seeps, they also act as a sink by absorbing and buffering large amounts of CO_2, present in the atmosphere (Ridgwell and Zeebe, 2005). Overall, the net absorption rate of oceans is currently 2.0±1.0 Gton CO_2-C y^{-1} (Takahashi et al., 2009). CO_2 is also removed from the atmosphere through weathering by silicate rocks and by burial in marine sediments, both are however slow processes (Reay and Grace, 2007). The rest of the produced CO_2 ends up in the atmosphere, enhancing the greenhouse effect.

I.2.2 Sources and sinks of CH_4

An important part of the carbon cycle takes place under anaerobic or anoxic conditions, whereby O_2 cannot serve as electron acceptor and only a partial oxidation is possible. Fermentation for example takes place when organic matter is decomposed in the absence of O_2 or other electron acceptors like nitrate, sulphate or ferric iron. Hereby, the substrate acts both as electron donor and acceptor and is transformed in fermentation products and CO_2 (Thauer et al., 1977). Anaerobic digestion on the other hand is a series of processes in which hydrolytic-fermentative organisms, acetate oxidizing organisms and methanogenic archaea work together to break down biodegradable material in the absence of O_2, finally resulting in the production of energy-rich biogas (Gujer and Zehnder, 1983). The latter typically consists of 40-70% (v/v) CH_4, 30-60% (v/v) CO_2 and 1-5% other gases like NH_3, H_2S and H_2 (Demirel et al., 2010, Long, 2000). These microbial processes, which are now also industrially applied for waste management, are the most important CH_4 source worldwide. Although the identity of the major global CH_4 sources is known, an estimation of their specific CH_4 emission is difficult (Figure I-2). About 69% of CH_4 emission is thought to result from microbial processes, next to 25% from purely anthropogenic activities like mining, and combustion, while 6% could result out of poorly understood chemical CH_4 producing processes in plant material (Conrad, 2009, Hsu et al., 2010, Vigano et al., 2008). Over the years, many natural CH_4 sources have been managed by humans for their benefit: the enormous growth of the cattle population is now responsible for ± 15% of the worldwide CH_4 production, as 15% of the feed intake ends up as natural gas after ruminant digestion (Kumar et al., 2009). Wetlands are the most important natural sources of CH_4, estimated to account for 70% of natural CH_4 emissions, but this number includes the large areas of man-made flooded rice fields (Anderson et al., 2010, Whalen, 2005). Entirely natural sources comprise all kinds of water bodies, gas hydrates in oceans and fermentation processes in termites (IPCC, 2007b, Milich, 1999). Conversely, anthropogenic sources include landfills, wastewater treatment plants and gas installations (Figure I-2) (IPCC, 2007b, Lechtenbohmer et al., 2007).

Man-made or managed systems are responsible for an increasing global emission of CH_4, currently in the order of 500-600 Mton CH_4 y^{-1} (Cakir and Stenstrom, 2005, Wang et al., 2004). However, CH_4 is less stable in the atmosphere than CO_2, as different chemical mechanisms assure that CH_4 molecules do not accumulate indefinitely in the atmosphere. Consequently, the estimated lifetime of CH_4 is only 8 to 12 years (Lelieveld et al., 1998, Lucas et al., 2007). The largest CH_4 sink is the photo-oxidative reaction of hydroxyl radicals, responsible for 85-90% of the current CH_4 removal (Huijnen et al., 2010, Letexier et al., 1988).

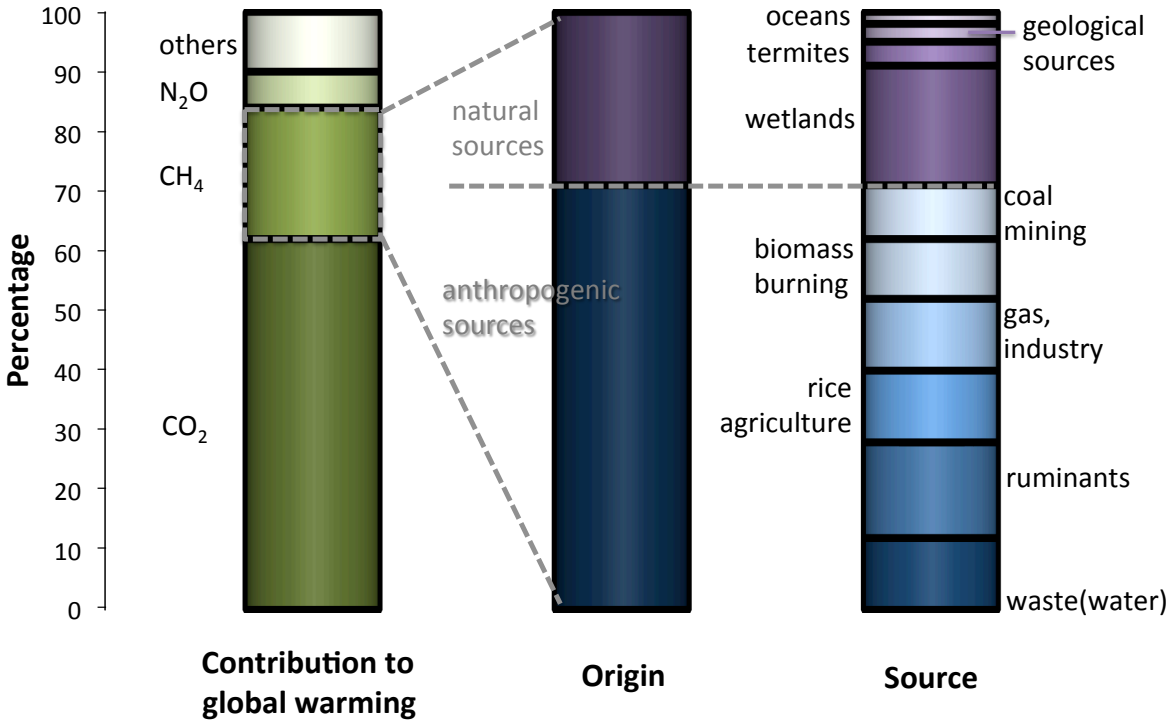


Figure I-2. Schematic overview of the contribution of the most important GHG to the increase in global warming (left), the ratio of natural and influenced CH_4 sources (middle) and an overview of the most important CH_4 sources (right). All percentages are estimations, based on data from Barker et al. (2007) and the USEPA (2008).

Conversely, the second most important CH_4 sink has a microbiological origin and concerns the methanotrophs, responsible for about 5-15% of CH_4 removal (Wahlen, 1993, Yoon et al., 2009). The methanotrophic activity in soils alone accounts for a removal of 30 Mton

CH_4 y^{-1} (Allan et al., 2005, Aronson and Helliker, 2010). It is hereby important to realize that a decrease of the current CH_4 emissions with only 6% (22 Mton CH_4 y^{-1}) would suffice to balance the global CH_4 budget (Jardine et al., 2004).

I.3 Methanotrophy

I.3.1 Anaerobic methane oxidation

Methane is a relatively stable molecule from a chemical point of view. It has a symmetrical structure, making it difficult to attack the molecule. Moreover, the four C-H bonds are notoriously unreactive as is illustrated by the fact that the removal of the first hydrogen atom in CH_4 demands 435 kJ mol^{-1} (Dalton, 2005). Hence, controlled chemical oxidation technologies are based on specific catalysts and elevated temperatures (Muradov, 2001). However, as microorganisms have billions of years of experience with CH_4, specific groups of bacteria and archaea have found ways to oxidize this molecule, in order to fulfill their carbon and energy needs (Hanson and Hanson, 1996, Knittel and Boetius, 2009).

A first group of methanotrophs is known as anaerobic methane oxidizing archaea in deep-sea environments. Although there is a large knowledge gap, it seems that they form a symbiotic consortium with sulphate reducing bacteria, oxidizing one mol CH_4 mol^{-1} reduced SO_4^{2-} (reaction (a) in Figure I-3) (Valentine, 2002). This yields only a small amount of 20 kJ mol^{-1} CH_4 Gibbs free energy, which is just enough to sustain activity (Thauer, 2011).

$$CH_4 + SO_4^{2-} \Rightarrow HCO_3^- + HS^- + H_2O \qquad \Delta G° = -20 \text{ kJ mol}^{-1} CH_4$$

Three phylogenetically different archeal groups have been identified, labeled as ANME-I, ANME-II and ANME-III (Garrett and Klenk, 2007). However, the biochemical mechanism of the enzymatic oxidation reaction is still unclear and none of the archaea has been isolated (Girguis et al., 2005, Nauhaus et al., 2007). These bacterial-archaeal communities form a major CH_4 sink, as they oxidize about 90% of CH_4 of oceanic origin before it reaches the surface (Knittel and Boetius, 2009). There are also indications that these consortia are present in sediments of marine, freshwater and soda lake environments (Nauhaus et al., 2007, Thauer and Shima, 2006). Very recently, it has been stated that anaerobic methane

oxidation (AOM) is not an obligate syntrophic process but is carried out by the archaea alone, resulting in a novel pathway (reaction (d) in Figure I-3) (Milucka et al., 2012).

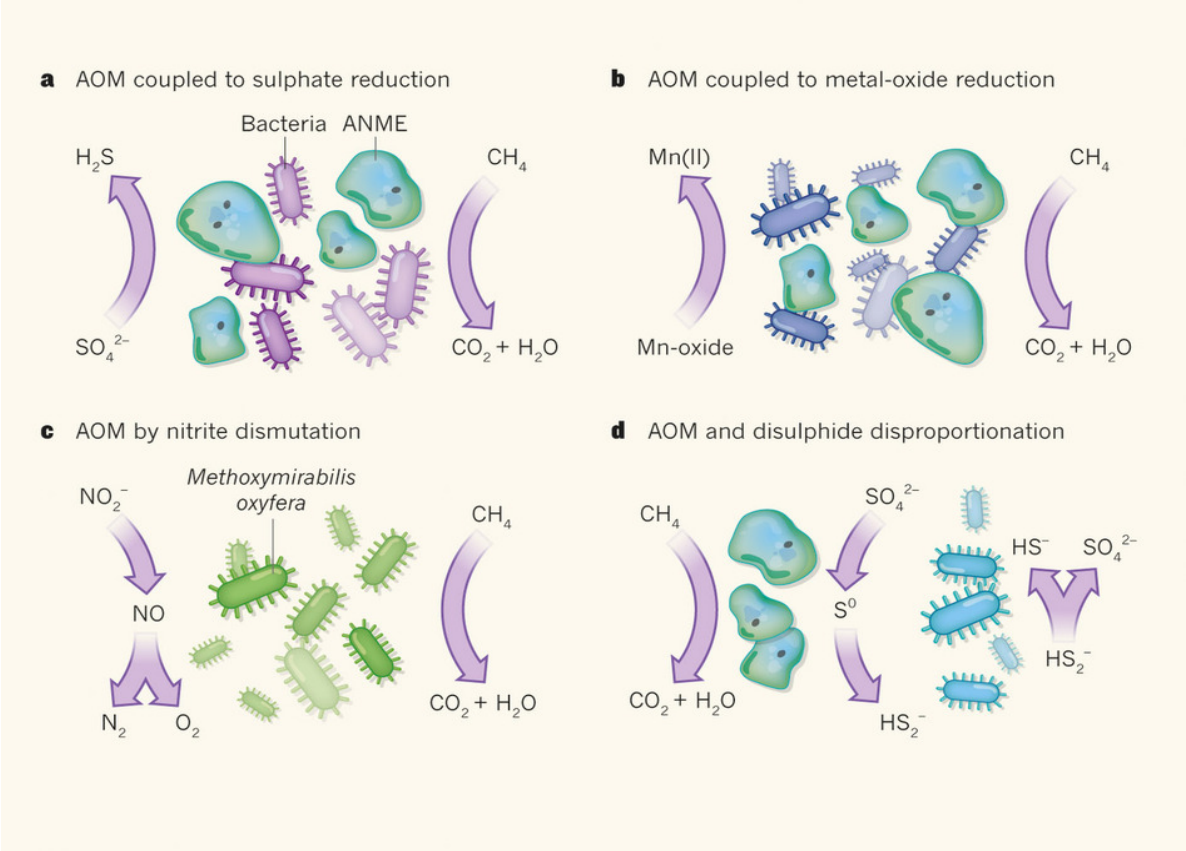


Figure I-3. Four pathways are known whereby microorganisms achieve anaerobic methane oxidation (AOM): (a) and (b) rely on obligate associations between two or more microbial partners, of which one performs oxidation and the other reduction. In (c) and (d), a single microorganisms performs both simultaneously. In (c), a bacterium is responsible for the methane oxidation, while in (d) this is an archaeon (Joye, 2012).

Recently, the first bacterial phylum was found with members able to oxidize CH_4 under anaerobic conditions. The *Candidatus Methylomirabilis oxyfera* of the *NC10* phylum couples CH_4 oxidation to NO_2^- reduction, with NO-radicals as central intermediates (Ettwig et al., 2010, Wu et al., 2011). Currently, more research is done on this intra-aerobic denitrification reaction ((c) in Figure I-3) (Ettwig et al., 2012):

$$3\ CH_4 + 8\ NO_2^- + 8\ H^+ \Rightarrow 3\ CO_2 + 4\ N_2 + 10\ H_2O \qquad \Delta G^\circ = -928\ kJ\ mol^{-1}\ CH_4$$

I.3.2 Aerobic methane oxidation

While anaerobic methane oxidation (AMO) is a relatively new finding, aerobic methane oxidation is known since 1906, when the Dutch microbiologist, N.L. Söhngen reported a bacterium capable of growth on CH_4 (Dalton, 2005). A real turning point came in 1970, when Whittenbury et al. (1970b) reported that they were able to isolate methane oxidizing bacteria under aerobic conditions without the addition of an organic C source. Indeed, except for the bacteria and archaea mentioned in the preceding paragraphs, all known methane oxidizing species are known to be facultative or obligate aerobic, applying O_2 as final electron acceptor. The Gram-negative alpha- and gamma-proteobacteria are by far the best known group of aerobic methanotrophic bacteria, and form the main subject of this dissertation. However, during the last years, members of novel phyla were described which are able to oxidize CH_4 (Khadem et al., 2011, Op den Camp et al., 2009). Methane oxidizing Verrucomicrobia have been found, thriving under acidophilic conditions, with optimal growth in a pH range of 1.0-3.5, a range wherein no alpha- and gamma-proteobacterium is known to be active (Islam et al., 2008, Pol et al., 2007). These species apply unknown pathways and are expected to play a major role in mitigating CH_4 emissions of peat lands and permafrost soils (Dedysh, 2009). Some sheathed gamma-proteobacterial species like *Clonothrix fusca* and *Crenotrix polyspora* were also added to the list of methanotrophs, but have never been isolated. All these new discoveries show that methanotrophy is taxonomically and ecologically more widespread than thought, and with the introduction of novel molecular techniques, it can be expected that more phyla are waiting to be discovered.

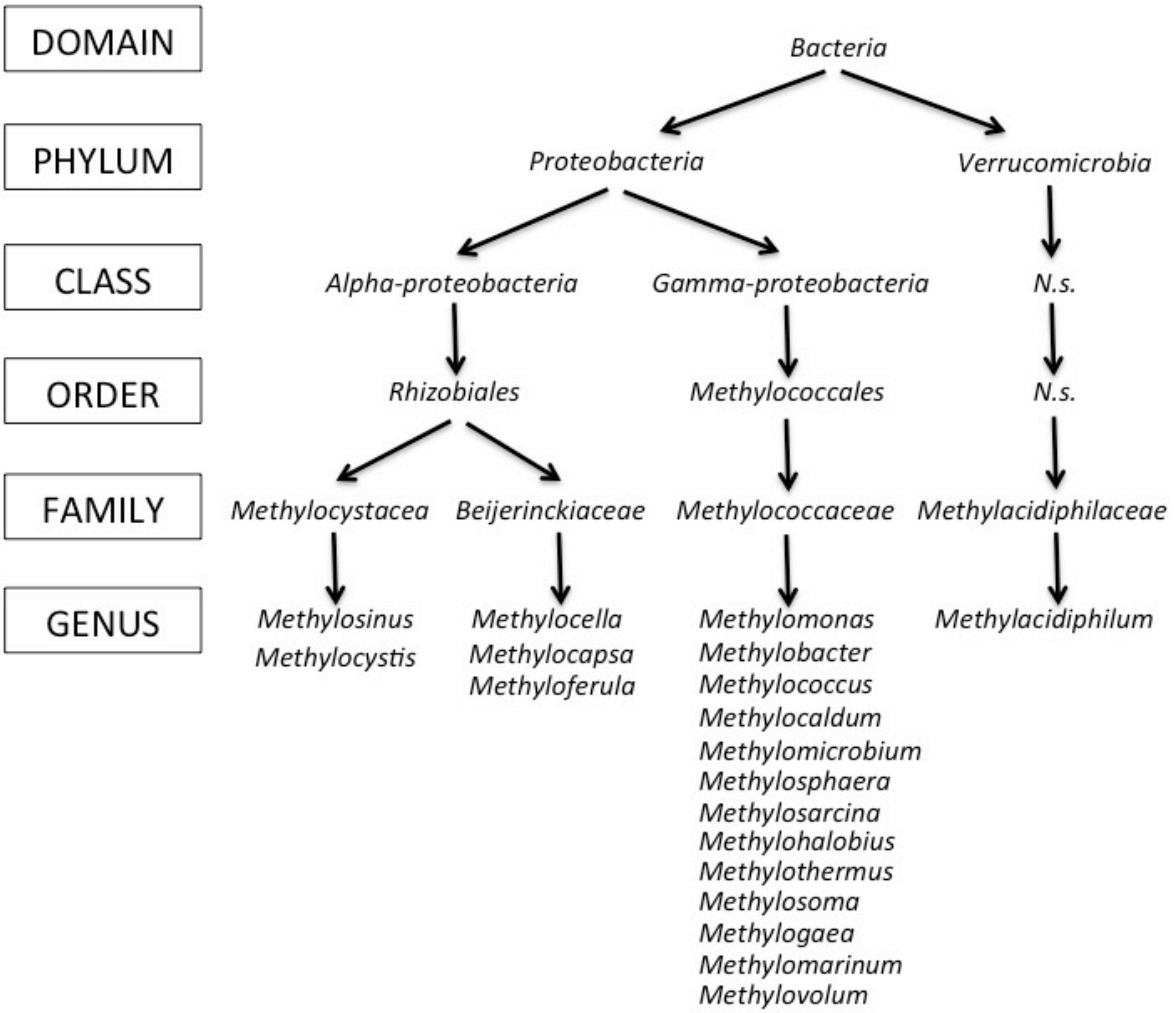
I.4 Methane oxidizing bacteria

The by far best described methanotrophic bacterial group comprises the alpha- and gamma-proteobacterial methane oxidizing bacteria (Hanson and Hanson, 1996). It was this group of methanotrophs that was under investigation in this dissertation. For clarity, they will be described from now on as methane oxidizing bacteria (MOB), while the larger group of organisms able to oxidize CH_4 will be annotated as methanotrophs.

I.4.1 Taxonomy

A good overview of the different milestones during the study of MOB can be found in a review by Trotsenko and Murell (2008). Up to a few years ago, the MOB were subdivided into three major groups, i.e. type I, type II and type X, based on taxonomic, physiological and biochemical characteristics (Hanson and Hanson, 1996). However, modern techniques like DNA sequencing have indicated that the subdivision into those three groups was not really accurate. From a taxonomical point of view, 18 genera can be currently described, belonging to three families. The five known type II MOB genera are now listed in the alpha-proteobacterial *Methylocystaceae* and *Beijerinkiaeae* families, while the 13 known gamma-proteobacterial type I and former type X genera belong to the family of the *Methylococcaceae* (Figure I-4) (Dedysh, 2009, Jiang et al., 2010).

Although many exceptions have been encountered, the subdivision between type I (gamma-proteobacterial) and type II (alpha-proteobacterial) is still useful to describe and understand the behavior of the different species. Differences are present in their carbon assimilation pathways, cellular structures, fatty acid composition, CH_4 affinity, copper dependence and N_2 fixation capacity, described in more detail later on (Bowman et al., 1993b, Dedysh, 2009, Hanson and Hanson, 1996, Wendlandt et al., 2010). As powerful high-throughput sequencing analyses find their way in microbiology, more knowledge is gained about the origin of MOB. Phylogenic 16S rRNA sequence trees show that type I MOB are quite closely related to beta-proteobacterial AOB, while type II MOB are already more distinct. Much more distinct are the *Verrucomicrobia* and *NC10* phylum, respectively (Figure I-5) (Vlaeminck et al., 2011).



. List of taxonomically described methanotrophic bacteria. Type II species

, while type I and X MOB belong to the

form a separate phylum. N.s. means not

,

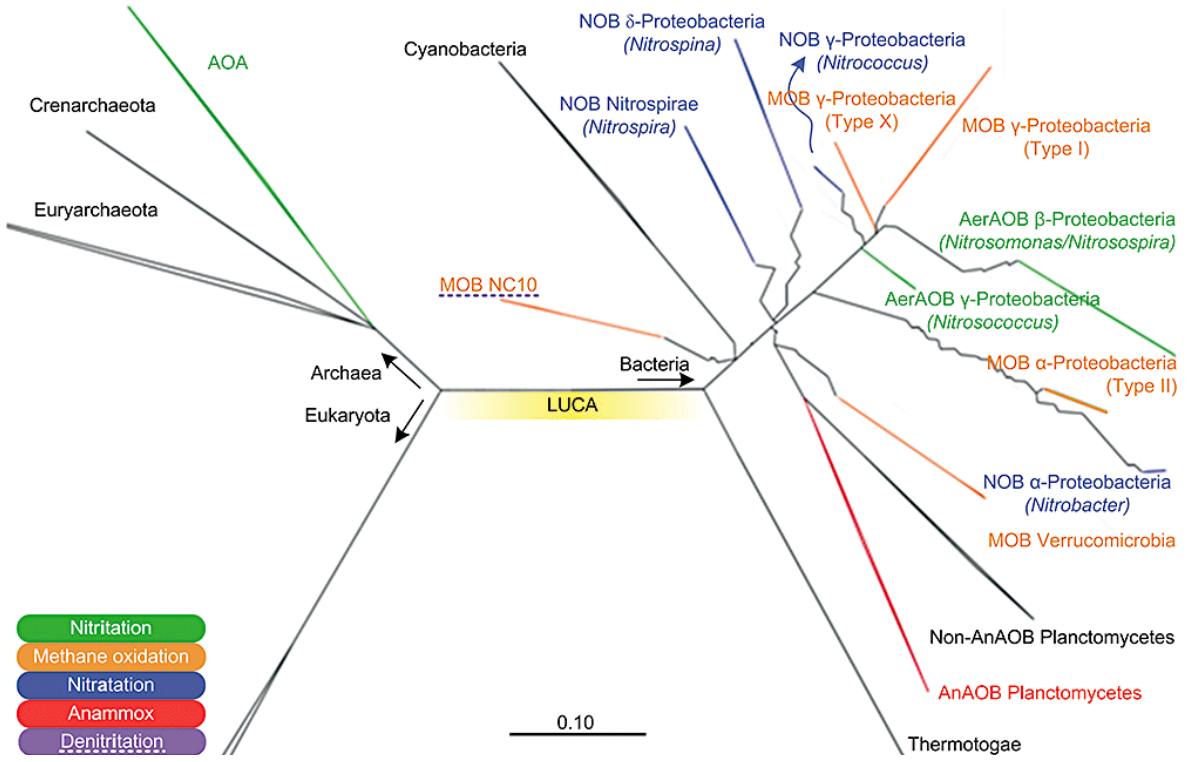
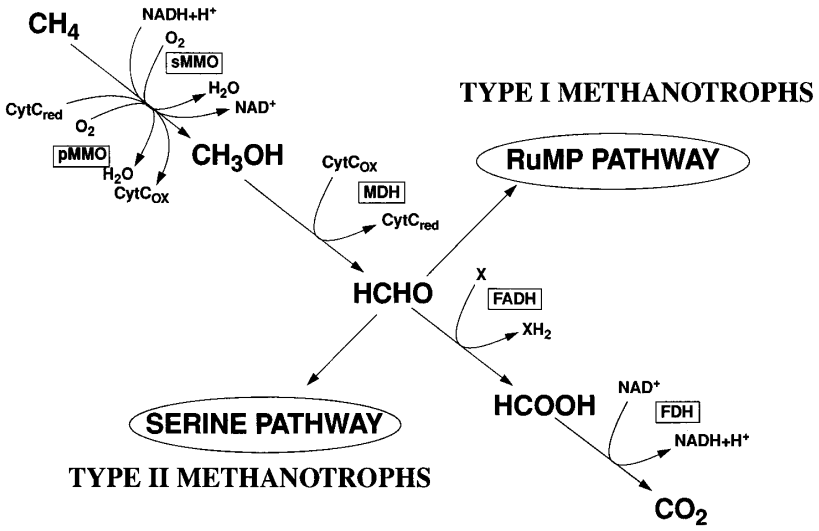


Figure I-5. Phylogenetic 16S rRNA sequence tree of life, displaying different important taxonomic groups in the N and C cycle in relation to the last universal common ancestor (LUCA). While type I and type II are relatively close, members from the *Verrucomicrobia* or the *NC10* phylum are already more distinct. 'The all-species living tree' project release LTP 100 (September 2009) was used as a backbone for the most up-to-date topology for the bacterial and archaeal domain (Vlaeminck et al., 2011).

I.4.2 Physiology and biochemistry

The aerobic methane oxidation process exists out of a sequel of oxidation steps, comparable to the principles of cytochrome P450, catalyst of the oxidation of organic molecules in most living organisms. Thanks to the enzyme methane monooxygenase (MMO), MOB can perform the difficult first and rate limiting oxidation step. This enzyme allows to split the O-O bond of O_2 into two reducing equivalents (Dalton, 2005, Yoon et al., 2009). While one oxygen atom is reduced to H_2O, the other atom is incorporated into CH_4 to form methanol (CH_3OH) (Tinberg and Lippard, 2011). The latter is then oxidized by periplasmic methanol dehydrogenase into formaldehyde (HCHO), an important

intermediate as it can be used for both catabolic as anabolic processes. It can be further oxidized to formate (HCOOH) and finally CO_2, hereby transforming CH_4 to its most oxidized state. A cascade of enzyme systems is hereby activated, including nicotinamide adenine dinucleotide (phosphate) or NAD(P) and NAD-dependant formate dehydrogenases (Figure I-6) (Hanson and Hanson, 1996).



Murrell, 2008, Ward et al., 2004). The stoichiometric reactions of the RuMP and serine pathway, respectively are as follows (Trotsenko and Murrell, 2008):

RuMP: $CH_4 + 1.50\ O_2 + 0.12\ NH_4^+ \rightarrow 0.12\ (C_4H_8O_2N) + 0.53\ CO_2 + 1.71\ H_2O + 0.12\ H^+$

Serine: $CH_4 + 1.57\ O_2 + 0.10\ NH_4^+ \rightarrow 0.10\ (C_4H_8O_2N) + 0.59\ CO_2 + 1.74\ H_2O + 0.10\ H^+$

Higher growth yields can be achieved by means of the RuMP pathway, as only 1 mol ATP per mol of C3 intermediate is needed, where the serine pathway consumes 3 mol ATP (Hanson and Hanson, 1996). MOB can regulate partially which amount of HCHO goes towards anabolic and catabolic processes, thereby influencing the O_2 consumption: while the complete oxidation of CH_4 to CO_2 requires 2 mol O_2 mol^{-1} oxidized CH_4, only 1 mol O_2 is required when formaldehyde is built in as biomass (Scheutz et al., 2009a).

I.4.3 The monooxygenase couple

MOB are actually a subgroup of the methylotrophs, defined as those bacteria that have the ability to use C1 compounds like CH_3OH as carbon and energy source (Madigan et al., 2012). Many MOB like *Methylosinus trichosporium* are able to grow on methanol as sole C source (4% v/v). For other MOB, even low concentrations of 0.01% (v/v) CH_3OH can cause a toxic effect (Best and Higgins, 1981, Malashenko et al., 2000). MOB distinguish themselves from the other methylotrophs by possessing the enzyme MMO. Two distinctively different types are known, both resulting in the same overall reaction. The first and most common type is particulate methane monooxygenase (pMMO), a membrane associated enzyme that has not been completely described yet, as isolation is difficult and the molecule extremely unstable (Kitmitto et al., 2005, Semrau et al., 2010). Active pMMO seems to include two Fe atoms and approximately 15 Cu atoms per molecule (Lieberman and Rosenzweig, 2004, Trotsenko and Murrell, 2008). Oxidation of CH_4 by pMMO takes place with reduced cytochrome C as electron donor. Also a small chromopeptide, called methanobactin, is important as it acts as a Cu shuttle, involved in the uptake and binding of Cu and possibly other metals (Choi et al., 2008, Kenney and Rosenzweig, 2012). Recently, two different *pmoA* genes were identified as being responsible for pMMO expression: the conventional *pmoA* and the novel *pmoA2*, which

seems to be essential for methane oxidation under atmospheric CH_4 concentrations (Kravchenko et al., 2010). Most MOB express pMMO, with some *Methylocella* and *Methyloferula* species as rare exceptions (Vorobev et al., 2011).

Soluble methane monooxygenase (sMMO) is the other known MMO. Expression of sMMO is not as widespread as of pMMO and seems more common in type II and type Ib MOB than in type Ia MOB (Semrau et al., 2010). In contrast to pMMO, it is not membrane bound but soluble in the cytoplasm (Semrau et al., 2010). Due to the Fe molecules in its center, Fe^{2+} availability is important, while at the same type Cu^{2+} can inhibit the enzyme. Moreover, the protein components and gene sequences differ significantly from pMMO (Martinho et al., 2007). The oxidation of CH_4 also differs, as sMMO applies a NAD(P)H- and O_2-coupled conversion of CH_4 to CH_3OH (Trotsenko and Murrell, 2008):

$CH_4 + NAD(P)H + H^+ + O_2 => CH_3OH + NAD(P)+ H_2O$

The expression of sMMO and pMMO is highly regulated by the Cu^{2+} concentration, with inhibition of sMMO often occurring at concentrations above 0.08-0.8 µM Cu^{2+} (Green et al., 1985, Tsien et al., 1989). At the same time, concentrations lower than 1 µM Cu^{2+} dramatically lower pMMO expression (Trotsenko and Murrell, 2008, Wendlandt et al., 2010). At such low concentrations, only those MOB that are able to express sMMO can sustain their metabolism. Due to the different nature of sMMO and pMMO, this also leads to different growth characteristics of the methanotrophic community. sMMO expressing MOB have a 35% lower growth yield, as NAD(P)H has a lower redox potential than the electron donor of pMMO, i.e. cytochrome C (Dalton, 2005). Moreover, sMMO expresses a lower affinity for CH_4 (Km = 3 µM vs 1-2 µM) and O_2 (K_m of 17 µM vs 0.1 µM) in comparison to pMMO (Trotsenko and Murrell, 2008). Therefore, pMMO has an advantage for MOB active at low O_2 or CH_4 concentrations. On the other hand, sMMO shows a higher robustness against inhibitors like chelating agents and cyanide, thus creating an advantage in some polluted environments (Yu et al., 2009).

I.4.4 Living on the edge

Two different adaptations by MOB are also worth mentioning, i.e. methanotrophy under atmospheric conditions and facultative behavior, respectively.

The atmospheric CH_4 concentration is currently on average 1.8 ppmv (WMO, 2012). Hence, at atmospheric pressure, only about 32 ng CH_4 is dissolved in one liter of water, making CH_4 a growth-limiting element for MOB. Therefore, MOB living at such low concentrations demand enzymes with a very high affinity for CH_4 (Conrad, 2009). Although not much is unraveled yet, such methanotrophs have been found. The strain *Methylocystis sp.* SC2 is one of the few high-affinity MOB so far isolated (Dunfield et al., 1999, Kolb, 2009). A special type of pMMO seems required to grow on low CH_4 concentrations of 1-100 ppmv, as typical pMMO is only active at higher CH_4 concentrations (>600 ppmv) (Baani and Liesack, 2008).

One of the strategies to survive periods with a low or no CH_4 provision is facultative methanotrophy. For a long time it was assumed that all methanotrophic bacteria were obligate methylotrophic, i.e. could only utilize C1 compounds. Recently however, type II MOB of the genera *Methylocystis, Methylocapsa and Methylocella* have been found to grow on CH_4 as well as alternative C sources (Dedysh et al., 2005, Dunfield et al., 2010). Some of them even showed a higher growth rate and yield when cultivated with organic C sources like acetate, pyruvate, succinate, malate, citrate, ethanol and methylamine instead of CH_4 (Belova et al., 2011, Dunfield et al., 2010, Im et al., 2011, Semrau et al., 2011). Moreover, both sMMO and pMMO were found in some of these strains. A lot of research is currently done on these strains, as they have a large biotechnological potential.

I.4.5 Gas dependency

The activity and community composition of MOB is influenced by many factors such as temperature, pH, moisture content, N and P source, copper/iron availability and the availability of CH_4 and O_2. Because of the gaseous character of both the C source, electron

donor and electron acceptor, gas phase transfer and composition are particularly important towards the activity and ecology of methanotrophic communities.

MOB have a broad optimal O_2 range, as changes between 0.45 and 20% (v/v) only lead to minor differences in methane oxidizing activity (Wilshusen et al., 2004a). Although not much research has been performed with O_2 concentrations above the atmospheric O_2 concentration (21% (v/v)), oxidative stress due to the accumulation of reactive O_2 radicals can occur when O_2 concentrations are high, especially when a large amount of light energy is present (Medvedkova et al., 2009).

The CH_4 range wherein CH_4 oxidation has been observed is even broader. Although most MOB show optimal activity between 5 and 50% (v/v), so called high affinity MOB have been found that can survive on concentrations as low as 1-100 ppmv, as was mentioned before (Dunfield et al., 1999, Knief and Dunfield, 2005, Kolb, 2009). As the activity and growth of MOB is often limited by the availability of the relatively poorly soluble O_2 and CH_4, good gas mixing should always be provided.

The concentrations of both mentioned gases also influence the community structure, as it has been observed that type I MOB prevail under CH_4 concentrations lower than 1% (v/v), when atmospheric O_2 concentrations occur. Conversely, type II MOB seem to have an advantage when higher CH_4 concentrations are present (Hanson and Hanson, 1996, Nyerges et al., 2010, Semrau et al., 2010). In tests with agar diffusion columns and counter gradients of CH_4 and O_2, bands of type I and type II MOB were present at different heights, depending on the gradient of both gases (Amaral and Knowles, 1995). In natural samples, this distribution is however not that sharp, and often both type I and type II are found together (Knief et al., 2006).

I.4.6 Growth factors

Typically, increasing temperatures result in a higher enzymatic activity. However, in case of MOB, this effect is partly countered as a higher temperature leads to a lower mass transfer of CH_4 and O_2 towards the water phase. Therefore, the Q_{10} value, indicating how

much the activity rate increases when the temperature rises with 10°C, has a relatively low value between 1.7 and 4.1 (Börjesson et al., 2004, Urmann et al., 2009). Most MOB are mesophilic with an optimum between 25°C and 35°C. However, extremophiles, almost all type I, are known living at temperatures as low as -5°C or as high as 72°C (Jiang et al., 2010, Khmelenina et al., 2007, Mohanty et al., 2007, Urmann et al., 2009). The optimal pH range for MOB is often between 6 and 7 and adaptation to fluctuating pH is common in a pH range between 5 and 8.5 (Hanson and Hanson, 1996, Melse and Van der Werf, 2005). While no MOB were found living at a pH below 3.8, extremophilic MOB are known withstanding a pH of 12 (Trotsenko and Khmelenina, 2002, Vorobev et al., 2011). The moisture content is also a steering factor for methanotrophic communities. A minimum of water is needed to maintain the cell functions. It is however of interest to limit the amount of water that the gases have to pass before reaching the bacteria, in order to increase the mass transfer rate (Melse and Van der Werf, 2005). As for all bacteria, phosphorus is an important macronutrient for MOB. A start concentration of only 1.5 mmol KH_2PO_4-P resulted in physiological stress during an incubation of a *Methylosinus trichosporium* strain (Zhang et al. (2008)). Due to the uncertainty in the needs, a wide range of concentrations and N:P ratios has been applied in literature (Nikiema et al., 2010).

A major steering factor is the availability, identity and quantity of N sources. As N is an important building block for their microbial cell structures, MOB require a rather high amount of 0.25 mol N mol^{-1} assimilated C (Anthony, 1982). Therefore, N becomes a limiting growth factor when the C:N ratio is above 10 (Bodelier and Laanbroek, 2004). The versatility of the N metabolism of MOB is higher than of most common bacterial groups (Hamer, 2010). All known MOB are able to use NH_4^+ as N source. Most MOB can also use NO_3^- as N source, hereby relying on catabolic nitrification (Modin et al., 2007, Nyerges and Stein, 2009). Although NO_2^- can also be applied, inhibiting levels are often as low as 1 mg NO_2^--N L^{-1} (Nyerges and Stein, 2009). Even N_2 fixation can occur, when no organic N source is available. This is however a highly energy-demanding process that is mainly applied by type II MOB (Hamer, 2010). Under certain conditions, MOB can be a source of NO_2^-, NO and N_2O, for example when NO_3^- is assimilated at low O_2 concentrations (Figure I-7) (Ren

et al., 2000, Stein et al., 2011). However, until now, no MOB has been found able to perform the complete denitrification pathway up to N_2 (Knowles, 2005).

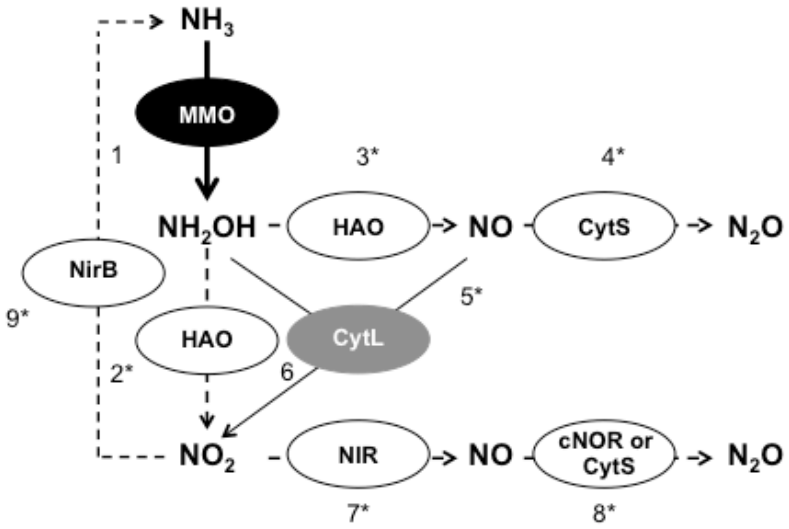


Figure I-7. Pathways of nitrification and denitrification processes identified in some gamma-proteobacterial MOB (full line, black oval) or deduced from *in vitro* biochemistry (thin full line, grey oval) and transcriptional data (dashed line, white oval). An asterix (*) indicates that the products are derived from transcript and/or cell studies without knowledge of methanotrophic enzymes. Acronymes of enzymes: HAO, NH_2OH oxidoreductase; CytS, cytochrome c0-b; CytL, cytochrome P460; NIR, NO-forming NO_2^- reductase (NirK, NirS or cytochrome c protein); cNOR, cytochrome c-dependent NO reductase; NirB, NH_3 forming siroheme NO_2^- reductase (Stein and Klotz, 2011).

Standard growth medium of MOB contains NO_3^--N or NH_4^+-N as N source. Carefulness is however needed when NH_4^+-N is applied. Although it can enhance the methane oxidizing activity when the N content is limiting, inhibiting effects have been observed, even at low concentrations of 25 mg NH_4^+-N L^{-1} (Begonja and Hrsak, 2001). In certain soils, an addition of 40 mg NH_4^+-N kg^{-1} soil resulted in a 96% reduction of the methane oxidation activity. Even after all the NH_4^+-N was nitrified, MOB activity was still 21% lower than before the treatment (Hutsch, 1998). Hereby, the prevailing pH is of importance, as NH_3 exerts a higher toxicity towards MOB than NH_4^+ (O'Neill and Wilkinson, 1977). The reasons for these inhibiting effects are not completely unraveled, but a good overview of current knowledge is given by Nyerges and Stein (2009).

I.4.7 Cultivation conditions and obstacles

As MOB can use CH_4 as their sole C source, a minimal mineral medium is commonly used to grow, enrich and isolate them. After extended testing by Whittenbury et al. (1970b), (diluted) nitrate or ammonium mineral salts medium (NMS/AMS) is typically used as growth medium. The MOB are incubated at their specific optimal temperature, commonly 28° or 37°C, and enrichments commonly occur under an atmosphere of 20-50% (v/v) CH_4 in air (Dunfield et al., 2007, Hanson and Hanson, 1996). Due to the formation of acidifying CO_2, a relatively strong phosphate buffer is used. The expression of the preferred MMO is altered by adapting the Cu^{2+} concentration. Enrichment of MOB for industrial applications is however a difficult task due to (a) the limited CH_4 and O_2 solubility of ± 9 mg L^{-1} at a concentration of 20% (v/v) (Hamer, 2010), (b) the slow mass transfer (Sander, 1999), (c) the relatively low specific growth rates (μ = 0.45±0.06 d^{-1} for an MOB isolate) (Hrsak and Begonja, 2000) and (d) the relatively low maximal cell density of cultures (e.g. 3.2 g CDW L^{-1} after 140 h cultivation) (Han et al., 2009). By adding a CH_4 vector such as paraffin oil, the gas transfer of CH_4 can be increased, leading to a higher solubility. These additions shorten the lag phase of the MOB and allow a higher cell density of 14 g CDW L^{-1} after 240 h cultivation, but create post-processing issues (Han et al., 2009, Jiang et al., 2010).

I.4.8 The close siblings: ammonium oxidizing bacteria

As can be observed in Figure I-5, MOB and ammonia oxidizing bacteria (AOB) share a close ancestor. Therefore, both groups still show some similarities (Madigan et al., 2012, Vlaeminck et al., 2011). One of them is the relatively high similarity between pMMO and the ammonium monooxygenase (AMO), used by AOB to oxidize NH_3 (Bedard and Knowles, 1989, Hanson and Hanson, 1996). However, this enzyme has a low specificity towards NH_3, resulting into oxidation of other compounds like CH_4. However, this is a co-metabolic reaction, as the AOB cannot take advantage of the released energy. The affinity of AMO for CH_4 is however low and therefore only a small CH_4 sink is assumed to be formed by AOB (Bedard and Knowles, 1989, Hanson and Hanson, 1996).

I.5 Biotechnological potential of MOB

I.5.1 Methane mitigation

From a biotechnological point of view, methane mitigation is the most important application that comes to mind. Although this is often a secondary goal, installations have been developed with the sole purpose to lower the CH_4 emissions of an industrial plant or ecosystem. Indeed, many natural anaerobic zones can be found worldwide where CH_4 formation takes place. Introducing or enhancing MOB close to these environments could lower the emission of this GHG to the atmosphere. As MOB are found in a large amount of soils and water bodies, the first task is often to find out what the growth limiting nutrient or condition is and how to improve the growth conditions (Bodelier et al., 2000, Noll et al., 2008). Carbon cycles in wetlands and rice fields have been a popular scientific subject, as these ecosystems are important CH_4 sources (Boeckx and Van Cleemput, 1996, Dalal and Allen, 2008). Some successes have been achieved by adapting the management of such diffuse systems. By draining wetlands, soils and sludge for example, the latter become more permeable, allowing O_2 to penetrate into deeper layers. This does not only inhibit methanogenesis, but also creates an environment wherein MOB can become more active (Dalal and Allen, 2008, Jackel et al., 2001).

Also in agricultural soils, awareness of the role of MOB is important. Agricultural techniques such as ploughing disturb the soil's vertical profile and cause a decrease in CH_4 oxidation (Hutsch, 2001). Even more important is the role of N based fertilizers. As ammonia and nitrite can exhibit a significant inhibitory effect on MOB, applying too high doses of such fertilizers decreases the CH_4 oxidizing capacity of such soils dramatically (Bodelier and Laanbroek, 2004, De Visscher and Van Cleemput, 2003). Therefore better agricultural practices should be implemented, whereby the type and dose of fertilizers and possible inhibitory products are better managed. At the same time, applied physical disturbances should be avoided as much as possible (Ho et al., 2011, Menyailo et al., 2008, Seghers et al., 2005).

Due to the diffuse type of previous emissions, engineered solutions are not straightforward. This is different for point sources, where controlled mitigation techniques can be applied (Jiang et al., 2010). The most straightforward physico-chemical treatment is based on conventional gas turbines or internal burning motors, combined with electricity production (Maigaard et al., 2003). However, this technique can only be used for gas flows containing more than 15% (v/v) CH_4. Sometimes the CH_4 content in incompatible gas flows can be increased, for example by means of cryogenic purification or via membranes (Su et al., 2005). When the concentrations in the gas flows are lower than 3% (v/v) CH_4, the treatment cost increases drastically. Techniques like reversal flow reactors and porous burners for example are quite expensive and have issues with variable gas flows or changing compositions (Gosiewski et al., 2008, Wood and Harris, 2008). In those cases, biotechnological approaches are often the best and only solution.

Most success has been booked on landfills, highly controlled and monitored environments producing important amounts of CH_4 (Gebert and Grongroft, 2006, Scheutz et al., 2009a). By installing a biocover on top of landfills, 95-99% of CH_4 can be remediated by means of a passive MOB biofilter (Gebert and Grongroft, 2006, Huber-Humer et al., 2008). A biofilter based on leaf compost resulted in stable CH_4 removal rates of 120 g CH_4 m^{-2} d^{-1} (Wilshusen et al., 2004b). The same approach can be used in manure pits, where natural or (semi-)artificial crusts can be created on top of the manure pit. The latter acts as a CH_4 sink, due to the methanotrophic activity (Petersen et al., 2009).

The development of a biofilter for CH_4 loaded gas treatment, as found in stables, mines and wastewater treatment plants has also received much attention in the past (Melse and Van der Werf, 2005, Nikiema et al., 2009). These biofilters often contain a packing material whereupon methanotrophic communities can attach and grow. In lab tests, an 85% reduction of the CH_4 emission has been observed (Melse and Van der Werf, 2005, Nikiema et al., 2005). However, CH_4 has a low solubility in water and no good stripping potential towards the water phase, as both the affinity for changes in salt concentration and pH are low. Therefore large reactor volumes are needed when treating larger flows of CH_4 enriched gases. Until better reactor configurations are developed for treatment of

poorly soluble gases, such bioreactors will not be easily economically feasible. Removal of 85% of the CH_4 emissions from a cow manure pit would cost for example about 25 EUR per ton CO_2-equivalents removed (Melse and Van der Werf, 2005, Yoon et al., 2009).

I.5.2 Bioremediation

The specific ability of MOB to oxidize CH_4 lies in the nature of the MMO, able to oxidize the strong C-H bonds. Like the related AMO, they are relatively unspecific (Dalton, 2005). Consequently, MOB can perform a wide range of co-metabolic processes whereby substrates are oxidized without gaining energy out of these processes. There is however a distinct difference between sMMO and pMMO in the type of substrates that can be co-metabolized. The substrate profile of pMMO is narrow and includes mostly linear hydrocarbons such as C1-C4 alkanes and alkenes (Bedard and Knowles, 1989, Jiang et al., 2010). The sMMO substrates include alkanes, alkenes, aromatic compounds, alicyclic hydrocarbons and halogenated aliphatics (Jiang et al., 2010, Sullivan et al., 1998, Yoon and Semrau, 2008). Most researchers have focused on the breakdown of chlorinated hydrocarbons, often used as solvents (Koh et al., 1993). These compounds have become one of the most widespread groundwater contaminants due to the frequent use, for example in dry cleaning processes. MOB have successfully oxidized toxic chlorinated hydrocarbons like trichloroethylene (TCE), 1,2-dichloroethane and chloroform (Hesselsoe et al., 2005, Wendlandt et al., 2010). Hereby, metabolites are often formed that are more susceptible to further biodegradation by heterotrophs (Dalton, 2005, Jiang et al., 2010, Trotsenko et al., 2005). The upscaling of these processes was however not as easy as thought. The reliability of the different processes taking place in the mixed community is insufficient for industrial applications. Moreover, a very strict monitoring of the different processes is needed (Hamer, 2010, Wendlandt et al., 2010). It is also important to bear in mind that these bioremediation processes are often tested with a small selection of model organisms. Due to cultivation problems and the microbiological fixation on monocultures, a large range of MOB has never been tested and could exhibit a higher activity and robustness under such harsh and specific conditions.

I.5.3 Production of added value compounds

The first intermediate of CH_4 oxidation is CH_3OH, a valuable product with many applications in the chemical industry, for example as alternative fuel source, antifreeze or as building block for other chemical compounds (Schrader et al., 2009). The physico-chemical techniques that allow to produce CH_3OH out of CH_4 have a high energy demand and are therefore costly. A biotechnological approach has the benefit that it can be carried out at room temperature and at atmospheric pressure (Xin et al., 2004). It is however difficult to induce this partial oxidation while at the same time avoiding inhibiting concentrations (Schrader et al., 2009).

MOB are also able to produce epoxypropane via co-metabolic oxidation of propene by sMMO and pMMO (Xin et al., 2010). Epoxypropane is an important building block for polyether polyols in the chemical industry (Dalton, 2005). Production of other bioproducts has also been studied. Different enzymes produced by MOB can serve as oxidoreductases and bioprotectants. A promising compound is ectoine, produced by halophylic MOB, which finds applications in the biotechnological, cosmetic and medical sector (Reshetnikov et al., 2011, Trotsenko et al., 2005). Also the production of single cell protein (SCP) is worth mentioning. The biomass of methylotrophs is comparable in essential amino acid composition to fish and soybean flour. Therefore, this so-called BioProtein, created with MOB cultures, finds use as protein source in animal feed (Bothe et al., 2002, Overland et al., 2010).

Lastly, MOB can be used to produce biopolymers such as poly-hydroxy-butyrate (PHB). Poly-3-hydroxy-butyrate is a high quality biopolymer, which can be produced by various bacterial species as a kind of intracellular energy and carbon storage (Halet and Defoirdt, 2007, Zhang et al., 2008). These bacteria can then use this PHB as a storage polymer when growth conditions become worse. PHB accumulation has been observed with MOB under deficiencies of nitrogen, phosphorus and magnesium (Wendlandt et al., 2001, Zhang et al., 2008, Zuniga et al., 2011). To increase the PHB content, often a feast-and-famine strategy is applied (Pieja et al., 2011): during the feasting period, the bacteria are active under

optimal conditions, allowing to achieve a large increase in the CDW concentration. When the exponential growth phase is finished, one of the mentioned nutrients is limited, which results in an increase of the internal PHB reserves. As only the bacteria with a high PHB content are best able to sustain this famine period, a natural selection for the best PHB producers takes place. By applying sequential cycles, the PHB content can increase to a concentration of 0.6 g PHB g^{-1} CDW (Wendlandt et al., 2001, Zhang et al., 2008). Although both the RuMP as the serine pathway can be used to produce PHB, type II bacteria appear to be the most effective PHB producers (Helm et al., 2006, Zhang et al., 2008). The resulting PHB has found many applications, for example as prebiotic (De Schryver, 2010), as slow-release application for medication (Wendlandt et al., 2010) and most interestingly as bioplastic (Lee et al., 2005, Salehizadeh and Van Loosdrecht, 2004). Remarkably, certain methylotrophs introduce hydroxyvalerate into the chains of PHB, resulting in a poly-hydroxy-butyrate/valerate (PHBV) mixture, thereby improving the physico-chemical properties (Trotsenko et al., 2005).

I.6 Interactions of MOB with other organisms

As MOB can fulfill their energy and carbon needs solely with CH_4, they are able to be part of ecosystems lacking organic C sources. Moreover, some MOB have such a high affinity for CH_4 that they can maintain their metabolism under atmospheric conditions, provided that the environment can fulfill their water and nutrient requirements. At the same time, MOB can form the basis of a whole microbial food web, as certain heterotrophic bacteria are capable of utilizing methanotrophic metabolites like methanol as energy source (Hrsak and Begonja, 2000, Modin et al., 2007, Murrell and Jetten, 2009). These cooperative bacteria possibly decrease the concentration of inhibitory intermediates and by-products like methanol and formaldehyde, which can negatively influence the activity of the MOB (Hesselsoe et al., 2005, Hrsak and Begonja, 1998, 2000, Megraw and Knowles, 1989, Modin et al., 2007). Also citrate, proteins, PHB, nucleic acids, carbohydrates and acetate have been suggested as leaking products from the methanotrophic metabolism (Costa et al., 2000, Eisentraeger et al., 2001, Modin et al., 2007). At the same time, heterotrophic

bacteria possibly produce growth factors, enhancing the metabolism of the MOB (Hesselsoe et al., 2005, Hrsak and Begonja, 2000). Although cobalamine has been the only component that has been identified, proteins and vitamins have been mentioned as most probable candidates (Hrsak and Begonja, 2000, Iguchi et al., 2011). An interesting application of this methanotrophic-heterotrophic interaction is AME-D: aerobic CH_4 oxidation coupled to denitrification (Modin et al., 2010, Modin et al., 2007). Hereby, organic compounds released by the MOB are used by heterotrophic denitrifiers as electron donor to fuel the transformation of NO_3^- to N_2 (Amaral and Knowles, 1995, Modin et al., 2007).

Also higher life forms show close interactions with MOB. Symbiotic relations have been observed between MOB and many marine invertebrates like snails, mussels, sponges and tubeworms (Dalton, 2005, Dubilier et al., 2008, Hanson and Hanson, 1996, Petersen and Dubilier, 2009). The host hereby provides the MOB with an ideal position in the mixing zone, where CH_4 rich fluids meet the aerated layer. In return, the MOB provide the host with carbon-rich metabolites. Interestingly, the host gains a lot of its energy needs from the MOB, hence, the MOB need to oxidize enough CH_4 to provide sufficient energy for itself as well as the host. Although none of these MOB have been successfully cultivated, sequencing results show that these bacterial communities almost entirely consist out of type I related MOB (DeChaine and Cavanaugh, 2005, Dubilier et al., 2008). Recent studies applying fatty acid isotope screening indicate that the MOB support whole aquatic food webs up to the fish level (Sanseverino et al., 2012). MOB were also detected in the gut system of termites, which are important CH_4 producers, (Leadbetter and Breznak, 1996). However, there have been doubts whether MOB indeed play a CH_4 mitigating role inside these termites (Pester et al., 2007).

As plants produce O_2 and consume CO_2, they are also candidates for interactions with MOB. Roots of the aquatic grass *Calamogrostis canadensis* seem to harbor methanotrophic communities (King, 1994a). The interior of aquatic macrophytes serves as transport channels for atmospheric O_2 to deeper zones, while at the same time CH_4 and other anaerobic gases are transported upwards. Recently, symbiotic interactions between

submerged *Sphagnum* mosses and both type I as well as type II MOB have been found. MOB deliver extra CO_2 to the mosses, while mosses in return provide O_2 and a growth surface for the MOB (Kip et al., 2012, Kip et al., 2010). This cooperation prevents a large fraction of CH_4 seeping from these peat bogs to the atmosphere (Raghoebarsing et al., 2005). Additionally, moss-MOB interactions were also observed in other biotopes, like lawns, pools and hummocks (Kip et al., 2010). Also floating plant material can result in significant rates of CH_4 oxidation. *Lemna minor* or little duckweed is a common inhabitant of freshwater ponds and can harbor large populations of MOB (Hanson et al., 1993). The same kind of interaction as with mosses can be suspected. This kind of plant-MOB interactions has also been observed in swamps. Harrison and Subramania-Aiyer (1914) discovered that roots of rice crops were covered by a microbial biofilm emitting O_2 rich gas while CH_4 was simultaneously oxidized. In this case, MOB are probably working together with O_2 producing algae in the biofilm (Hamer, 2010).

I.7 Microalgae

I.7.1 Taxonomy

As is the case with the term 'methanotrophs', also the definition of 'algae' is prone to confusion. All microorganisms that are able to photosynthesize are referred to as algae. This however includes next to the well known eukaryotic 'algae' also the prokaryotic cyanobacteria (Barsanti and Gualtieri, 2006, Herrero, 2008). Although the taxonomy of the latter changes regularly, two large subgroups can be distinguished, i.e. the *Cyanophyta* and *Prochlorophyta* (Herrero, 2008). The eukaryotic algae are typically taxonomically grouped according to their photosynthetic pigments, flagellation, life cycle and cell structure (Barsanti and Gualtieri, 2006). The most important divisions are the green *Chlorophyta*, the red *Rhodophyta*, the gold-brown *Heterokontophyta*, the *Phyrrhophyta* and the *Euglenophyta* (Barsanti and Gualtieri, 2006, Wang et al., 2008). The term microalgae is used for all algae with a size below 100-200 µm, mostly unicellular and without cell differentiation (Mata et al., 2010, Olaizola, 2003).

I.7.2 Oxygenic photosynthesis

Photosynthesis is one of the most important processes on Earth as it allows to capture light energy and subsequently transform it into chemical energy, applicable for living organisms. In short, the energy present in sun radiation is used to produce carbohydrates out of CO_2 and H_2O (Barsanti and Gualtieri, 2006). The photosynthetic pigment that allows to convert solar energy into chemical energy is chlorophyll a, which all algae posses (Evangelista et al., 2006). However, also other pigments and assisting pigments can occur (Evangelista et al., 2006, Richmond, 2004).

Photosynthesis actually comprises two closely linked processes: the light-dependent reactions taking place in photosynthetic thylakoid membranes and the light-independent reactions occuring in the stroma (Barsanti and Gualtieri, 2006, Richmond, 2004). During the light-dependent reactions, light energy is captured and transformed into energy-rich compounds: nicotinamide adenine dinucleotide phosphate (NADPH) and ATP (Finazzi et al., 2010, Rost et al., 2006). Two large protein complexes, named photosystem I (PSI) and photosystem II (PSII) absorb the photonic energy and induce excitation of two types of pigment, i.e. P700 and P680. When an excited electron falls along the electron transfer chains (ETC), $NADP^+$ is reduced to NADPH. These chains mainly consist of ferredoxin, cytochromes and plastoquinones. At the same time, an electrical potential and difference in proton concentration is created over the thylakoid membrane, leading to ATP production via ATP synthase. The electron transfer chain is represented in Figure I-8.

These light-dependent reactions lead to an uptake of CO_2, while H_2O is split and O_2 released (Barsanti and Gualtieri, 2006). During the light-independent reactions, the CO_2 is then fixed and reduced to carbohydrates, often starch or oils (Carlsson et al., 2007). The energy is provided by the NADPH and ATP, generated during the light-dependent reactions. The different light-independent processes are referred to as the Calvin Benson Bassham cycle, which consists of three main phases: first carboxylation takes place, merging CO_2 with ribulose biphosphate (RuBP), a C5 acceptor molecule.

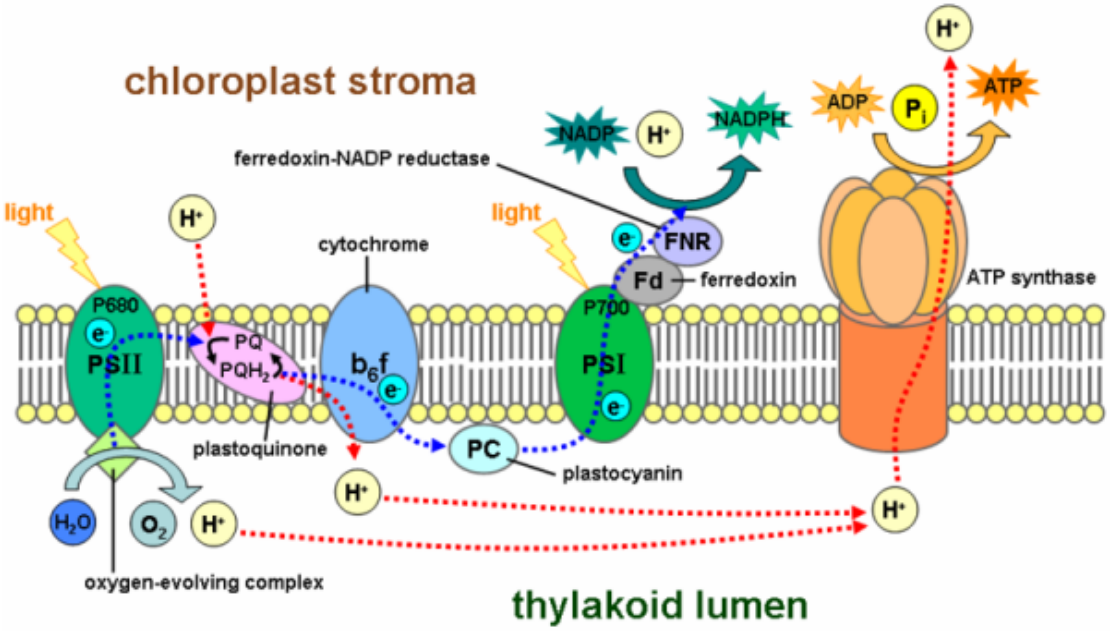


Figure I-8. The light-dependent reactions of photosynthesis, showing the electron transfer chain in algae cells (CAPP, 2012).

The reaction is catalyzed by the ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO) enzyme, the most abundant protein on Earth. The resulting C6 molecule is then split into C3 products, called 3-phosphoglycerate. In a second phase, the latter is reduced to the carbohydrate level, requiring extra ATP and NADPH. In the last phase, the CO_2 acceptor RuBP is regenerated. It takes six rounds of the cycle to produce one mol of hexose, leading to an energy balance as shown by following equation (Barsanti and Gualtieri, 2006):

$$6\ CO_2 + 12\ H_2O + 18\ ATP + 12\ NADPH \Rightarrow C_6H_{12}O_6 + 6\ H^+ + 18\ ADP + 18\ Pi + 12\ NADP$$

I.7.3 Light as driving force

Chlorophyll-containing organisms depend highly on the incoming light energy. The algae can however only use photons with a wavelength between 400 and 700 nm, defined as the photosynthetically active range (PAR) (Barsanti and Gualtieri, 2006). Although plants and algae have a certain adaptation potential, an optimal intensity range exists for their

photosynthetic processes. For every algae or plant, a light-saturation curve can be plotted, whereby the O_2 evolution is plotted in relation to the light intensity (Figure I-9).

When there is no light energy available, no light-dependent reactions can take place. Therefore no energy is available to fixate CO_2, leading to an uptake of O_2 due to respiratory activity. When sufficient light energy is available in the applicable wavelength range, the light-dependent reactions start up. With low light intensities, a linear relationship is observed between the light intensity and O_2 evolution, as 9.7 photons are needed to produce 1 mol of O_2 (Melis, 2009). A maximum solar energy conversion is observed in this lower range. The photosynthetic activity is limited by the light reactions until the saturation point is reached. For algae, this is often around 400 µmol PAR m^{-2} s^{-1} (Kumar et al., 2011, Melis, 2009). After that, light saturation conditions are reached and a plateau is observed (Figure I-9).

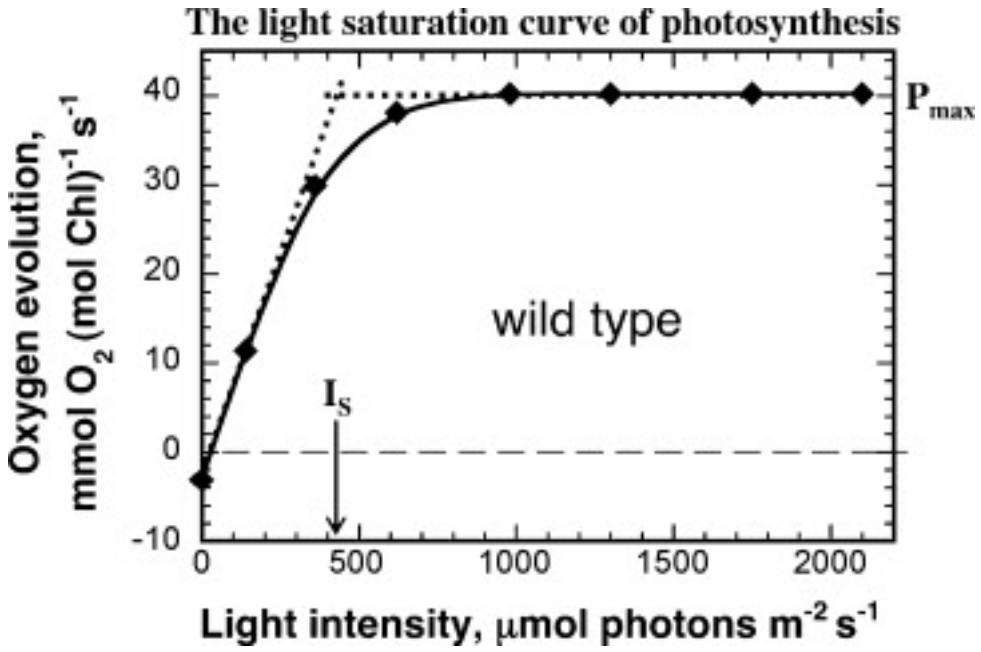


Figure I-9. The light-saturation curve of photosynthesis obtained with wild type microalgae. The rate of photosynthesis (O_2 evolved mol^{-1} Chl s^{-1}) is plotted as a function of light intensity (Melis, 2009).

The underlying reason for this saturation lays in the slow catalytic rate of B6f and RuBisCO during the carbon reactions (Melis, 2009). While enzymes often perform more than 1000 actions in a second, RuBisCo only transforms 3 mol s^{-1} (Barsanti and Gualtieri, 2006). When the light intensity increases even more, damage can occur as PSII gets damaged (Barsanti and Gualtieri, 2006). This often occurs when the light intensity is higher than 1500 µmol PAR m^{-2} s^{-1} and the O_2 concentration exceeds 200% (Molina et al., 2001). Algae can protect themselves to a certain extent via photo-adaptation, hereby regulating the amount and size of pigments (Niyogi, 1999).

Although saturation takes place at about 400 µmol PAR m^{-2} s^{-1}, sunlight intensities can reach values of 2500 µmol PAR m^{-2} s^{-1}. Often, photosynthetic systems are saturated during a large part of the day, leading to a loss of about 80% of absorbed solar irradiance via non-photochemical quenching (Melis, 2009). The ±20% that is absorbed cannot be used completely by the organisms. Indeed, each step of the photosynthetic reactions has its own efficiencies and losses. Therefore the best-case solar-to-biomass energy conversion efficiency is estimated to be 18% of PAR or 8-10% of total solar radiation (Wijffels, 2008). In practice, efficiencies were achieved of about 2% of total solar radiation for the algal model organism *Chlorella* sp. For other tested species, measured values are even lower. Although some species prefer a 16:8 or 12:12 light regime (light:dark), most algae species can be cultivated under continuous light, without a huge loss of activity (Rost et al., 2006).

I.7.4 Oxygen sensitivity

The photosynthetic system is also sensitive to increased O_2 concentrations as the RuBisCo enzyme is versatile and capable of catalyzing the oxygenation of RuBP. This photorespiration is possible because O_2 can bind with RuBisCo on the active site. At the same time RuBisCo has a low binding affinity of 8.8 mg L^{-1} for CO_2 (McNevin et al., 2006). Due to the competition with carboxylation, high concentrations of O_2 lower the photosynthetic efficiency. Algae can counteract this shift by activating the 'CO_2 concentration mechanism', which leads to an increased concentration of CO_2 around the RuBisCo (Giordano et al., 2005). These photorespiration processes whereby O_2 is taken up

and CO_2 released occur predominantly when there is a high O_2:CO_2-ratio and during the night period (Barsanti and Gualtieri, 2006).

I.7.5 Metabolic diversity

Some algae are strictly photoautotrophic: they can only use photosynthesis in order to reduce CO_2 into organic compounds. However, many algae are mixotrophic, combining photoautotrophy with heterotrophy (Richmond, 2004). Heterotrophy allows species to utilize exogenous organic compounds as their carbon and energy source. Instead of producing their own C source through photosynthesis, they take them up from the environment. Also other combinations of carbon and energy metabolisms exist, leading to a classification into four main groups based on their nutritional capacities:

- **Obligate phototrophic algae** normally strive for light energy, but can sustain themselves under light limiting conditions by taking up organic compounds by means of osmotrophy (uptake of dissolved substances) or phagotrophy (uptake of bacteria and other cells) (Becker, 1994).
- **Obligate heterotrophic algae** primarily behave heterotrophically, but can sustain themselves by phototrophy.
- **Facultative mixotrophic algae or amphitrophic algae** are capable of growth either by phototrophy or heterotrophy. The preferred pathway depends on the availability of light energy and organic compounds.
- Lastly, **obligate mixotrophic algae** only use heterotrophy to provide essential substances for growth. This strategy is for example necessary for photoauxotrophic microalgae, which are not capable of synthesizing essential components such as certain vitamins or fatty acids themselves.

Moreover, many microalgae are capable of metabolic shifts in response to the environment. The model species *Chlorella vulgaris* and *Scenedesmus sp.* are examples of algal species capable of growing under photoautotrophic, heterotrophic and mixotrophic conditions (Barsanti and Gualtieri, 2006, Larsdotter, 2006).

I.7.6 Growth factors

Next to light energy, autotrophic microalgae need sufficient CO_2, water, nutrients and trace elements. The Redfield-ratio gives an indication of the amount of macronutrients needed for optimal activity. The ratio of C:N:P is stated to be 106:16:1, but can vary with different species and growth conditions (Redfield, 1958). Phosphorus is in most natural environments the growth limiting macronutrient (Klausmeier et al., 2008).

The most common C source for algae is CO_2. However, when CO_2 dissolves in water, it will be hydrolyzed and depending on the pH of the medium, the equilibrium will shift to CO_3^{2-}, HCO_3^- or H_2CO_3. Although free CO_2 is commonly the preferred C source, certain algae are able to utilize also these mentioned C species. Some cyanobacteria have an increased photosynthetic efficiency when grown on HCO_3^-, while a higher maximal biomass concentration was found for *Chlorella vulgaris* when carbon was amended as CO_3^{2-} (Yeh et al., 2010). This C species is however taken up less efficiently by most algae, and can even become toxic (Khalil et al., 2010, Lehman, 1978). Although many algae are negatively influenced by CO_2 concentrations above 5% (v/v), some types are able to grow at concentrations of 70 and even 100% CO_2 (Carlsson et al., 2007, Olaizola, 2003).

Algae can utilize different N species. Often NH_4^+-N is preferred, followed by NO_3^--N (Bhaya et al., 2000). Also organic forms like urea and small amounts of NO_2^- can be used (Lourenco et al., 1998). When applying equal amounts of NH_4^+, NO_3^- and urea ($CO(NH_2)_2$) as N source to a *Scenedesmus* sp. culture, specific growth rates of 0.82 d^{-1}, 0.54 d^{-1} and 0.75 d^{-1} were found, respectively, indicating that NH_4^+ is the preferred N source (Hamilton et al., 2001, Li et al., 2010a). The pH is of high importance when NH_4^+-N is amended, as the equilibrium shifts quickly from NH_4^+ to NH_3 when the pH increases above 8. Not only does the NH_3 escape from the medium, it also exhibits a higher toxicity, as it decouples the electron transport of PSII and competes with the oxidation of H_2O (Becker, 1994, Munoz and Guieysse, 2006). As microorganisms typically use NH_4^+ as building blocks for their cell structures, nitrite- and nitrate reductase is expressed when NO_3^--N or NO_2^--N are applied, leading to a pH increase and consumption of 8 mol electrons mol^{-1} reduced NO_3^--N

(Becker, 1994). The uptake of NH_4^+-N on the other hand leads to a pH decrease, as protons are released during assimilation (Molinuevo-Salces et al., 2010). NO_2^- is more toxic for cyanobacteria than the eukaryotic algae, and a concentration of 14 mg NO_2^--N L^{-1} (pH 6) can already inhibit carbon uptake (Wodzinski et al., 1978). Some algae are able to utilize NO radicals as N source (Richmond, 2004). The latter is an intermediate in the nitrification process, which some algae are able to perform and can result in the production of the strong GHG N_2O. Lastly, nitrogen fixation is only observed with some cyanobacteria (Barsanti and Gualtieri, 2006).

About 1% of algal CDW consists of phosphorus, which they preferably take up as PO_4^{3-} (Barsanti and Gualtieri, 2006). Algae and cyanobacteria often perform so called luxury uptake, whereby a surplus of phosphorus is stored in polyphosphate bodies (Oliver and Ganf, 2000). This approach allows the algae to use these storage compounds in times that phosphorus becomes limiting (Richmond, 2004). Lastly, some micro-nutrients are essential for most microalgae, i.e. S, Na, K, Fe, Mg, Ca, B, Cu, Mn, Mo, Co, V, Se and Si (Richmond, 2004).

I.8 Biotechnological potential of algae

I.8.1 Wastewater treatment

Microalgae are successfully applied for wastewater treatment. Wastewater Stabilization Ponds (WSP) are in general cheap and simple constructions for wastewater treatment in developing countries (Hill et al., 2006, Johnson and Wen, 2010, Mara, 2004). They function particularly well in warmer regions, where enough light and heat is present (Mara, 1992). Generally, these WSP exist out of a facultative pond, wherein wastewater is pumped to the bottom. Under anaerobic conditions, methanogens will convert the biodegradable fraction to biogas, which escapes to the atmosphere or is contained in an anaerobic digester (Mara, 2005). At the surface of the facultative pond, algae can provide O_2, which allows aerobic bacteria to oxidize the rest of the biological oxygen demand (BOD) in the wastewater (Pearson et al., 1987). The facultative pond effluent can then be directed towards High Rate Algal Ponds (HRAP), where the wastewater is polished by suspended

algae. Nitrogen and phosphorus are hereby removed, therefore decreasing the risk of eutrophication (Figure I-10) (Craggs et al., 2012, Garcia et al., 2000). At the same time, organic contaminants, heavy metals and pathogens are partly removed due to the algal metabolism and additionally, the resulting high pH above 9 (Elhamouri et al., 1994, Toumi et al., 2003). The algal biomass can sometimes be harvested and used as low-cost fertilizer or animal feed (Mata et al., 2010, Munoz and Guieysse, 2006).

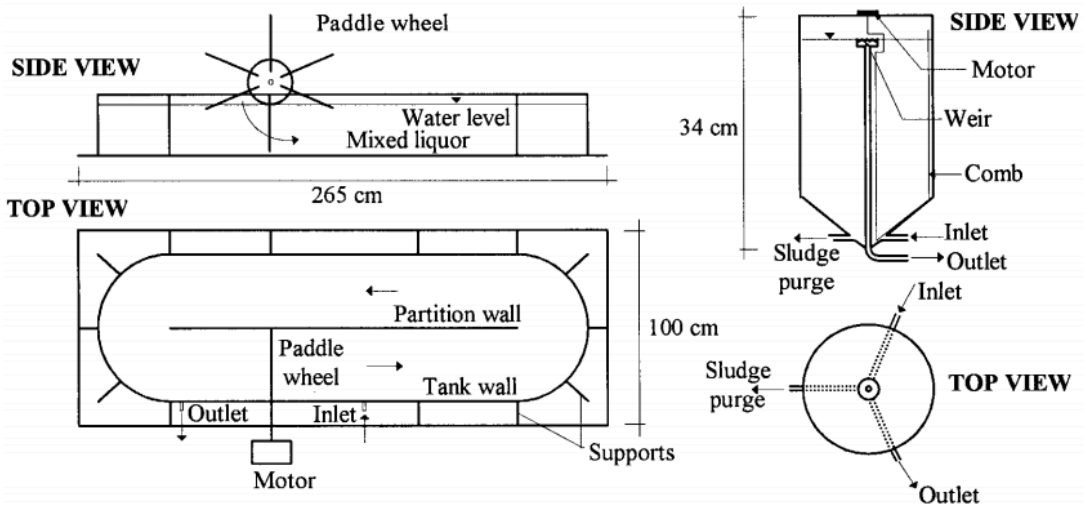


Figure I-10. Diagram of top and side views of an experimental HRAP and clarifier (Garcia et al., 2000).

I.8.2 Biofuels

Our current society relies heavily on fossil fuels. This energy source is however non-renewable and contributes to the global warming phenomena, due to the emissions of greenhouse gases. Biofuels are generated out of crops and therefore evaluated as renewable, GHG-neutral and a good alternative for fossil fuels (Hill et al., 2006). Currently the market is in transition, whereby second and third generation biofuels are - or should be - produced from non-food feedstocks such as algal biomass (Johnson and Wen, 2010, Mata et al., 2010, Singh and Bhaduri, 2010). Even under normal growth conditions, 0.22 g lipids g^{-1} CDW are detected in *Chlorella vulgaris*, next to about 0.55 g proteins, 0.15 g carbohydrates and about 5% nucleic acids (Singh and Bhaduri, 2010). Moreover, the lipid

content of algae can be increased considerably under certain inducing conditions. The potential oil productivity of microalgae therefore exceeds the productivity from oilseed crops (Metting, 1996). The harvesting and post-processing is however a point of concern as the current processes are very energy-demanding (Mata et al., 2010, Singh and Bhaduri, 2010). Therefore, optimization of the algae cultivation, lipid production and harvesting processes are needed before the ultimate goal is achieved: the development of algae farms in order to lower the need for agricultural land with an ever growing population. It is however possible to combine the production of algal biomass with other biotechnological processes like sequestration of greenhouse gases, wastewater treatment or fish-farms. As such, the production of biofuels with algae could be integrated in a biorefinery concept.

I.8.3 Production of added value compounds

Microalgae have the capacity to synthesize all amino acids, including the essential ones for humans and animals. At the same time, proteins can represent up to 60% of their CDW, which is more than vegetable sources. Therefore, they are considered a valuable protein source (Delanoue and Depauw, 1988). They are also valuable sources of essential vitamins and polyunsaturated fatty acids (PUFA's) of which the omega-3 and omega-6 types are the most known and valuable representatives (Ahlgren et al., 1992). Moreover, they are often rich in valuable pigments such as chlorophyll, phocobiliproteins and carotenoids like astaxanthin (Cardozo et al., 2007). Many of the fine chemicals and bioactive compounds present in algae are beneficial for human health. Consequently, more than 70 companies are cultivating the model organism *Chlorella* sp. to take benefit of its health promoting effects (Spolaore et al., 2006).

Microalgal cells and extracts are also of interest as feed component for a range of animals. For example for rearing larvae and molluscs, crustaceans, fish and pets (De Schryver et al., 2008). Microalgae can also be added to cattle feed in order to reduce the CH_4 production during rumen fermentation. The PUFA are inhibitors for the methanogenic activity in the rumen and an 80% reduction of CH_4 emission by cows has been observed (Fievez et al.,

2007). Screening is however a necessity, as the nutritional value should be high enough, in order to assure that the price of investment can be paid back. Lastly, when used for any commercial purpose, an extended quality control is needed, as some microalgae are known to take up toxins and heavy metals (Munoz and Guieysse, 2006) or produce toxins (Singh et al., 2005).

I.9 Rationale and objectives of this study

Little is known about methanotrophic microbiomes, i.e. entire microbial communities directly or indirectly depending on CH_4 for their growth and activity. In this dissertation, two factors were examined that are important for utilizing these communities for applications in environmental biotechnology. The first factor concerns external parameters that influence the activity and structure of such communities (**Chapter III & IV**). The other factor relates to the interactions taking place within the methanotrophic microbiome. The latter includes both the interaction between MOB and the associated heterotrophic community (**Chapter V and VI**), as well as the specific interaction between MOB and microalgae, defined as the *methalgae* interaction (**Chapter VII, VIII and IX**).

Firstly, a high-throughput method will be developed to isolate MOB with specific characteristics, in order to facilitate these examinations (**Chapter II**). Thereafter, the effects of certain growth parameters on methanotrophic communities are investigated (**Chapter III and IV**). In **Chapter III**, the focus lies mostly on the influence of Cu^{2+} concentration on the resilience towards salt additions. In **Chapter IV,** the emphasis lies on the influence of NH_4^+-N. Further chapters focus on the interactions within the methanotrophic microbiome. Firstly, interactions between MOB and heterotrophs are evaluated at different Cu^{2+} concentrations (**Chapter V**). Thereafter, the occurrence of carbon fluxes in the methanotrophic microbiome will be studied (**Chapter VI**). Lastly, symbiotic effects between MOB and algae are investigated, with a focus on biotechnological applications (**Chapters VI, VII and VIII**).

In **Chapter II**, an optimized and miniaturized extinction culturing method is presented and compared with conventional dilution plating. A better isolation efficiency is hereby achieved, allowing to isolate and characterize certain MOB with desired characteristics.

In **Chapter III**, the effects of certain growth parameters like the salt concentration, the N source and the addition of Cu^{2+} are investigated. The study shows that higher Cu^{2+} concentrations increase the methane oxidizing activity and salt resistance significantly.

In **Chapter IV**, the adaptation capacity of methanotrophic communities is tested towards high loads of NH_4^+. It shows that if enough adaptation time is given, methanotrophic communities can adapt to high loads of NH_4^+.

In **Chapter V**, the focus lies on the microbial methanotrophic food web that forms under different Cu^{2+} concentrations. Proof is given of metabolic networking, whereby MOB provide heterotrophic bacteria of a carbon source.

In **Chapter VI**, a closer look is taken on the methanotrophic-heterotrophic interactions. By means of stable isotope probing, the flux of CH_4 derived C within the methanotrophic microbiome is evaluated. Moreover, the concept of metabolic networking was tested by coculturing an MOB strain with a methylotrophic yeast strain, relying on CH_4 as sole carbon source.

In **Chapter VII**, insights are provided about the interactions between MOB and microalgae. Algae hereby provide MOB *in situ* of O_2, allowing MOB to be active in anoxic environments.

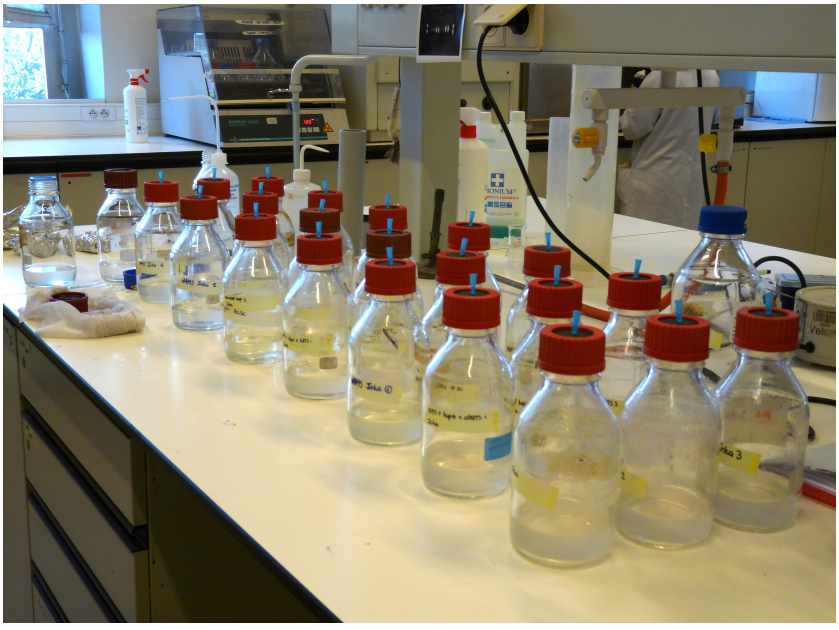
In **Chapter VIII**, the symbiotic interaction between MOB and algae is identified in a facultative pond. It was observed that algae have an essential supportive role in the methane oxidation processes of such ponds.

In **Chapter IX**, the interaction between algae and MOB will be exploited to develop a new microbial sink for biogas by converting it into valuable biomass.

Finally, a general discussion on the obtained results is provided in **Chapter X**, presenting future perspectives and possible applications.

CHAPTER II

MINIATURIZED ISOLATION OF METHANE OXIDIZING BACTERIA



Methanotrophic communities, LabMET

I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

Marie Curie

Chapter II. Miniaturized isolation of methane oxidizing bacteria

Abstract

Methane oxidizing bacteria (MOB) have an important potential as a microbial sink for the greenhouse gas methane as well as for biotechnological purposes. However, their application in biotechnology has so far been hampered, in part due to the relative slow growth rate of the available strains. To enable the availability of novel strains, this study compares the isolation of MOB by conventional dilution plating with miniaturized extinction culturing, both performed after an initial enrichment step. The extinction approach rendered 22 MOB isolates from four environmental samples, while no MOB could be isolated by plating. In most cases, extinction culturing immediately yielded MOB monocultures making laborious purification redundant. Both type I (*Methylomonas* spp.) and type II (*Methylosinus* sp.) MOB were isolated. The isolated methanotrophic diversity represented at least 11 different strains and several novel species based on 16S rRNA gene sequence dissimilarity. These strains possessed the particulate (100%) and soluble (64%) methane monooxygenase gene. Also, 73% of the strains could be related to a highly active fast-growing methanotrophic community. In conclusion, miniaturized extinction culturing was more efficient in rapidly isolating numerous MOB requiring little effort and fewer materials, compared with the more widely applied plating procedure. This miniaturized approach allowed straightforward isolation and could be very useful for subsequent screening of desired characteristics, in view of their future biotechnological potential.

Redrafted after:

Hoefman, S.[*], van der Ha, D.[*], De Vos, P., Boon, N., Heylen, K. (2012) Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast-growing methane-oxidizing bacteria. Microb. Biotechnol. 5: 368-378. ([*] contributed equally).

II.1 Introduction

Despite the multifunctional potential of MOB, there are still several factors limiting large-scale applicability in industrial processes, which are mostly related to the MOB themselves, i.e. slow growth rates and low substrate affinity (Jiang et al., 2010b). In order to achieve the full potential of MOB and communities for such biotechnological applications, a straightforward isolation technique is of high value, as it allows the optimization of the growth conditions of the MOB of interest. However, their cultivation is still laborious (Bowman, 2006). To date, only few MOB have been studied and examined thoroughly for further biotechnological applications (Jiang et al., 2010b). The examined cultures were often not selected for their optimal use in these bioprocesses but were just the only available methanotrophic cultures at the time.

It was not until 1970 that Whittenbury and co-workers established a successful procedure to isolate and characterize methane oxidizing bacteria by plating on Nitrate- or Ammonium Mineral Salts (NMS/AMS) medium (Whittenbury et al., 1970). Since then, novel research on MOB mainly encompassed exploring new environments and using cultivation conditions adapted to the corresponding environment (Bussmann et al., 2004, Dedysh et al., 1998b, Svenning et al., 2003, Wise et al., 1999b). Most of the methanotrophic diversity thus obtained could be phylogenetically positioned within the classes of the *Alpha-proteobacteria* (such as *Methylosinus* (type II)) and the *Gamma-proteobacteria* (such as *Methylomonas* (type Ia) and *Methylococcus* (type Ib)) (Dedysh, 2009). Since the recognition of "The Great Plate Count Anomaly" (Staley and Konopka, 1985), alternative ways to increase the general cultivability of the microbial diversity were explored, for example by application of growth conditions that closely mimic the natural environment (Dedysh et al., 1998b), prolonged incubation at low temperatures (Song et al., 2009) or the use of alternative gelling agents replacing agar (Dedysh et al., 2007a, Janssen et al., 2002, Stott et al., 2008, Tamaki et al., 2009). Another approach was extinction culturing by diluting a sample to the point of extinction and thereby purifying to a less complex sample containing only one or a few organisms (Button et al., 1993b, Schut

et al., 1993), which was further optimized by the development of high-throughput culturing methods (Connon and Giovannoni, 2002a, Rappe et al., 2002, Stingl et al., 2007). To date, MOB are still mostly isolated via plate methods, whereby liquid enrichment steps are followed by a serial dilution onto plates (Dedysh et al., 2004, Dunfield et al., 2003, Heyer et al., 2005, Tsubota et al., 2005a, 2005b, Wartiainen et al., 2006). However, these procedures are very laborious and almost always require elaborate purification.

The main objective of this study was to combine and optimize several of the mentioned cultivation approaches for the specific isolation of MOB, and to compare the isolation efficiency of the resulting two-step liquid isolation procedure, consisting of an initial prolonged enrichment and subsequent miniaturized extinction culturing, with conventional dilution plating. Initial enrichment followed by extinction culturing greatly simplified purification procedures and easily rendered novel MOB strains, which were further characterized by sequence analysis of the 16S rRNA gene, repetitive element sequence based PCR fingerprinting (rep-PCR), *pmoA* and *mmoX* gene amplification (encoding for pMMO and sMMO, respectively) and sMMO activity assays. Most of the strains were traced back to highly active and fast-growing methanotrophic communities through denaturing gradient gel electrophoresis (DGGE) targeting the *pmoA* gene.

II.2 Experimental section

II.2.1 Characterization of the sampling sites

Samples were taken from (i) the top layer of a denitrification tank of a wastewater treatment plant (WWTP, Ossemeersen, Gent, Belgium), (ii) a covered but aerobic slurry pit of a cow stable (Melle, Belgium), (iii) the top litter layer of a wetland (Bourgoyen, Gent, Belgium) and (iv) the biofilter of an anaerobic digester (DRANCO, Brecht, Belgium). The concentrations of the most important macro- and micronutrients were determined for all four samples and are represented in Table II.1. The ammonium concentration was determined by steam distillation, according to Greenberg et al. (1992). Filtered samples (0.45 µm filter, Millipore, Brussels, Belgium) were analyzed for Cl^-, NO_3^-, NO_2^-, SO_4^{2-} and

PO_4^{3-} by means of an 761 Compact Ion Chromatograph, equipped with a conductivity detector (Metrohm, Zofingen, Switzerland). The Fe- and Cu-concentrations were analyzed using ICP-OES spectrometry (Vista MPX, Varian, St-Katelijne Waver, Belgium).

Table II-1. The concentration (mg L^{-1}) of NH_4^+-N, NO_3^--N, NO_2^--N, PO_4^{3-}-P, SO_4^{2-}, Cl^-, Cu and Fe in the samples from the WWTP, slurry pit and wetland, respectively. For the biofilter sample, concentrations are expressed as mg kg^{-1} biofilter material. Therefore 10 g of sample was diluted in 100 ml distilled water and shaken for 2h (120 rpm) according to Alexander *et al.* (2004). The liquid phase was analyzed similarly to the other samples.

	WWTP	Slurry pit	Wetland	Biofilter
NH_4^+-N	7.17	462	2.75	1034
NO_3^--N	0.19±0.01	0.68±0.00	0.13±0.01	<0.01
NO_2^--N	0.12±0.09	1.34±0.09	0.09±0.01	<0.01
PO_4^{3-}-P	13.0±0.2	78.9±0.7	19.0±0.2	1135±5
SO_4^-	0.06±0.03	<0.01	0.17±0.12	3.80±1.56
Cl^-	65.4±1.8	449±3	59.1±3.5	997±4
Cu	0.34	0.38	0.87	1.02
Fe	2.45	7.12	4.20	49.2

II.2.2 Isolation of methane oxidizing bacteria

On the day of sampling, 3 g of each sample was homogenized in 27 mL of a 5 times diluted Nitrate Mineral Salts (dNMS) medium (Dunfield et al., 2003), with a 2 mM Na_2HPO_4/KH_2PO_4 buffer and a modified copper concentration of 0.8 µM (50 µg Cu^{2+} L^{-1}). The pH of the medium was adjusted to the pH of each sample (biofilter material: pH 7.8; other samples: pH 6.8). Dilution series of samples (10^{-2} up to 10^{-11}) were prepared in dNMS medium in triplicate. The resulting 120 vials were sealed and 20% (v/v) CH_4 was added to the headspace. The cultures were incubated for five weeks at 20°C while shaken (90 rpm). Weekly, concentrations of CH_4, O_2, CO_2 and N_2O in the headspace were analyzed

with a Compact GC (Global Analyser Solutions, Belgium). Dilutions were considered positive for methanotrophic growth when CH_4 and O_2 were consumed (drop below initial average subtracted by five times the standard deviation), with subsequent rise in CO_2 levels, and observation of turbidity. This information was used to estimate the abundance of cultivable MOB using Most Probable Number (MPN) tables (USDA-FSIS, 2008). To retrieve MOB from the enrichment cultures showing methanotrophic activity, dilution plating and extinction culturing were carried out in parallel. A schematic overview of the followed methodology is shown in Figure II-1.

Dilution plating was performed by inoculation of the enrichment cultures (10^{-2}, 10^{-4} & 10^{-6}; 100 µL $plate^{-1}$) on dNMS medium with 0.9% gellan gum and 1% $MgSO_4.7H_2O$. After incubation for 2 weeks at 20°C in gas-tight jars under a CH_4:air (1:1) atmosphere, 200 colonies were randomly selected and subcultured to purity. The isolates were checked for methanotrophy by (i) growth on solid dNMS with CH_4 in the headspace, (ii) absence of growth on solid dNMS under air and (iii) absence of growth on 1/10 Trypticase Soy Agar (TSA) under air.

Extinction culturing was performed in duplicate by serially diluting the enrichment cultures (10^{-2} to 10^{-9}) with liquid dNMS medium (dilution-to-extinction) in sterile 96-well microtiter plates. After incubation for two weeks at 20°C under a CH_4:air (1:1) atmosphere, turbidity indicative of growth was checked visually and by measuring the optical density at 600 nm. For each dilution series, the highest dilution showing growth was (i) confirmed for CH_4 oxidation by GC analysis (see above), (ii) plated on solid dNMS medium (with gellan gum) and subcultured to purity if necessary. MOB purity was evaluated by (i) colony morphology, (ii) phase contrast microscopy and (iii) absence of growth on 1/10 TSA and dNMS plates supplemented with 0.1% glucose, 0.1% fructose and 0.1% yeast extract under air. The isolates were confirmed for CH_4 oxidation by GC analysis.

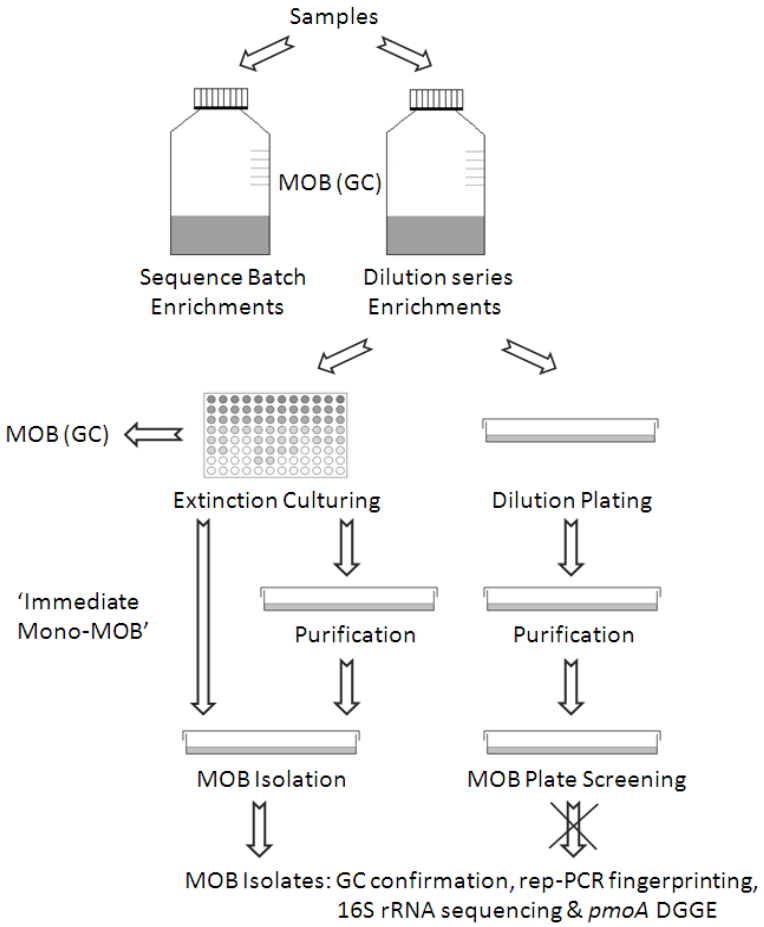


Figure II-1. Flow chart of the followed enrichment and isolation strategies.

II.2.3 Methane oxidation rate of sequence batch enrichments

In parallel with the dilution series enrichments used for MOB isolation, sequence batch enrichments using the same cultivation conditions were set-up in triplicate on the day of sampling for each of the four original samples, allowing the estimation of methane oxidation rates (MOR) of the methanotrophic communities cultivable under set conditions. Gastight Schott-bottles with a total volume of 1150 mL were filled with 200 mL dNMS medium. Inoculation was performed with 2 mL of the original sample. For the sample of biofilter material, 0.5 g of inoculum was added. After sealing the reactors, 20% (v/v) CH_4 was added to the headspace (950 mL). The cultures were placed on a shaker (100 rpm) at 20°C and GC analysis of the headspace was performed daily. When the activity dropped to

almost zero, reactors were opened under non-sterile conditions after which 160 mL of liquid phase was removed for physico-chemical analysis or stored at -20°C for *pmoA* gene DGGE analysis (see further)**.** The remainder of the liquid phase (40 mL) of the triplicate sequence batch enrichments was merged together and subsequently distributed equally over the three reactors. Freshly made dNMS medium was added to a total volume of 200 mL after which 20% (v/v) CH_4 was again added to the headspace. In total, this cycle was repeated two times. The hydraulic retention time (HRT) and sludge retention time (SRT) for each cycle were 90h.

II.2.4 Identification and characterization of MOB isolates

Dereplication of all isolates was performed to assess genetic heterogeneity and group isolates with identical genomic fingerprints, further referred to as strains. Rep-PCR was performed as described by Ghyselinck et al. (2011) with a $(GTG)_5$-primer (Versalovic et al., 1994). The clustering method was supported by visual inspection: isolates were considered as genomically identical when they demonstrated identical fingerprints, which led to a cut-off value of 93%. For each group, a representative strain was selected randomly and deposited in the BCCM/LMG Culture Collection (LMG 26258-26263 & LMG 26612-26616).

Each strain was identified to the genus level through 16S rRNA gene sequence analysis. PCR amplification and sequencing of the 16S rRNA gene was performed as described by Heyrman and Swings (2001). The sequences were analyzed using a 3130 XL Genetic Analyzer (Applied Biosystems, USA) and assembled with BioNumerics 5.1 software (Applied Maths, Belgium). A reliable genus identification was obtained in two steps: (i) query in the “Classifier” program of the Ribosomal Database Project II (Cole et al., 2005) of the 16S rRNA gene sequence of each new strain, (ii) all type strains of all species of all genera mentioned in the Classifier report were compared in an exhaustive pair wise manner with the query sequence of each new strain in BioNumerics 5.1. Strains were

provisionally assigned to the genus of their closest type strain based on the obtained 16S rRNA gene sequence.

A slightly modified version of the naphthalene oxidation assay of Brusseau et al. (1990) was used to measure sMMO activity of the MOB strains. A crystal of naphthalene was added to 5 mL freshly grown culture, in dNMS without copper addition, and incubated at 28°C on a shaker (150 rpm) for 2h. After incubation, 20 µL of freshly prepared tetrazotized-o-dianisidine solution (2.68 g L^{-1}) was added to 180 µL of each cell suspension in duplicate in microtiter plates, and the formation of a colored diazo-dye was immediately monitored by recording the absorbance at a wavelength of 525 nm via spectrophotometry. The assay was validated using four MOB reference type strains that possess sMMO (DSM 17706^T, DSM 15673^T, DSM 18500^T, NCIMB 11131^T) and four that only possess pMMO (DSM 13736^T, DSM 17261^T, NCIMB 11914^T, NCIMB 11130^T). Primers described in literature for amplification of the *mmoX* gene were also tested using these type strains. Primers described by Hutchens et al. (2004) were selected for *mmoX* gene amplification of the isolates positive for the sMMO activity assay. Amplification was confirmed by subsequent sequencing of the *mmoX* gene.

II.2.5 *pmoA* gene DGGE analysis

Primers described in literature were tested for suitability for DGGE analysis targeting either 16S rRNA or *pmoA*, since most known MOB, except for members of *Methylocella* and *Methyloferula* (Dedysh et al., 2005, Vorobev et al., 2011), as well as all novel strains from this study contained *pmoA*. Thirteen type strains (six type I and seven type II MOB) were used as positive controls for the evaluation. The PCR mix and temperature-time profiles from the original description were tested, as well as the PCR mix used for 16S rRNA gene amplification (Heyrman and Swings, 2001). Only one *pmoA* primer set (A189f/mb661r) could correctly detect the pMMO gene in all tested strains. The 16S rRNA gene could be amplified in all strains with both the type IF/type IR and type IIF/type IIR sets of Chen et al. (2007), however, amplicons were too long for DGGE analysis. The *pmoA* set A189f/mb661r (Costello and Lidstrom, 1999) with GC clamp 5'-

CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3' was selected for further DGGE analysis.

The DNA extraction procedure was adapted from Gevers et al. (2001) and El Fantroussi et al. (1999). DGGE analysis of the PCR amplicons was performed with an INGENY phorU2X2 DGGE-system (Goes, The Netherlands). A 6.5% (w/v) polyacrylamide gel with a 30 to 80% denaturing gradient (a 100% denaturant solution contains 7 M urea and 40% (w/v) formamide) was applied. Gels were run in 1x TAE buffer for 16h at 150 V and stained afterwards with SYBR Green I nucleic acid gel stain. The resulting DGGE patterns were processed using BioNumerics 5.1. Band position analysis was used to track the obtained isolates back to the sequence batch cultures and the dilution series enrichments used for MOB isolation and was performed by visual comparison of band location in the gel.

II.2.6 Nucleotide sequence accession numbers

The 16S rRNA gene sequence data generated in this study has been deposited in GenBank/EMBL/DDBJ with accession numbers FR798952 to FR798973.

II.3 Results

II.3.1 Isolation of fast-growing MOB

Four environmental samples (wastewater treatment plant, wetland, biofilter and slurry pit) were serially diluted and enriched, while monitoring CH_4 and O_2 consumption and CO_2 production, respectively. During the first four weeks, higher dilutions were gradually found positive with each subsequent measurement, stabilizing by the fifth week (Figure II-2).

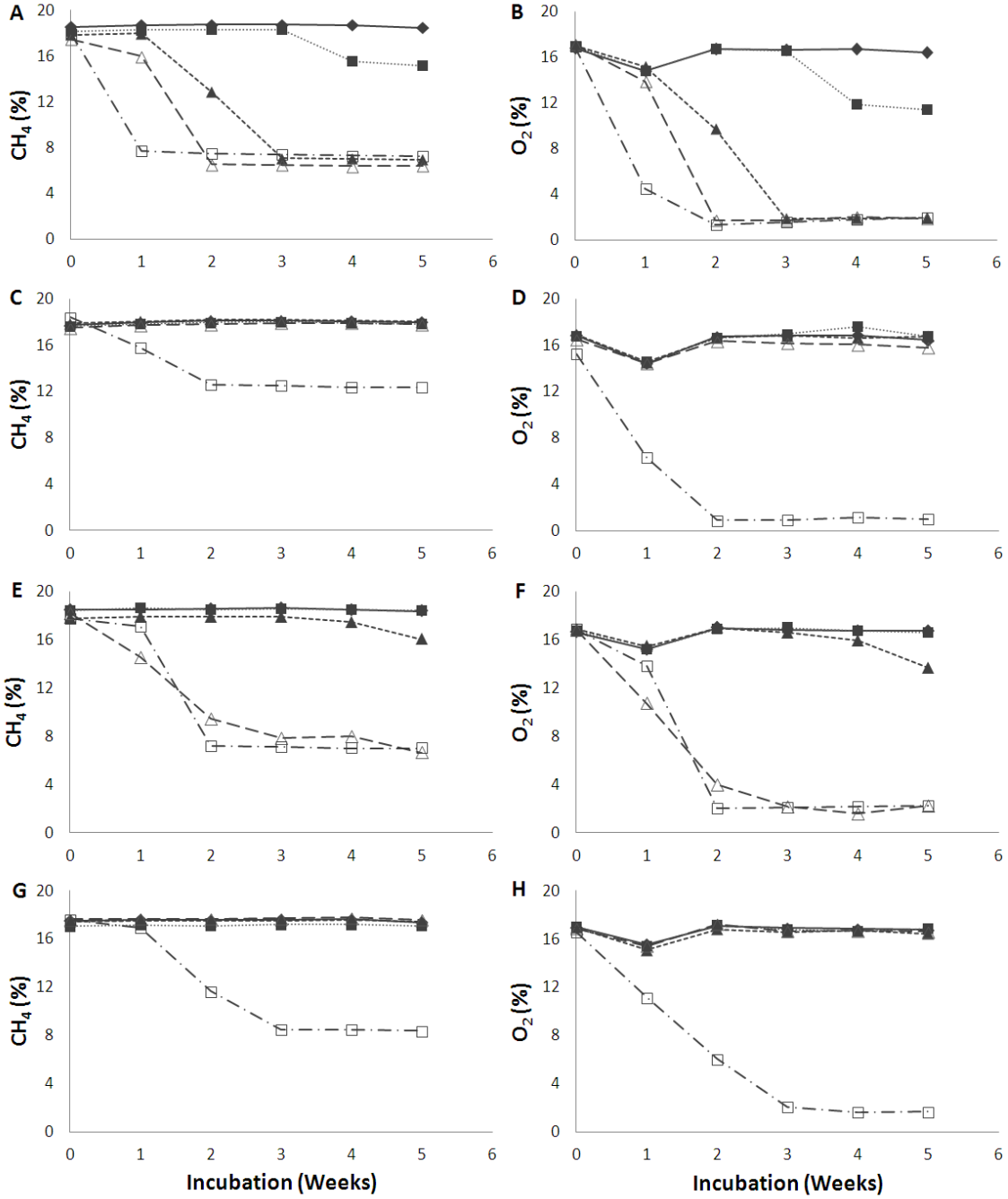


Figure II-2. Average methane (A, C, E, G) and oxygen (B, D, F, H) levels (%) during 5 weeks incubation in dNMS at 20°C (90 rpm) under a CH_4:air (1:4) atmosphere for the WWTP (A, B) slurry pit (D, E), wetland (G, H) and biofilter sample (J, K) for the dilution series 10^{-2} (white square), 10^{-3} (white triangle), 10^{-4} (black triangle) 10^{-5} (black square) and 10^{-6} (black diamond) in triplicate (error bars not shown for clarity).

The extinction culturing procedure was performed in duplicate to isolate MOB from these enrichment cultures, resulting in 46 extinction series, of which only four did not show growth after two weeks of incubation. The highest dilutions of the remaining 42 series were transferred to gas-tight vials and dNMS medium solidified with gellan gum for confirmation of CH_4 consumption and purity check, respectively. Methane oxidation was observed in 27 series, of which 14 were immediately monocultures. Seven CH_4 oxidizing cultures consisted of an MOB in coculture with a non-methanotrophic bacterium (identified as a member of *Nocardioides* with 16S rRNA gene sequence analysis) forming a distinct colony morphology surrounding the methanotrophic colonies. From the remaining six CH_4 oxidizing cultures, several different colony morphologies were found upon plating. Subsequent purification of these MOB was achieved after a maximum of 3 sub-cultivation steps. One dilution series resulted in a monoculture (identified as *Ancylobacter* with 16S rRNA gene sequence analysis), but did not oxidize CH_4. Fourteen dilutions were discarded since these were not able to oxidize CH_4 and did not result in a pure culture upon plating.

Dilution plating was performed in parallel. The 23 initial CH_4 oxidizing enrichments were diluted, plated on dNMS (solidified with gellan gum) and incubated under atmospheric conditions supplemented with CH_4. Randomly, 200 colonies were picked up and purified. Almost all purified isolates (197 out of 200) showed heterotrophic growth on diluted TSA without CH_4 and were not considered as potential MOB. The three remaining isolates, not able to grow on diluted TSA, also failed to grow on dNMS with or without CH_4 added to the headspace. To confirm these results, eight randomly selected isolates obtained via dilution plating, were identified to the genus level through 16S rRNA gene sequence analysis and were affiliated with *Nocardioides*, *Zoogloea*, *Rhizobium*, *Pseudomonas*, *Polaromonas*, *Rhodobacter* and *Enterobacter* genera, not harboring known MOB.

II.3.2 Identification and characterization of MOB isolates

In total, 22 purified MOB isolates were retrieved from four different samples (Table II-2). Dereplication with rep-PCR fingerprint analysis grouped the isolates into 11 distinct clusters, representing 11 unique strains (Figure II-3).

Table II-2. Genus assignment of 11 representative MOB strains (rep-PCR; Figure II-3) based on 16S rRNA gene sequence analysis (> 1,400 bp). Similarity values of 16S rRNA gene sequence to closest type strain, origin of the strains and results of naphthalene oxidation assay and *mmoX* gene amplification are given.

Sample	Representative Strain	sMMO & *mmoX*	Genus identification	Type strain with highest 16S rRNA gene sequence similarity to query sequence			
				Species name	Strain	Sequence	Accession
WWTP	D1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.5%	AF304196
	C1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.4%	AF304196
	G1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	E1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	H1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.6%	AF304196
	K1	Yes	*Methylomonas*	*Methylomonas methanica*	NCIMB 11130^T	98.3%	AF304196
	B1	No	*Methylomonas*	*Methylomonas scandinavica*	SR5^T	97.5%	AJ131369
Slurry	I1	No	*Methylomonas*	*Methylomonas scandinavica*	SR5^T	97.5%	AJ131369
Wetland	A6*	No	*Methylomonas*	*Methylomonas fodinarum*	ACM 3268^T	96.2%	X72778
	J1	No	*Methylomonas*	*Methylomonas fodinarum*	ACM 3268^T	95.6%	X72778
	F1	Yes	*Methylosinus*	*Methylosinus sporium*	NCIMB 11126^T	98.9%	Y18946
Biofilter	A6*	No	*Methylomonas*	*Methylomonas fodinarum*	ACM 3268^T	96.2%	X72778

*Five wetland and two biofilter isolates clustered together according to rep-PCR, wetland isolate A6 was randomly selected as representative strain.

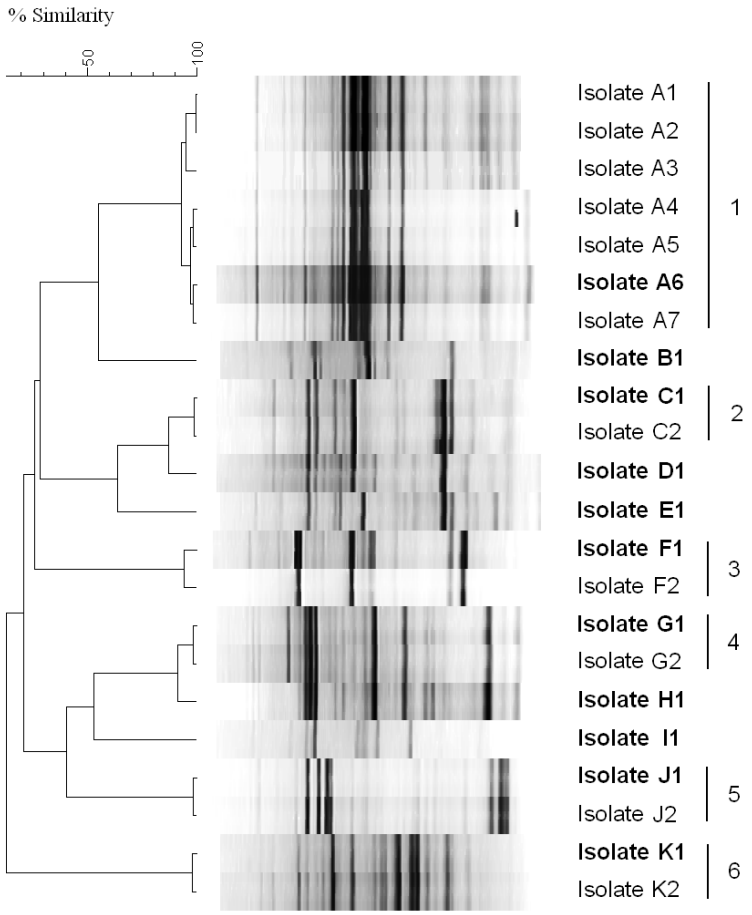


Figure II-3. GTG_5 rep-PCR fingerprinting using Pearson product moment correlation coefficient and UPGMA. Five isolates showed unique profiles (B1, D1, E1, H1 and I2) and 6 groups of isolates (1-6) showed identical profiles. F1 and F2 isolates belonged to the genus *Methylosinus*, all the other isolates were identified as *Methylomonas* spp. (Table II-2).

Randomly chosen representatives of each strain were further identified to the genus level with 16S rRNA gene sequence analysis. Ten out of eleven methanotrophic strains were assigned to the gamma-proteobacterial genus *Methylomonas*, while the remaining strain was assigned to the alpha-proteobacterial genus *Methylosinus* (Table II-2). Pairwise comparisons of the 16S rRNA gene sequences of the 10 newly isolated strains with the type strains of all species of *Methylomonas* suggested that representatives of potentially novel *Methylomonas* species were isolated from all four environments, with 16S rRNA

gene sequence similarities below 98% (Stackebrandt and Ebers, 2006). The eleven reference strains contained the *pmoA* gene, seven of which also harbored the *mmoX* gene and showed sMMO activity (Table II-2): six *Methylomonas* strains isolated from the WWTP sample as well as the *Methylosinus* sp. strain.

II.3.3 Detection of the isolated MOB in fast-growing methanotrophic communities

Sequence batch enrichments from the original samples were set up in parallel with the dilution series enrichments used for MOB isolation, with the same cultivation conditions.

To evaluate the presence of the isolates, under conditions selecting for highly active fast-growing MOB in a mixed community, a sequence batch set-up with a relatively low sludge retention time of 90 h was chosen. All sequence batch enrichments showed CH_4 oxidation activity, although the moment that a significant CH_4 oxidation (a drop below the initial average CH_4 concentration subtracted by five times the standard deviation) was observed differed between samples: 72h for the WWTP cultures, 96h for the wetland cultures and 144h for the cultures inoculated with samples from the slurry pit or the biofilter material (Figure II-4).

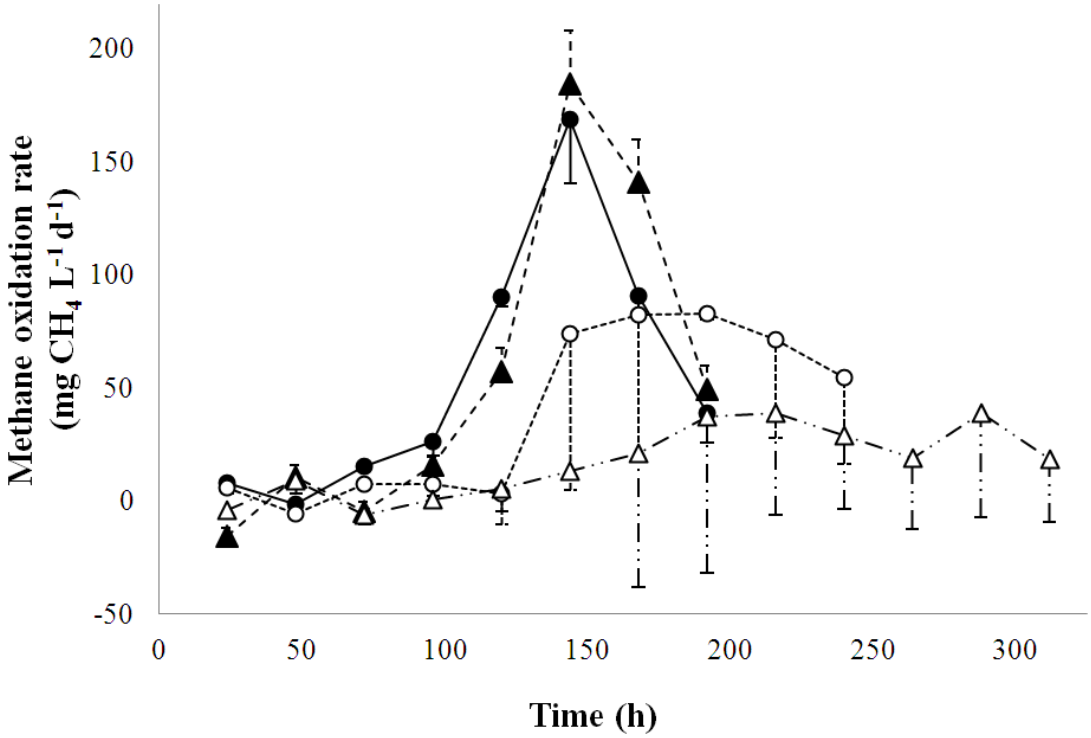


Figure II-4. The average methane oxidation rate (*n*=3, mg CH_4 $L^{-1}_{liquid\ phase}$ day^{-1}) of sequence batch enrichments of the original samples during the first cycle, for the sample of the wastewater treatment plant (black circle), slurry pit (white circle), wetland (black triangle) and biofilter material (white triangle). One-sided error bars are shown for clarity, with the same line type.

A steep rise in the methane oxidation rate over time was observed, with a maximum after 144 h of 169±28 mg CH_4 L^{-1} day^{-1} and 184±24 mg CH_4 L^{-1} day^{-1} for the WWTP and wetland cultures, respectively. The maximal methane oxidation rate (MOR) was lower for the two other cultures, with 83±3 mg CH_4 L^{-1} day^{-1} after 192 h and 39±45 mg CH_4 L^{-1} day^{-1} after 216 h for the slurry pit cultures and the biofilter cultures, respectively. When the O_2 concentration in the reactors became limiting, a decrease in the MOR was observed and a second cycle was started. The observed daily MOR for the second cycle was of the same order for all four inocula with a minimum of 108±16 (biofilter) and a maximum of 266±7 (wetland) mg CH_4 L^{-1} day^{-1} (Table II-3).

Table II-3. Overview of activity parameters of enriched cultures during the second cycle in sequence batch reactors: the methane oxidation rate (mg CH_4 L^{-1} liquid day^{-1}) during the first and second day of the second cycle for the four samples, the ratio of produced CO_2 over consumed CH_4 (mg CO_2-C mg^{-1} CH_4-C), the ratio of produced volatile suspended solids over consumed CH_4 (mg VSS mg^{-1} CH_4-C) and the ratio of consumed CH_4 over consumed NO_3^- (mg CH_4-C mg^{-1} NO_3^--N).

	WWTP	Slurry pit	Wetland	Biofilter
MOR_{day1}	182±29	203±20	266±7	108±16
MOR_{day2}	165±25	91±18	110±10	194±10
CO_2-C:CH_4-C ratio	0.56±0.02	0.53±0.04	0.55±0.02	0.51±0.03
VSS:CH_4-C ratio	0.60±0.09	0.77±0.19	0.68±0.12	0.79±0.14
CH_4-C:NO_3^--N ratio	15.7±1.3	13.0±3.3	15.6±2.4	16.5±3.5

Furthermore, *pmoA* DGGE analyses (Figure II-5) were performed on the eleven representative strains selected, following rep-PCR fingerprinting (Figure II-3) and SBR enrichments after the first cycle of each environmental sample. DGGE profiles of the WWTP and wetland enrichments were more diverse than those of the biofilter and slurry pit. From the seven *Methylomonas* strains isolated from the WWTP only one (represented by isolate K1) could not be traced back to the complete profile of the SBR enrichment, while this was the case for the other six strains, suggesting that these were dominantly present. It is however clear that different strains (isolates C1, D1, G1, E1 and H1) as proven by rep analysis, did show a *pmoA* band at a similar height, indicating that one band of the complete profile from the SBR enrichment covered a diversity of different methanotrophic strains. From the wetland enrichment, the two retrieved *Methylomonas* strains (represented by isolate A6 and J1) could be traced back to the SBR enrichment, while this was not the case for the single *Methylosinus* strain (isolate F1).

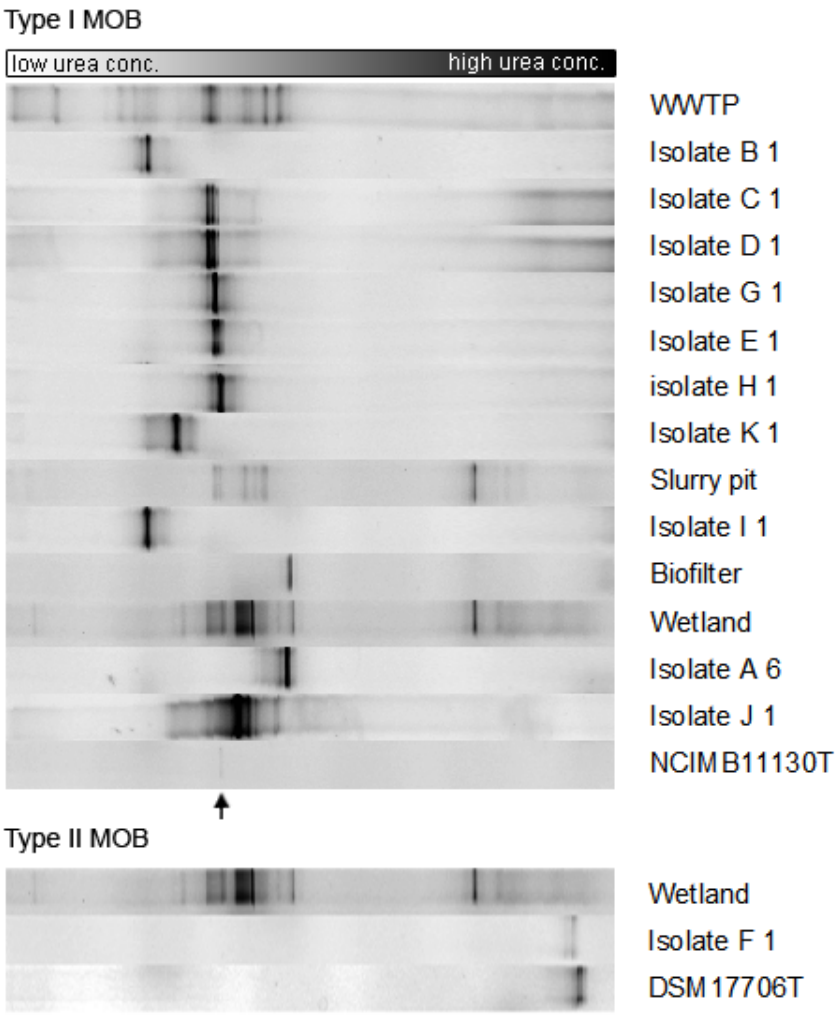


Figure II primer set A189fGC/mb661r for type I and type II MOB, respectively. The community in the active enrichment from one sequence batch reactor is shown after the first cycle for each of the four samples, i.e. a sample from the wastewater treatment plant (WWTP), a slurry pit, biofilter material and a wetland. The 11 representative strains selected based on rep-PCR fingerprinting (Figure II-3) are shown in relation to their corresponding active enrichment. Strains NCIMB11130^T (*Methylomonas methanica*, band indicated by arrow) and DSM17706^T (*Methylosinus sporium*) are shown as reference. Based on the band position of the strains, eight out of eleven representative strains could be linked to their active community, demonstrating the abundance of these strains in a mixed community selecting for fast-growing methane oxidizing bacteria.

The two isolates retrieved from the biofilter formed a single stable rep cluster with isolates from wetland, represented by isolate A6. The *pmoA* band of this strain was also observed in the DGGE profile of the biofilter, again suggesting the dominant presence in the SBR enrichment of this sample. From the slurry pit, only one strain was isolated for which the matching *pmoA* band could not be observed in the DGGE profile of the SBR enrichment. However, the *pmoA* band of this strain had a similar GC content as isolate B1 from WWTP, of which rep analysis already showed that they represented different strains.

II.4 Discussion

Methane oxidizing bacteria can serve as important sinks for the greenhouse gas CH_4 or as key players in different biotechnological industries (Semrau et al., 2010). Currently, their applicability is limited by the number of suitable strains readily available, which were not specifically isolated for this purpose, and the lack of the necessary properties for efficient use in large-scale industrial applications (Jiang et al., 2010a). Therefore, novel methanotrophic strains need to be easily obtained in culture and characterized. However, the isolation of MOB with the conventional plating approach is laborious and time consuming, requiring one or more liquid enrichment steps followed by serial dilution plating and extensive purification (Bodrossy et al., 1995, Bowman et al., 1993, Dedysh et al., 1998a, Iguchi, 2010, Whittenbury et al., 1970). Therefore, in this study, a simple and miniaturized isolation protocol was applied to efficiently isolate MOB by a combination of several recently developed cultivation procedures: a prolonged initial enrichment at low temperatures (Song et al., 2009) and adaptation of incubation conditions for desired MOB (Dedysh et al., 1998b), followed by high-throughput extinction culturing (Bodelier et al., 2005, Button et al., 1993a, Connon and Giovannoni, 2002b, Wise et al., 1999a) and subsequent purification using gellan gum plates (Dedysh et al., 2007b, Janssen et al., 2002). Other high-throughput culturing approaches were designed to favor the isolation of abundant bacteria *in situ* (Connon and Giovannoni, 2002b, Rappe et al., 2002, Song et al., 2009, Stingl et al., 2007), while our protocol specifically targeted a certain sub-

population of a specific functional group with a custom-made prolonged enrichment. This made the organisms of interest, in this case fast-growing MOB, abundant *ex situ* before extinction culturing. This approach resulted in immediate pure cultures, avoiding elaborate purification, which is known to be problematic for the isolation of MOB (Bowman, 2006). Without this approach, no immediate MOB pure culture would be obtained since heterotrophic bacteria are more abundantly present in environmental samples (Wise et al., 1999b). In perspective of biotechnological applications, varying the cultivation parameters of the initial enrichment in combination with miniaturization in 96-well plates has the potential to select for MOB with specific desired characteristics. As such, these wanted MOB can become abundant, even if non-abundant *in situ*, and can then be rapidly isolated via miniaturized extinction culturing. For example, the cultivation conditions chosen in this study, a diluted NMS medium under a high concentration of CH_4 and a relatively low hydraulic retention time, are known to select for highly active fast-growing MOB, a characteristic which is important for industrial use of bacteria (Begonja and Hrsak, 2001, Schrader et al., 2009, Wendlandt et al., 2010). Indeed, the mixed SBR communities showed a high CH_4 removal rate from 108±16 to 266±7 mg CH_4 L^{-1} day^{-1} respectively, which is in the range of reported highly active CH_4 oxidizing communities (Gebert and Grongroft, 2006, Melse and Van der Werf, 2005, Nikiema et al., 2005, Scheutz et al., 2009). By band position analysis of *pmoA*-targeted DGGE, most isolated MOB could be traced back to this active community, demonstrating that the isolated strains were able to rapidly oxidize CH_4 and grow to higher densities in a competitive setting. Most isolated MOB were indeed closely related to *Methylomonas* (type Ia), known to harbor fast-growing MOB with a short generation time of 3.5 h (Whittenbury et al., 1970). Fast-growing type Ib MOB (f.e. *Methylococcus*) with similar doubling times (Whittenbury et al., 1970) were not expected to be retrieved because they require higher isolation temperatures, while type II MOB are generally known to grow slower, with generation times ranging from 5h up to several days (Dedysh et al., 2002, Dedysh et al., 2000, Vorobev et al., 2011, Whittenbury et al., 1970). Since DGGE patterns mainly represent the major constituents of a community (Muyzer and Smalla, 1998), the few strains that could not be traced back by band position analysis probably

were expected to have longer generation times and as such were not abundantly present in the highly-active CH_4 oxidizing communities. Not all *pmoA* bands in the mixed communities had isolated representatives, therefore an up-scaling of the isolation campaign, with more extinction cultures per enrichment, would detect more novel MOB cultivable under the set conditions. This was confirmed as extinction series from initial enrichments did lead to the isolation of two different MOB strains (strains E1 & H1 and strains B1 & D1).

In total, 22 MOB isolates belonging to eleven distinct strains were obtained, which is a relatively high number compared to other studies (Auman et al., 2000, Bussmann et al., 2004, Dianou and Adachi, 1999, Miller et al., 2004). Members of *Methylomonas* (type I MOB) were isolated from all four samples, while *Methylosinus* representatives (type II MOB) were only obtained from the wetland sample. Despite that isolates were assigned to known genera, they represented at least 10 different *Methylomonas* strains, several of which belonging to novel species within the genus based on 16S rRNA gene sequence dissimilarity with *Methylomonas* type strains. Currently, four species have been validly described, although only the type strain of *Methylomonas methanica* (NCIMB 11130^T) is still accessible for the scientific community. This issue on availability of fastidious microorganisms such as MOB is a widely recognized problem and greatly hampers the in-depth investigation of their biotechnological potential, especially because properties such as substrate affinity, growth rate, substrate range or degradation of xenobiotics are strain-dependent features. For example, six different *Methylomonas* strains isolated in this study possessed sMMO, in addition to pMMO, while the type strain of the genus does not possess sMMO (Koh et al., 1993). Both sMMO and pMMO are known to degrade pollutants, such as chlorinated hydrocarbons (Bowman et al., 1993, Jiang et al., 2010b): sMMO has a broader substrate range and is known to rapidly degrade pollutants, pMMO degrades compounds at slower rates but over an extended time frame (Semrau et al., 2010). While common in type II & Ib MOB (Hanson and Hanson, 1996), few type Ia MOB such as *Methylomonas* contain sMMO, although it has been reported (Auman et al., 2000, Bussmann et al., 2006, Koh et al., 1993, Shen et al., 1997). Since type I MOB have a

higher efficiency in carbon conversion (Scheutz et al., 2009), these *Methylomonas* isolates, which possess both sMMO and pMMO, could particularly be of interest to screen for degradation of recalcitrant compounds.

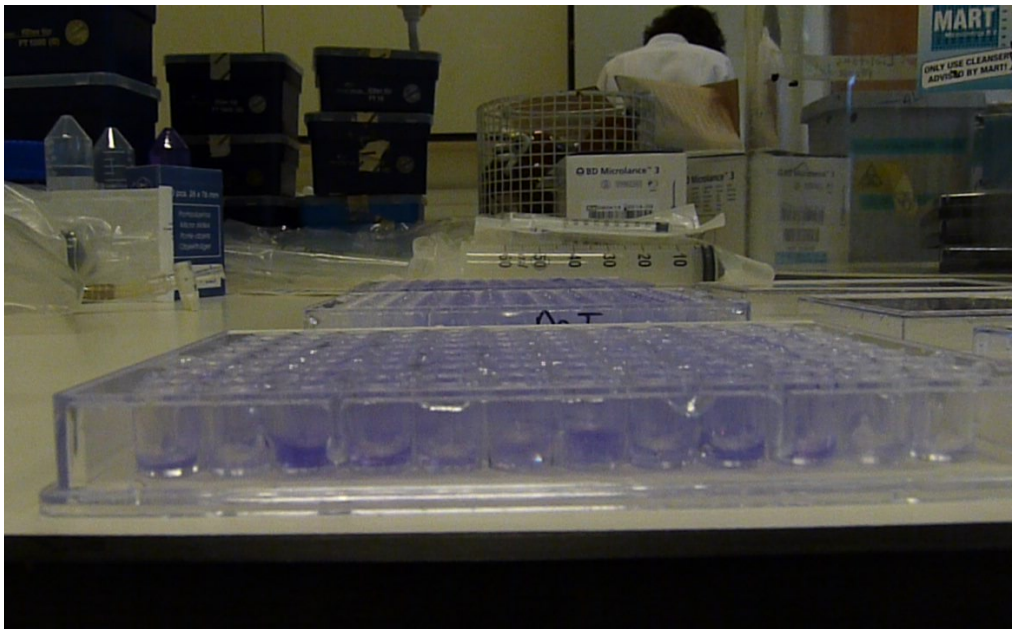
In this study, the isolation efficiency for the retrieval of fast-growing MOB between miniaturized extinction culturing and conventional dilution plating was compared. To our knowledge, such a comparison has not been previously reported. The plating approach, which is the most applied method for isolation of MOB (Dedysh et al., 2004, Dunfield et al., 2003, Heyer et al., 2005, Tsubota et al., 2005b, Wartiainen et al., 2006), did not render any MOB in this study when performed in parallel with extinction culturing from the same initial enrichments. However, the applied methodology should allow their isolation since MOB isolates obtained from extinction culturing and reference strains from public bacteria collections were cultivated successfully on solid medium in the same manner. Therefore, it is likely that additional plating trials investigating more colonies would allow the isolation of MOB. However, even then, miniaturized extinction culturing from initial enrichments will be more time and labour efficient than conventional plating in retrieving numerous methane oxidizing bacteria with specific desired characteristics.

Acknowledgements

DVDHA, SH, NB and KH designed the experiments. DVDHA and SH performed the experiments, analyzed the data and wrote the paper. DVDHA was the main responsible for the PCR-DGGE and activity tests. SH was the main responsible for the miniaturized extinction culturing and isolation. KH, NB and PDV commented on the manuscript. This work was funded by the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005) and a PhD grant for David van der Ha from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen, SB-83259). The authors gratefully thank Willy Verstraete, Suzanne Read and Jonas Ghyselinck for proof reading the manuscript. Tim Lacoere and Joke Buyse are greatly acknowledged for their help with the molecular analyses.

CHAPTER III

COPPER ENHANCES THE ACTIVITY AND SALT RESISTANCE OF METHANE OXIDIZING COMMUNITIES



Molecular work, LabMET

When the heart speaks, the mind finds it indecent to object.

Milan Kundera

Chapter III. Copper enhances the activity and salt resistance of methane oxidizing communities

Abstract

Effluents of anaerobic digesters are an underestimated source of greenhouse gases, as they are often saturated with methane. A post-treatment with methane oxidizing communities could mitigate diffuse emissions at such sites. Semi-continuously fed stirred reactors were used as model systems to characterize the influence of the key parameters on the activity of these mixed methanotrophic communities. The addition of 140 mg L^{-1} NH_4^+-N had no significant influence on the activity, nor did a temperature increase from 28°C to 35°C. On the other hand, an addition of 10 µM Cu^{2+} increased the methane removal rate by a factor of 1.5 to 1.7, since the activity of particulate methane monooxygenase (MMO) was enhanced. The effect of different concentrations of NaCl was also tested, as effluents of anaerobic digesters often contain salt levels up to 10 g NaCl L^{-1}. At a concentration of 11 g NaCl L^{-1}, almost no methane oxidizing activity was observed in the reactors without copper addition. Yet, reactors with copper addition exhibited a sustained activity in the presence of NaCl. Soluble methane monooxygenase (sMMO) was inhibited by copper, suggesting that the pMMO was the active enzyme and thus more salt-resistant. The results obtained demonstrate that the treatment of methane saturated effluents, even those with increased ammonium (up to 140 mg L^{-1} NH_4^+-N) and salt levels, can be mitigated by implementation of methane oxidizing microbial consortia.

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III.1 Introduction

Effluents of anaerobic digester installations are often oversaturated with methane (CH_4) (Hartley and Lant, 2006). When treating low strength wastewaters, up to 25% of the produced CH_4 is lost in the effluent (Cakir and Stenstrom, 2005, Hartley and Lant, 2006). It is important to control these CH_4 emissions, as this greenhouse gas is 25 times more effective at trapping heat than carbon dioxide (CO_2) (Boucher et al., 2009). Methane oxidizing communities could be used to mitigate CH_4 saturated effluents. Growth factors such as micro- and macronutrients, gas concentrations and physical parameters have an important influence on the activity and growth rate of MOB (Begonja and Hrsak, 2001, Bender and Conrad, 1995, Scheutz et al., 2009a). Generally, MOB have the highest activity in a pH range between 6 to 7 and a temperature range between 25 to 35°C (Hanson and Hanson, 1996, Scheutz et al., 2009a, Whittenbury et al., 1970b). Although nitrate is commonly used as N source in growth media for methanotrophs, ammonium can be an alternative. However, it is still unclear which concentrations of ammonium have a stimulating or inhibitory effect on the activity (Bodelier and Laanbroek, 2004).

MOB possess two CH_4 oxidizing enzyme systems: the particulate methane monooxygenase (pMMO), present in most MOB, and the soluble methane monooxygenase (sMMO), mostly precent in alpha-proteobacterial MOB (Dalton, 2005, Hanson and Hanson, 1996). The similarity between those methane oxidizing enzyme systems is low, although they perform the same task, i.e. oxidizing CH_4 (Dalton, 2005). The soluble form, sMMO has a non-heme diiron active site (Merkx et al., 2001). This cytoplasmic enzyme is mostly found in alpha-proteobacterial MOB under copper-limiting growth concentrations up to 1 µM Cu^{2+} (Jahng and Wood, 1996, Koh et al., 1993). The pMMO, which has only been studied to a limited extent, has a copper active site (Balasubramanian and Rosenzweig, 2008, Dalton, 2005). pMMO is often the only active methane monooxygenase above a concentration of 8 µM Cu^{2+} (Hakemian and Rosenzweig, 2007). In between these values, a gradual switch between both enzymes is observed (Hakemian and Rosenzweig, 2007, Shah et al., 1992). The differential expression of both

enzyme systems is thus controlled by the Cu^{2+} concentration. However, the exact mechanism of this 'copper switch' is still not known (Begonja and Hrsak, 2001, Hakemian and Rosenzweig, 2007).

The responses to elevated salt concentrations remain subject of discussion. Gulledge and Schimel (1998) and Whalen (2000) came to the conclusion that the inhibitory effect of ammonium on CH_4 oxidation was due to Cl^-, the counter ion of NH_4^+. De Visscher and Van Cleemput (2003) observed a high chloride sensitivity for alpha-proteobacterial MOB in soils. Other authors found no growth inhibition for 135 methanotrophic strains in a medium with 10 g NaCl L^{-1} (Bowman et al., 1993b).

Most research in the field of MOB focuses on soil processes. Up till now, tests with bioreactors were mostly performed with axenic cultures. Yet, methanotrophs are more active when grown in communities together with other bacteria (Begonja and Hrsak, 2001, Helm et al., 2006). The aim of this study was to examine the influence of copper and salt addition on the CH_4 oxidation activity in mixed communities. Moreover, the effect of temperature and N source was investigated. The outcomes of this study provide a first attempt towards process development for a CH_4 oxidizing bioreactor.

III.2 Experimental section

III.2.1 Sampling and enrichment

As sources for MOB, samples were taken on sites expected to contain an active and abundant methanotrophic community: an anaerobic sludge effluent tank, an open pig manure container, the top of a composting heap, an effluent vessel of an anaerobic digester, a water sample out of a vessel with rotting plant material and a soil sample. MOB were enriched in each sample: they were inoculated separately in nitrate mineral salts medium (NMS), without Cu^{2+} addition (Whittenbury et al., 1970b). The enrichment cultures were placed on a shaker (120 rpm) during 10 days under standard conditions (28°C, pH 7, 20% (v/v) CH_4 in air). Therefore airtight bottles, with a volume of 1150 mL

were used (Schott AG, Mainz, Germany), which were closed with a rubber butyl stopper (Ochs GmbH, Bovenden, Germany).

III.2.2 Experimental set-up to assess the influence of temperature, nitrogen source and copper addition (test 1)

All the enriched cultures were merged together in equal volumes (1% in total) to four identical airtight bottles of 1150 mL. The latter were filled with NMS-medium up to a volume of 400 mL and were placed on a shaker at 120 rpm (28°C, pH 7, 20% (v/v) CH_4 in air). However, one parameter was altered for each reactor. Reactor A was kept under the standard conditions as mentioned before. Reactor B was placed at a higher temperature of 35±1°C. In reactor C, the nitrate was replaced by ammonium (140 mg NH_4^+-N L^{-1}), which can be used as alternative N source for methanotrophs. To reactor D, 10 µM Cu^{2+} was added as $CuSO_4$, as this can be assumed to stimulate the expression of pMMO (Shah et al., 1992).

After each cycle of 70h, half of the well-mixed liquid phase was replenished with freshly made medium. Hence, the hydraulic retention time (HRT) and sludge retention time (SRT) were both 140h. The addition of new medium was performed under non-sterile conditions. The reactors were open for at least 30 minutes, which allowed the gas phase to equilibrate with the outside air. A volume of 150 mL of CH_4 (99.95% pure, Air Liquide, Luik, Belgium) was then added to the closed reactors. During the whole test, the pH of all reactors remained in the range of 6 to 7, which is optimal for most MOB (Hanson and Hanson, 1996).

III.2.3 Experimental set-up to assess the influence of a higher copper concentration (test 2)

Enrichment and inoculation was identical to test 1. Six reactors were placed under the standard conditions of test 1 (28°C, shaker at 120 rpm, NMS-medium without copper addition). However, to three of them, 10 µM Cu^{2+} was added. Half of the medium was replenished after 46h (one cycle), unless there was still more than 4% (v/v) oxygen present

in the reactor. This gives a HRT and SRT of 92h. From cycle 7 on, NaCl (Normapur, VWR, Leuven, Belgium) was added to the reactors at different concentrations. As a control, reactors without salt addition were maintained as in the previous cycles. After 16 cycles, half of the medium of each reactor was replaced as mentioned before. Then, they were autoclaved (20 min, 121°C, 1 bar). The airtight bottles were cooled down to room temperature and placed on a shaker, while new CH_4 was added. The CH_4 oxidation rate was followed up during 9 days.

III.2.4 Gas composition analyses

For every cycle, a gas sample was taken right after the addition of CH_4, after 1 day and at the end of each cycle, by use of a gastight syringe (Hamilton, Sigma Aldrich, Bornem, Belgium). Samples were stored in vacutainers, under atmospheric pressure (Labco limited, Buckinghamshire, UK). Gas phase analyses were performed with a Finnigan Trace GC Ultra (Thermo Fisher Scientific, Zellik, Belgium). CH_4 concentrations were measured with a Flame Ionisation Detector (FID), while CO_2 and O_2 were measured with a Thermal Conductivity Detector (TCD). The methane oxidation rate (MOR) was expressed as the chemical oxygen demand of methane (CH_4-COD) over time. The COD-value of CH_4 is 4 mg COD mg^{-1} CH_4.

III.2.5 Anion concentrations and pH

At the end of each cycle, samples were taken for further analysis. Filtered samples (0.45 µm filter, Millipore, Brussels, Belgium) were analyzed for Cl^-, NO_3^-, NO_2^-, SO_4^{2-} and PO_4^{3-}, by means of an 761 Compact Ion Chromatograph, equipped with a conductivity detector (Metrohm, Zofingen, Switzerland). Ammonium concentrations were determined by means of steam distillation, according to Greenberg et al. (1992). The pH was determined with an SP10B pH electrode, connected to a C532 multimeter analyzer (Consort, Turnhout, Belgium).

III.2.6 Biomass assessment

To correlate the CH_4 oxidation with the biomass growth, the latter was followed up by measuring the COD content in the water phase at the beginning and end of each cycle, according to Bullock et al. (1996). This analysis was performed with a kit, based on spectrophotometry (Hanna Instruments type HI 93754B-25 MR, Temse, Belgium). In test 2, cell dry weight (CDW) was assessed based on a determination of total solids and volatile solids according to Greenberg et al. (1992). To correlate both methods, simultaneous tests were done on all types of the ongoing reactors.

III.2.7 Naphtalene oxidation assay

A slightly modified version of the naphthalene oxidation assay of Brusseau et al. (1990) was used to quantitatively measure the sMMO activity. A crystal of naphthalene was added to 25 mL cell suspension and incubated at 28°C on a shaker for 2h. After incubation, 250 μl of freshly prepared tetrazotized-o-dianisidine solution (1.0 g L^{-1}) was added to each test tube, and the formation of a colored diazo-dye was immediately monitored by recording the absorbance at a wavelength of 525 nm via spectrophotometry; as described by Koh et al. (1993). The protein content of the cell suspension was determined by the total protein kit of Sigma-Aldrich (Bornem, Belgium) after lysis of the cells. The specific activity of sMMO is expressed as nanomoles of naphthol formed, per milligram of cell protein per hour.

III.2.8 Statistical analyses

Statistical analysis was performed with SPSS for Windows, version 15 (SPSS Inc., Chicago, USA). Linear regression and ANOVA were performed, after tests for homogeneity and normal distribution. A significance level of 0.05 was used (LSD).

III.2.9 DNA extraction, polymerase chain reaction (PCR) amplification and DGGE analysis

In test 2, samples were taken from the reactors, respectively after 1, 6 (just before salt addition) and 15 cycles, and stored at -20°C. DNA-extraction was performed according to Boon et al. (2000). Gel electrophoresis was performed to check if DNA was present. As gamma-proteobacterial MOB were not assumed to play an important role in the total activity (Henckel et al., 2000), primers specific for the alpha-proteobacterial MOB were chosen (Bodelier et al., 2005). These primers target a large part of the type II MOB diversity and allow detection of *Methylosinus* and *Methylocystis* species. Fragments of the 16S rRNA gene fragment were amplified using nested polymerase chain reaction (PCR), according to Bodelier et al (2005). The total amount of cycles was lowered to 15 and 25, respectively for the first and second round. An INGENY phorU2X2 DGGE-system (Goes, The Netherlands) was used for running 8% (w/v) polyacrylamide DGGE gels with a denaturating gradient ranging from 40% to 60% (Bodelier et al., 2005). The obtained DGGE patterns were subsequently processed, using Bionumerics software version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium).

III.3 Results

III.3.1 Effect of temperature, ammonium and copper on the methane oxidation activity

In test 1, four reactors with the same inoculum were incubated under different conditions: (A) a control reactor at 28°C, (B) a reactor at an elevated temperature (35°C), (C) a reactor with ammonium as N source (28°C) and (D) a reactor with Cu^{2+} addition (10 µM Cu^{2+}, 28°C). For reactors A, B and D, nitrate mineral salts (NMS) medium was used. In reactor C, NO_3^--N was replaced by the same concentration of NH_4^+-N (140 mg L^{-1}).

There was no significant difference in CH_4 oxidation activity observed between the reactors under the different conditions of N source or temperature (reactors A, B and C). From cycle four on, the average methane oxidation rate (MOR) of these three reactors

was 393±109 mg CH_4-COD L^{-1} d^{-1} (Table III-1). This was observed for eleven consecutive cycles. The average biomass growth, expressed as cell dry weight (CDW), also stabilized from the fourth cycle on, with an average value of 162±17 mg CDW-COD L^{-1} $cycle^{-1}$. The average growth yield of these reactors was 0.25±0.04 g CDW-COD g^{-1} CH_4-COD. The pH-value over the whole test period was 6.4±0.2 for all three reactors. The average nitrogen consumption of the three reactors was 18±4 mg N L^{-1} per cycle (70h). In most cases, the nitrite concentration was lower than 2 mg NO_2^--N L^{-1}. In the reactor with NH_4^+ as N donor, NO_3^--N and NO_2^--N concentrations stayed below the detection limit (1 mg NO_2^- L^{-1}).

Table III-1. Influence of the addition of 10 µM Cu^{2+} on the average and maximum MOR (mg CH_4-COD L^{-1} d^{-1}) during the first day of each cycle, the biomass growth (mg CDW-COD L^{-1} $cycle^{-1}$), the growth yield (mg CDW-COD mg^{-1} CH_4-COD) and pH during test 1, over 11 consecutive cycles. For the reactors without copper, the average values of reactors A (control), B (35°C) and C (NH_4^+-N) are given.

	no Cu^{2+}	with Cu^{2+}
Average CH_4 oxidizing activity	393±109	582±109
Maximum CH_4 oxidizing activity	703±89	1053
Biomass growth	162±17	225±23
Growth yield	0.25±0.04	0.26±0.03
pH	6.4±0.2	6.3±0.2

In reactor D, the CH_4 oxidizing activity was significantly influenced by a Cu^{2+} addition of 10 µM Cu^{2+} ($p \leq 0.001$). The average oxidation rate of 582±109 mg CH_4-COD L^{-1} d^{-1} was a factor of 1.5 higher than the average of the other three reactors. Nevertheless, this higher activity had no significant effect on the biomass growth, nor growth yield (Table III-1). The final gas concentration at the end of each cycle did not differ significantly between the four tested reactors. At the end of each cycle, O_2 levels dropped to values lower than 1% (v/v). The gas phase concentration of CH_4 and CO_2 at the end of each cycle was 4.2±1.1 and 5.3±1.2% (v/v) respectively. During a cycle of 70h, on average 861±144 mg CH_4-COD L^{-1} was oxidized.

III.3.2 Effect of copper on the methane oxidation activity and the microbial community

To confirm the influence of Cu^{2+} addition on the CH_4 oxidation activity, a new test was performed. In test 2, six reactors were set-up as before. In three of them, 10 µM Cu^{2+} was supplemented; the other three reactors served as controls without Cu^{2+} addition. During the first 96h after inoculation, the biomass concentration increased from less than 22 to 231±45 mg CDW-COD L^{-1}, without significant differences between the reactors (p<0.05). From the second cycle on, the average biomass growth was stable, with average values of 200±9 and 237±42 mg CDW-COD L^{-1} $cycle^{-1}$ for the reactors without and with Cu^{2+} respectively (Table 2). There was no significant difference in growth yield, nor C:N ratio, between the reactors with and without Cu^{2+} addition (Table 2). The activity was a factor of 1.7 higher in the reactors with Cu^{2+} addition. An average oxidation rate of respectively 452±79 (without Cu^{2+}) and 755±40 (with Cu^{2+}) mg CH_4-COD L^{-1} d^{-1} was found over the last five cycles (Table 2). The CH_4 concentration after 46h was generally around 3.5% (v/v) for the reactors with Cu^{2+} addition, while the O_2 level dropped below 0.5% (v/v). The reactors without Cu^{2+} addition did not reach these low levels after 46h. There was still about 2% (v/v) O_2 and 5% (v/v) of CH_4 present in the gas phase.

No sMMO was detected in the reactors with Cu^{2+} addition. For the reactors without Cu^{2+} addition, a naphthalene oxidation rate of 121±26 nmol mg^{-1} protein h^{-1} was observed.

Changes in the alpha-proteobacterial MOB composition of the reactors were evaluated by means of DGGE analysis . After six cycles, different CH_4 oxidizing species became dominant in the reactors with and without Cu^{2+} respectively, which is reflected in a low Pearson correlation of less than 60%. The communities in the reactors with the same treatment were similar to a high extent, with a Pearson correlation in all cases higher than 80%.

Table III-2. Influence of the addition of 11.2 g NaCl L^{-1} salts on the average (cycle 11 to 16) and maximum MOR (mg CH_4-COD L^{-1} d^{-1}), the biomass growth (mg CDW-COD L^{-1} $cycle^{-1}$), the C/N ratio (mg NO_3^--N mg^{-1} CDW-COD) and the sMMO activity (nmol mg^{-1} proteine h^{-1}), for the reactors with and without 10 µM Cu^{2+} during test 2.

	no Cu^{2+}		with Cu^{2+}	
	no NaCl	**with NaCl**	**no NaCl**	**with NaCl**
Average CH_4 oxidizing activity	452± 79	177±111	755±40	396±95
Maximum CH_4 oxidizing activity	549±46	439±114	1147±19	474±61
Biomass growth	134±9	122±13	159±28	162±10
C/N-ratio	20±4	17±5	17±4	17±4
sMMO	120	111	not	not

III.3.3 Effect of salt addition on the methane oxidation activity and the microbial community

An addition of 7 g NaCl L^{-1} to the reactors of test 1, did not result in a significant decrease of activity (p<0.05). However, an addition of 8.5 g NaCl L^{-1} resulted in a decreased MOR in all four reactors. The activity decreased with a factor four in the reactors without Cu^{2+} addition, where it lowered only with a factor two in the reactor with Cu^{2+} addition. This salt concentration was also tested in the reactors of test 2. Addition of 8.5 g NaCl L^{-1} lowered the MOR to the same extent as in test 1 (Figure III-1). From the eight cycle on, the sodium chloride concentration was increased again, to a final value of 11.2±0.5 g NaCl L^{-1}. This led to a decrease in activity in all the reactors. The highest decrease was observed in the reactors without Cu^{2+} addition. The activity of the latter was a factor 10 lower, with an average MOR of 39±12 mg CH_4-COD L^{-1} d^{-1}, immediately after the salt addition. The MOR was lower than 60 mg CH_4-COD L^{-1} d^{-1} during the first ten days following the salt addition. Moreover, one of the reactors did not show any oxidation during those ten days. After this period, these reactors showed an increasing performance with a maximum oxidation rate of 439±59 mg CH_4-COD L^{-1} d^{-1}. However, their activity showed a large variation.

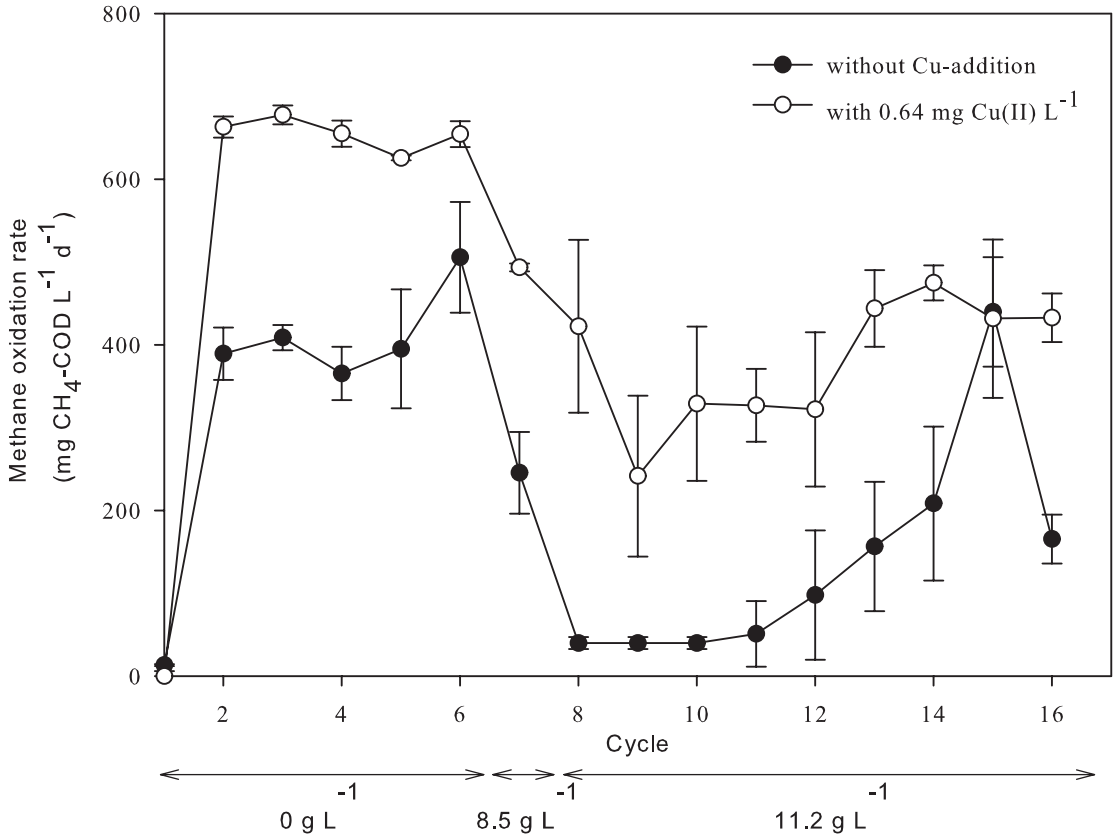


Figure III-1. The influence of salt addition on the methane oxidation rate of reactors without (black circle) and with (white circle) Cu^{2+} addition (10 µM) during test 2. The average MOR (mg CH_4-COD L^{-1} d^{-1}) and the standard error were calculated for the first day of each cycle. During the seventh cycle, the concentration of NaCl was 8.5 g L^{-1}. From cycle 8 on, the NaCl concentration was 11.2 L^{-1}.

The decrease in activity was less in the reactors with Cu^{2+} addition. The MOR decreased 41%, from 715±11 to 422±180 mg CH_4-COD L^{-1} d^{-1} when 8.5 g NaCl L^{-1} was added (cycle 8). In the next cycle (cycle 9), the MOR dropped to 241±168 mg CH_4-COD L^{-1} d^{-1} (Figure III-1). However, in the following cycles, these reactors showed a stable performance between 325 and 475 mg CH_4-COD L^{-1} d^{-1}, with a maximum of 474±61 mg CH_4-COD L^{-1} d^{-1} in the eight cycle after the first salt addition (cycle 14) (Figure III-1).

The salt addition had no influence on the N demand. However, in the reactors with Cu^{2+} addition, nitrite concentrations up to 40 mg NO_2^--N L^{-1} were detected. The increase of the nitrite concentration corresponded with the decrease in the nitrate level.

The salt addition altered all alpha-proteobacterial MOB communities to a large extent. In all reactors, expression of new DGGE bands was observed. The dominant alpha-proteobacterial MOB species before salt addition were less abundant after 9 cycles of higher salt concentrations, while other alpha-proteobacterial MOB species became dominant (Figure III-2).

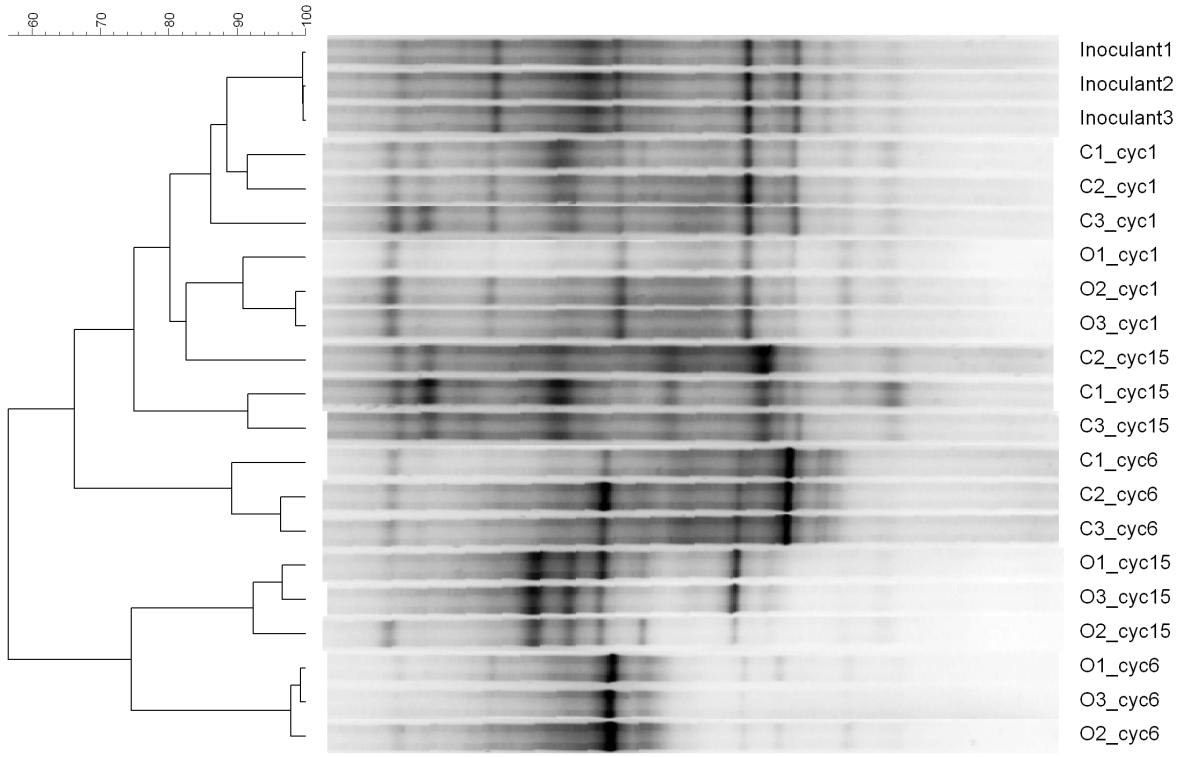


Figure III-2. Changes in DGGE patterns of the alpha-proteobacterial MOB community, in the different reactors of test 2, resulting from the addition of Cu^{2+} and/or salts. DGGE clustering was performed, based on the Pearson correlation (given as a percentage). The reactor samples are labelled as follows: inoculum (in triplicate), without Cu^{2+} (O1 to O3) and with Cu^{2+} (C1 to C3), after 1 cycle without salt (cyc1), after 6 cycles without salt (cyc6) and 15 cycles (9 cycles with salt addition) (cyc15).

There was no significant difference in biomass growth between the reactors with and without salt addition nor between the reactors with and without Cu^{2+} (Table III-2). When the cell dry weight was compared with COD measurements, a ratio of 1.49±0.12 mg COD mg^{-1} CDW was found for samples with, as well as without salt addition. The growth yield was independent of the Cu^{2+} and salt concentration, and was on average 0.22 mg CDW-COD mg^{-1} CH_4-COD.

From cycle 17 on, the concentration of salt was adjusted to 30 g NaCl L^{-1}. During the test period of ten days, the MOR were in all reactors below 50 mg CH_4-COD L^{-1} d^{-1}. No CH_4 oxidation could be detected after heat killing the active communities.

III.4 Discussion

In this study efforts are made to identify important parameters that affect CH_4 oxidizing microbial systems, applicable for the treatment of CH_4 containing effluents. It was found that the MOR was not influenced by either increased temperature, or by the presence of exogenous ammonium supplementation. The addition of Cu^{2+} or NaCl, however, had a significant influence on the MOR.

III.4.1 The effect of temperature and ammonium

A temperature increase from 28° to 35°C did not significantly affect the activity or growth rate of the MOB. It has been reported that most MOB have an optimal temperature of 30°C, which explains why an increase to 35°C was not accompanied by a higher activity (Park et al., 2005, Scheutz and Kjeldsen, 2004). Consequently, treatment of effluents in the range between 28°C and 35°C should not give problems.

Changing the N source from nitrate to ammonium also had no significant influence on the MOR. This result contrasts with those reported by other research groups, who observed inhibition of CH_4 oxidation at ammonium levels of 140 mg N L^{-1} (Begonja and Hrsak, 2001, Dalton, 2005, Hanson and Hanson, 1996, King and Schnell, 1998, Nyerges and Stein, 2009). These conflicting observations suggest that more insight is needed into the specific effects

that ammonium has on the microbial CH_4 oxidation process, especially in mixed culture conditions.

The communities in the different reactors behaved similarly under the different environmental conditions. Hence, stable CH_4 oxidizing communities were present. This was confirmed by DGGE analysis and the presence of active sMMO (Figure III-2). These communities reached a stable MOR after a start-up period of less than 72h (Table III-1). The observed activity, growth yield and nitrogen consumption were similar to previously described methanotrophic-heterotrophic communities, and confirm the presence of high methanotrophic activity in mixed communities (Helm et al., 2006, Hrsak and Begonja, 2000, Melse and Van der Werf, 2005). The used semi-continuous model system seems a good model for CH_4 oxidizing bioreactors, since the removal rates were similar (Melse and Van der Werf, 2005). Fluorescent *in situ* hybridisation revealed that only a small amount of alpha-proteobacterial MOB was present in the microbial community (data not shown). The CH_4 concentration at the end of each cycle was, in agreement with the results of all tested reactors, never lower than 3.5% (v/v). It seems that the observed O_2 concentration of 0.5% (v/v) at the end of most cycles, was the lowest O_2 level that allowed CH_4 oxidation. This is in agreement with other authors, who found maximum rates of CH_4 oxidation in a range of 0.45 to 20% (v/v) O_2 (Henckel et al., 2000, Wilshusen et al., 2004).

III.4.2 The effect of copper

Copper had a significant positive effect on the MOR. The addition of 10 µM Cu^{2+} increased the average oxidation rate in test 2 by a factor of 1.7 to 755±40 mg CH_4-COD L^{-1} d^{-1} (Table 2). This is slightly more than reported in studies with axenic methanotrophic cultures, where a factor of 1.2 to 1.5 has been found (Begonja and Hrsak, 2001, Joergensen and Degn, 1987). Oxygen became a limiting factor for growth before the end of each cycle. Therefore, the higher methane oxidation rate after Cu^{2+} addition did not lead to a significantly higher biomass concentration at the end of each cycle. However, no conclusions can be made about the specific growth yield of the MOB, as only the biomass growth of the whole bacterial community was measured.

The addition of Cu^{2+} had a distinct effect on the alpha-proteobacterial MOB community. DGGE analyses of test 2 revealed that the profiles of replicate treatments were very similar throughout the entire test period, although there was no direct cross-inoculation (Fig. 2). In contrast, Cu^{2+} addition had a distinctive selective power, as it shifted the alpha-proteobacterial MOB community to an important extent. Moreover, the Cu^{2+} addition had an influence on the MMO-enzymes. No sMMO activity was detected in the reactors with Cu^{2+} addition. The latter is in agreement with former observations, where a concentration of 10 µM Cu^{2+} had an inhibitory effect on sMMO expression, while stimulating the pMMO expression (Hakemian and Rosenzweig, 2007, Jahng and Wood, 1996, Shah et al., 1992).

III.4.3 The effect of salt addition

The addition of NaCl up to a concentration of 7 g NaCl L^{-1} had no significant influence on the MOR. However, a concentration of 8.5 g NaCl L^{-1} led to a decrease in CH_4 oxidizing activity in all reactors. Immediately after the addition of 11.2 g NaCl L^{-1}, the reactors without Cu^{2+} showed almost no activity (< 50 mg CH_4-COD L^{-1} d^{-1}) for ten days (Figure III-1). These results are in agreement with Schnell & King (1996), who found a 35% lower CH_4 oxidation when a salt concentration of 10 g NaCl L^{-1} was amended to the alpha-proteobacterial *M. trichosporium* OB3b, although these results contrast with the results of Bowman et al. (1993b), who did not observe a high sensitivity for NaCl.

Interestingly, the MOR remained above 250 mg CH_4-COD L^{-1} d^{-1} in the reactors which received both Cu^{2+} and NaCl (11.2±0.5 g L^{-1}). The different response to increased salt concentrations could be a result of either community and/or enzyme level effects. At the community level, a population shift was seen during the cycles without salt addition. It is possible that a fortuitously selection took place towards more salt tolerant species. This, however, appears unlikely, as the dominant alpha-proteobacterial MOB species before and after salt addition were completely different (Figure III-2). Moreover, since no significant activity was seen at a concentration of 30 g NaCl L^{-1}, it is clear that truly halophilic methanotrophs were not present in considerable amounts. However, over time,

the fact that the MOR recovered partly in all reactors (Figure III-1) suggests that salt tolerant species were enriched and became more abundant, as shown by DGGE analysis.

The fast decrease in activity after salt addition is probably due to differences at the enzymatic level. The immediate effect of the salt addition clearly depended on the Cu^{2+} concentration (Table 2). As copper is the key element in the expression of both sMMO and pMMO, the differential effect of salt on reactor performance could be related to the type of methane monooxygenases present in the different reactors: the reactors without Cu^{2+} were enriched with methanotrophs that showed sMMO activity, whereas the reactors with added Cu^{2+} addition did not show any sMMO activity. As the only other enzyme in MOB known to oxidize CH_4 with such a high rate is pMMO, the MOB in these reactors appear to have pMMO as the active methane monooxygenase (Begonja and Hrsak, 2001, Hakemian and Rosenzweig, 2007, Shah et al., 1992). This suggests that sMMO and pMMO possess different sensitivities towards NaCl. To our knowledge, these findings have not previously been reported and need to be confirmed by means of pMMO and sMMO expression assays. However, they are consistent with the work of Trotsenko and Khmelenina (2002), who found that only 2 of the 30 salt-resistant methanotrophs they analyzed produced sMMO. This difference in sensitivity can also be due to the different locations of the monooxygenase enzymes in the bacterial cells, rather than the intrinsic tolerance of the enzyme itself. pMMOs are embedded in intraplasmatic membranes, which might offer protection against the increased salt concentrations, where sMMOs have less osmotic protection in the cytoplasm (Colby et al., 1977, Dalton, 2005). If location is more important than enzyme structure itself, then the difference in sensitivity might also extend to other types of salts or solutes.

After ten cycles with salt addition, the MOR were still a factor of two lower than the controls. It has been reported that bacteria use additional energy to regulate their metabolism under hyperosmotic conditions (Csonka, 1989). That could lead to a lower bacterial activity. The formation of nitrite could also be a reason for the lower activity. Nitrite concentrations up to 40 mg NO_2^--N L^{-1} were observed in the reactors where Cu^{2+} and salt were amended. Although the reason for the high nitrite concentration is unknown,

similar levels of nitrite have been shown to reduce the CH_4 oxidation activity of at least three axenic MOB strains (Nyerges and Stein, 2009).

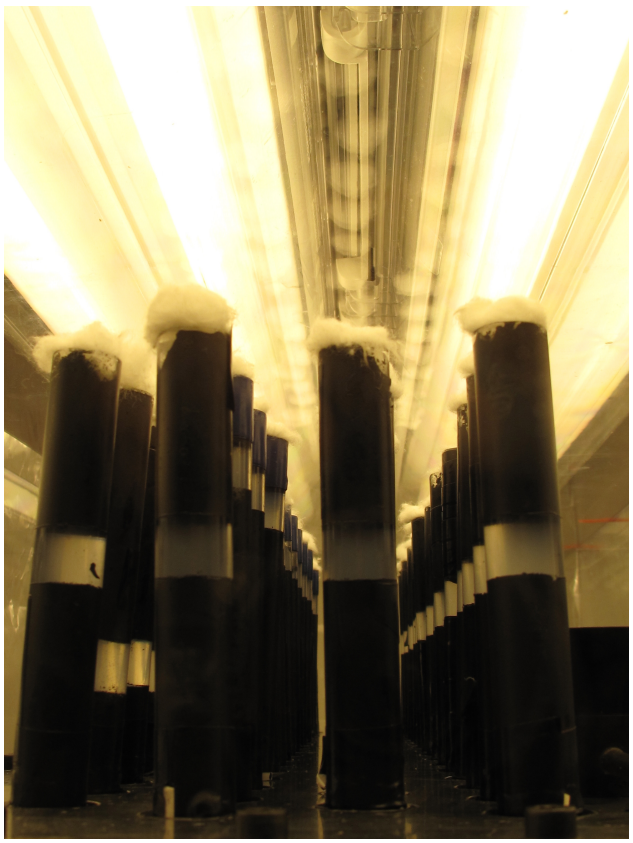
The findings of this work provide a first attempt for optimizing microbial CH_4 removal systems. The maximum MOR achieved was 280 mg CH_4 L^{-1} reactor d^{-1}. As the maximum solubility of CH_4 in water is 26 mg CH_4 L^{-1} (22°C) (Melse and Van der Werf, 2005), a hydraulic retention time of 2.2 h would be required to treat CH_4 saturated effluents. Because of the low solubility of both CH_4 and O_2, treatment of the aerated water phase as such would be necessary. Since the MOR is comparable at temperatures of 28°C and 35°C, good CH_4 removal can be achieved at lower temperatures. In situations where increased Cu^{2+} concentrations are acceptable, addition of Cu^{2+} to methanotrophic bioreactors should be considered, as this increased the MOR by more than 50%. Anaerobic effluents can contain salt concentrations of up to 10 g NaCl L^{-1} (Gourdon et al., 1989, Ismail et al., 2008). As concentrations of up to 7 g NaCl L^{-1} and 140 mg NH_4^+-N L^{-1} did not seem to have a negative influence on the MOR, treatment of CH_4 saturated effluents, rich in salts and high in ammonium should be possible.

Acknowledgements

DVDHA, WV and NB designed the experiment. DVDHA performed the experiments, analyzed the data and wrote the manuscript. PB gave input on the experimental design, helped with the gas analyses and commented on the manuscript. SH maintained the MOB cultures. SH, WV and NB commented on the manuscript. This work was supported by a Ph.D. grant (no. 83259) from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen), a research grant from the Flemish Fund for Scientific Research (FWO-Vlaanderen, 3G070010) and by the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005). Tim Lacoere is greatly acknowledged for the help with the molecular analyses. Also thanks to Jan Vermeulen and Samuel Bodé for their support with GC-analyses and Vicky D'havé for her assistance. Furthermore, Anthony Hay, Joachim Desloover, Yu Zhang and Willem De Muynck are gratefully appreciated for critically reading the manuscript.

CHAPTER IV

SELECTION OF A METHANE OXIDIZING CONSORTIUM RESISTANT TO HIGH AMMONIUM LOADS



Microsensor setup, DTU, Copenhagen

De wereld is verdergegaan.

Roland Deschain

Chapter IV. Selection of a methane oxidizing consortium resistant to ammonium loads

Abstract

Methane oxidizing bacteria (MOB) form a biological methane sink, thereby providing a key ecosystem service of global importance. Their resilience to ammonium amendments has been examined repeatedly, but it is unclear if the variation in response of methanotrophic communities to increased ammonium concentrations is mainly due to the community structure or due to adaptation at a physiological level. Here, the activity and composition of a methanotrophic community was assessed over time, in order to compare the influence of a sudden and a gradual increase of the ammonium concentration, respectively. In contrast to a sudden increase, MOB showed remarkable resilience to gradually increasing ammonium loads. For the first time, substantial methane oxidation activity was observed for a methanotrophic culture exposed to ammonium loads up to 2500 mg NH_4^+-N L^{-1}. Analyses based on the 16S rRNA gene diversity by denaturing gradient gel electrophoresis and a *pmoA*-based microarray showed that the composition of the methanotrophic community was affected by high ammonium amendments, favoring type Ia *Methylosarcina*-like MOB. The ability of a methanotrophic community to withstand a gradual increase of the ammonium concentration is important with regard to fertilizing programs in agriculture; a gradual increase of ammonium levels allows the MOB to adapt and could help to preserve the methane sink in agricultural soils.

Redrafted after:

van der Ha, D., Gorteman, D., Ho, A., Hoefman, S., Schneider, B., Frenzel, P., De Vos, P. and Boon, N. Selection and characterization of a methane oxidizing consortium resistant to high ammonium loads. Env. Microbiol. Rep. (submitted).

IV.1 Introduction

Proteobacterial methane oxidizing bacteria (MOB) are one of few bacterial groups that are able to use CH_4 as sole carbon and energy source and are therefore key players in mitigation strategies for this greenhouse gas (Denman et al., 2007). These MOB, allocated within the class of the *Gamma-proteobacteria* (type Ia/Ib MOB) and the *Alpha-proteobacteria* (type II MOB) are together with the Verrucomicrobia the best known aerobic methane oxidizing species. The MOB possess methane monooxygenase enzymes (MMO), existing in a soluble and particulate form, allowing them to oxidize CH_4 (Dalton, 2005). These enzymes have a low specificity for CH_4, resulting into co-metabolic oxidation of NH_3 among other chemicals (Dalton, 1977; Jiang et al., 2010). However, the NH_3 oxidation products hydroxylamine and nitrite can constrain the MOB, while at the same time a competitive inhibitory effect occurs (King and Schnell, 1994; Nyerges and Stein, 2009). Then again, NH_3/NH_4^+ can stimulate the activity and growth of MOB, as the bacteria use the N as building block for their cell structures (Anthony, 1982; Bodelier and Laanbroek, 2004). Therefore, ammonium amendments can lead to a decrease as well as increase of the methane oxidation, depending on the applied concentration, the duration and other affecting factors (De Visscher and Van Cleemput, 2003; Stein and Klotz, 2011). One example is the effect of the prevailing pH on the inhibitory effect; as NH_3 is more toxic than NH_4^+ towards MOB (pKa = 9.23), an increased inhibition occurs at higher pH values (O'Neill and Wilkinson, 1977).

Amendments of NH_3/NH_4^+ also influence the community structure, as MOB show differences in their resilience, resulting into selection for NH_3/NH_4^+-tolerant strains (Nyerges and Stein, 2009). These strain-specific characteristics also explain partly why test results with axenic cultures do not correlate well with these of mixed cultures in natural environments (Nyerges et al., 2010). While some MOB, often type II species, are known to be highly sensitive to NH_4^+ loads of only 125 mg NH_4^+-N L^{-1}, others do not experience any inhibitory effect with such concentrations (Begonja and Hrsak, 2001; Nyerges and Stein, 2009; Veillette et al., 2011). Various reasons have been put forward to explain these at

first sight contradictory results: (i) physiological differences without taxonomic correlation (Nyerges and Stein, 2009), (ii) ratio of applied NH_4^+ over available CH_4 (King and Schnell, 1994; Bodelier et al., 2000), (iii) osmotic effects of salts (King and Schnell, 1998), (iv) physiological mechanisms regulating the NO_2^--production (King and Schnell, 1994), (v) presence of N-fixing MOB or nitrifying bacteria in the community (Bodelier and Laanbroek, 2004; Noll et al., 2008) or (vi) N limited growth conditions (Bodelier and Laanbroek, 2004). However, as all these mechanisms occur simultaneously, it is difficult to differentiate among the various effects.

The main goal of the present study is to determine the capacity of a methanotrophic community to adapt to increasing NH_4^+-N loads. Therefore, sequential enrichments were performed with increasing NH_4^+-N loads up to a concentration of 2500 mg NH_4^+-N L^{-1}. Changes in methanotrophic activity and community composition were determined using methane measurements and molecular techniques, respectively. This approach allowed to evaluate the adaptation capacity of a methanotrophic community and to identify NH_4^+ tolerant species.

IV.2 Experimental section

IV.2.1 Inoculation and cultivation of methanotrophic communities

To achieve a high methanotrophic diversity, the starting inoculum was assembled by merging samples from a compost heap, top soil with leaf litter, anaerobic sludge and the top layer of a denitrification tank of a wastewater treatment plant (Ossemeersen, Gent, Belgium), all enriched for 72h with 20% (v/v) CH_4 in air. Ammonium mineral salts medium (AMS) containing 125 mg NH_4^+-N L^{-1}, amended with a buffer of 2 mM $Na_2HPO_4.12H_2O$ and 2 mM KH_2PO_4 was used as growth medium (10 times dilution of original samples). Then, 5 mL of each sample was merged and enriched in 100 mL AMS for another 72h in order to have an adapted mixed community.

This inoculum was then diluted in AMS medium (85 mg COD L^{-1}) and subjected to three different treatments (Figure IV-1). During the first cycle, the starting inoculum was suddenly exposed to different NH_4^+-N concentrations, varying from 125 to 2000 mg NH_4^+-N L^{-1}. During nine more cycles, the three evolved cultures with 125 mg NH_4^+-N L^{-1} (AMS medium) were used as controls whereby the NH_4^+ concentration was kept at that level. The incubations with 250 mg NH_4^+-N L^{-1} were used to evaluate the influence of a gradually increasing N concentration. All tests were performed in 595 mL Schott bottles filled with 100 mL AMS and 20% CH_4 (v/v) in air, shaken (120 rpm) in the dark at 28°C. During all cycles, the activity was followed up by measuring the CH_4 and O_2 consumption by means of GC analyses (van der Ha et al., 2011) and compared to the control bottles. A cycle was stopped when the concentration of O_2 in the gas phase dropped below 5% (v/v). In the incubations with a gradual NH_4^+-N increase, the NH_4^+ concentration was increased each cycle with 250 mg NH_4^+-N L^{-1} up to a value of 2500 mg NH_4^+-N L^{-1}.

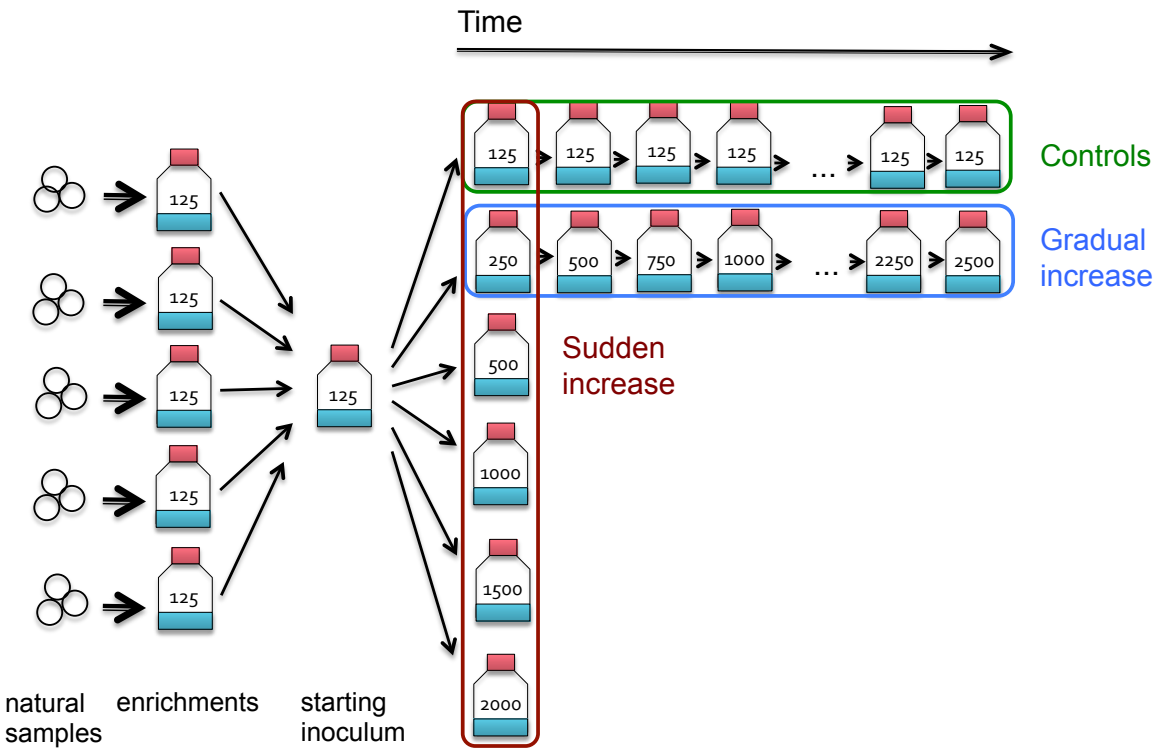


Figure IV-1. Schematic overview of the inoculation strategy and the three applied treatments.

In all tests, standardization of the starting concentration of bacterial mass occurred (85±13 mg COD L^{-1}) by means of chemical oxygen demand (COD) measurements, as this allowed to analyze in a quick and accurate way the amount of present biomass. Simultaneous cell dry weight (CDW) measurements (Greenberg et al., 1992) showed that these values correlated with 43±10 mg CDW. The starting pH was between 6.2 and 6.8, depending on the NH_4Cl-amendment.

IV.2.2 Physico-chemical analyses

The start concentrations of NH_4^+-N were followed up in 96 well plates by means of a colorimetric method (Taylor et al., 1974). The NH_4^+-N consumption was less than the error on the analysis (5% of value) and could therefore not be evaluated. The concentrations of dissolved NO_3^- and NO_2^- were followed up by ion chromatography (van der Ha et al., 2011), but were for all cycles and treatments lower than 2 mg NO_3^--N or NO_2^--N. All results were statistically analyzed by an independent two-sample Student t test with equal variance and at a significance level of 95%

IV.2.3 Molecular techniques

DNA was extracted by means of a FastDNA SPIN kit for soil (MP Biomedicals, USA) and PCR products were obtained by means of the specific MOB primer set A189f/mb661r, targetting the *pmoA* gene (Costello and Lindstrom, 1999), in accordance with van der Ha et al. (2012). DGGE was performed with an INGENY phorU2x2 System (Goes, Nederland) and analyzed by means of BioNumerics software version 5.10 (Applied Maths, Belgium) (van der Ha et al., 2012).

The microarray analysis was performed with minor modifications as described before (Bodrossy et al., 2003, Ho et al., 2011), using the A189f/T7-A682r primer combination. Hybridization signal was summed up, and the mean of the triplicates was used for standardization. Analysis of the standardized microarray data was performed in *R ver.2.10.0* (Oksanen et al., 2010) and implemented in *heatmap 2* in *gplots* ver.2.7.4. The

microarray has a wide probe coverage targeting known type I and type II MOB (Bodrossy et al., 2003). Probes grouped as 'Others' consist of *amoA* (a gene encoding for ammonia monoxygenase), *pmoA2* (an isozyme of the *pmoA*; Baani and Liesack (2008)); Verrucomicrobial methanotrophs, and other environmental clusters without a defined function (methane or ammonium oxidizers).

IV.3 Results

IV.3.1 Influence of increasing NH_4^+ amendments on the methanotrophic activity

A methanotrophic starting inoculum was incubated under three different conditions (Figure IV.1). The first set of conditions concerned a sudden increase of the NH_4^+ concentration from initially 125 mg NH_4^+-N L^{-1}, as typically applied in methanotrophic growth medium (AMS), up to a concentration of 2000 mg NH_4^+-N L^{-1}. Sudden increases up to concentrations of 500 mg NH_4^+-N L^{-1} did not result in an extended lag phase or a significant decrease of the methane oxidation rate (MOR) (Figure 1). However, from a concentration of 1000 mg NH_4^+-N L^{-1} onwards, significantly lower MOR were observed (p<0.05, n=3), and the variance between triplicates increased with higher NH_4^+ concentrations. At the same time, higher concentrations led to extended lag phases (Figure IV-2).

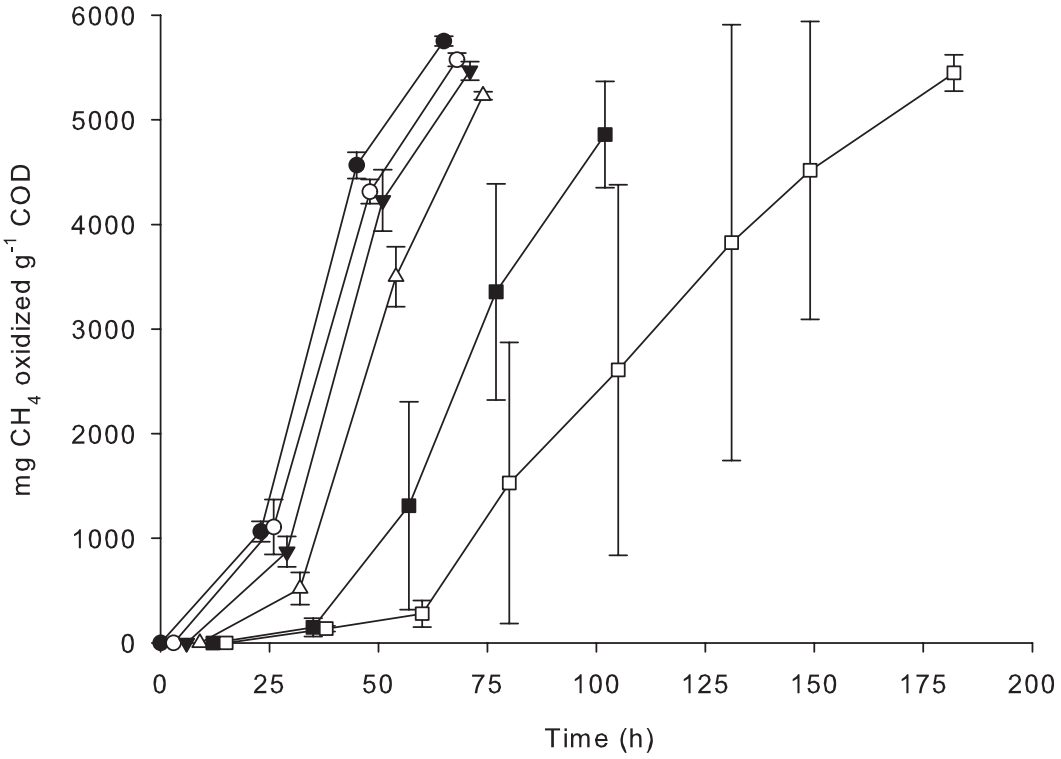


Figure IV-2. Cumulative methane consumption (mg CH_4 g^{-1} chemical oxygen demand or COD) after a sudden increase in NH_4^+ concentration from 125 (control, filled circle) to 250 (empty circle), 500 (filled triangle), 1000 (empty triangle), 1500 (filled square) and 2000 (empty square) mg NH_4^+-N L^{-1}, respectively (*n*=3). For clarity, each curve was shifted 3h.

After this first cycle, the two other conditions were tested. For ten cycles, the NH_4^+-N concentration of control incubations was kept at 125 mg NH_4^+-N L^{-1}. In parallel, a sequential incubation with a gradually increasing NH_4^+ concentration was applied. Therefore, the NH_4^+-N concentration was increased from 250 mg NH_4^+-N L^{-1} up to 2500 mg NH_4^+-N L^{-1}, in steps of 250 mg NH_4^+-N L^{-1} $cycle^{-1}$. During these series of incubation cycles, no significant difference in MOR (after 48h) was observed between the control treatment and the gradually increasing treatment, up to a concentration of 2000 mg NH_4^+-N L^{-1} (Figure IV-3). Above this concentration, one of the triplicates showed a low activity, leading to a high variance between the triplicates and a significantly lower methane MOR compared with the controls (p<0.05).

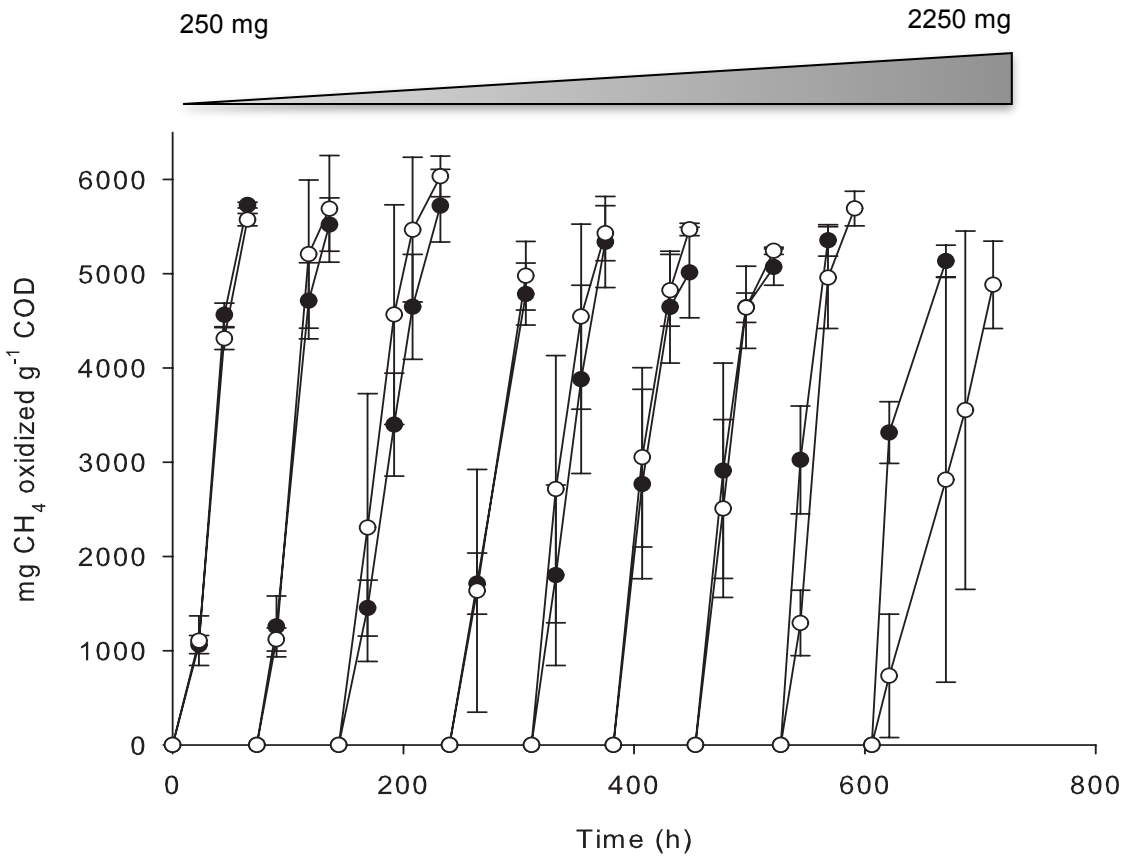


Figure IV-3. Cumulative methane consumption (mg CH_4 g^{-1} COD) during nine incubation cycles, with a gradual increase of the NH_4^+ concentration (empty circles) from 250 to 2250 mg NH_4^+-N L^{-1} (*n*=3, steps of 250 mg NH_4^+-N L^{-1} per cycle). Due to technical issues, no daily measurements could be completed during the 10th cycle with 2500 mg NH_4^+-N L^{-1}. The average MOR was however not significantly different in comparison with the former cycle with 2250 NH_4^+-N L^{-1}. In the control bottles (*n*=3, filled circles), the concentration of NH_4^+ was kept at 125±14 mg NH_4^+-N L^{-1}.

In a final attempt to investigate the effect NH_4^+-N L^{-1} concentration, the control bottles were also shock loaded with 2250 mg NH_4^+-N L^{-1} after the tenth cycle. On average 706 mg CH_4 was removed g^{-1} COD d^{-1} (*n*=3) after 48h, significantly less than the 1468 mg CH_4 g^{-1} COD d^{-1} observed for the gradually adapted communities at that concentration. Nevertheless, NH_4^+ resilient MOB were still present, as a significant MOR was observed and the lag phase was shorter than 24h.

IV.3.2 Effect of increasing NH_4^+ amendments on the methanotrophic community

Specific PCR-DGGE and a diagnostic microarray analysis targeting the *pmoA* gene were used to characterize the methanotrophic community. For all treatments, a high similarity in composition was observed between communities undergoing identical conditions, even if they differed significantly in activity. DGGE analysis revealed a temporal shift in the MOB community of the control incubations, leading to the dominance of three bands on the DGGE profile; two of these already dominantly present in the starting inoculum (Figure IV-4). In contrast, both the sudden and gradual NH_4^+ increase resulted into dominance of one specific band, also present in the DGGE profiles of the starting inoculum and the control incubations.

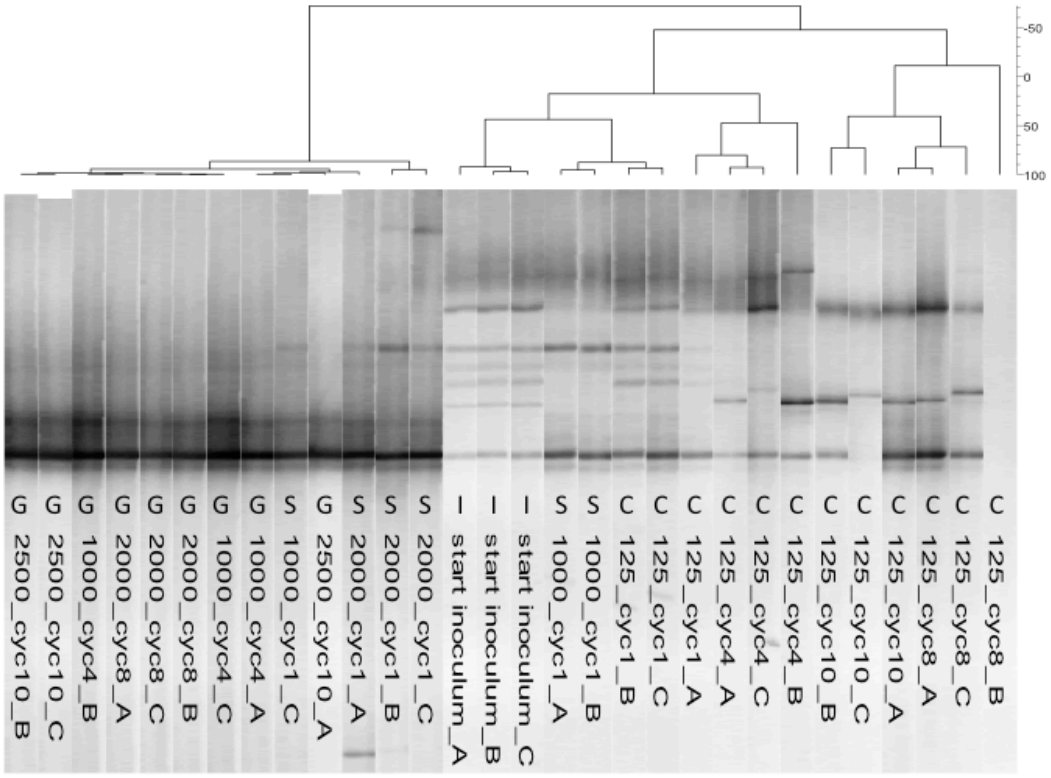


Figure IV-4. *pmoA*-based DGGE analyses revealed clustering of the MOB in the cultures undergoing a sudden (indicated by 'S') and gradual (G) increase of the NH_4^+ concentration, respectively, while the starting inoculum (I) and control cultures (C) clustered separately.

The *pmoA*-based microarray analysis allowed identification of dominant genera in the different MOB communities (Figure IV-5). Even after ten cycles, the MOB community in the control incubations were similar to the starting inoculum, and dominated by type II MOB. In contrast, the community shifted towards type I MOB when the NH_4^+-N load was gradually increased. With these samples, the dominant probes (Mmb304, P_Mmb259, Mmb303, O_Mmb562) were indicative for *Methylosarcina*-like MOB belonging to the type Ia subgroup, although type Ia MOB only represented a minority in the starting inoculum.

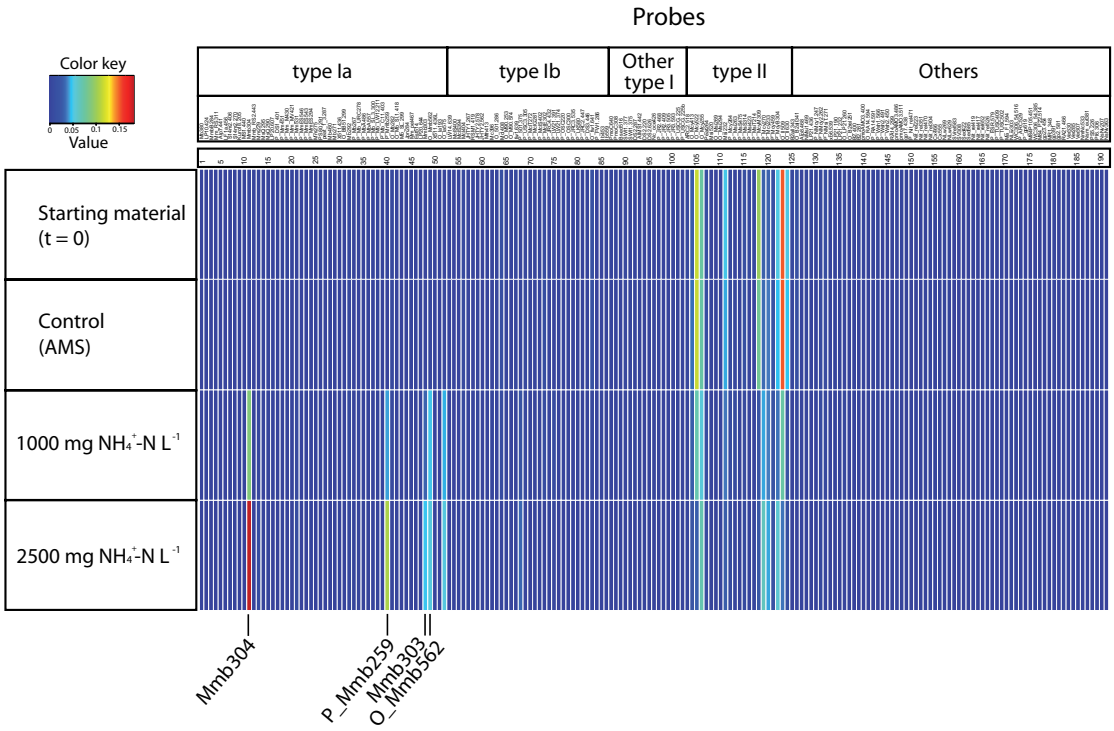


Figure IV-5. Diagnostic microarray analysis based on the *pmoA* gene showing the response of the methanotrophic community to ammonium amendments. Red and blue in the color key denote high, and no signal intensities, respectively.

IV.4 Discussion

When exposing the methanotrophic community without prior adaptation to high NH_4^+-N concentrations, no significant decrease in methanotrophic activity was observed up to a concentration of 500 mg NH_4^+-N L^{-1}. Above this concentration, a significant decrease in methane oxidizing activity was detected. At the same time, higher concentrations led to

extended lag phases (Figure IV-2); a phenomenon also observed by Park et al. (2005) and Yang et al. (2011), who applied high doses of 1000 mg NH_4^+-N kg^{-1} soil. Still, even with a concentration of 2000 mg NH_4^+-N L^{-1}, a substantial MOR was observed, indicating that the starting inoculum contained MOB able to maintain their metabolic activity while exposed to such high NH_4^+ concentrations.

When a gradual increase of the NH_4^+-N concentration was applied, the methanotrophic community was able to maintain its full methanotrophic activity up to concentrations of 2000 mg NH_4^+-N L^{-1}. In contrast with the experiments with a sudden NH_4^+-increase, no loss of methanotrophic activity nor an extended lag phase was observed when then NH_4^+ concentration was increased gradually, showing that some members of the methanotrophic community could adapt to concentrations of 2000 mg NH_4^+-N L^{-1} without MOR reduction. Thus, in order to allow the MOB to adapt to increased NH_4^+-concentrations and/or to allow shifts in the community towards more resilient species, it is important that an increase of NH_4^+ loads occurs gradually. Other research groups (Nyerges et al., 2010, Veillette et al., 2011, Yang et al., 2011) also investigated the effect of high NH_4^+ concentrations (up to 700 mg NH_4^+-N L^{-1}) on methanotrophic communities, but a non-inhibiting NH_4^+ concentration of 2000 mg NH_4^+-N L^{-1} is the highest reported so far. At such high NH_4^+ concentrations, the ratio of dissolved NH_4^+:dissolved CH_4 is 256, while the amount of dissolved NH_3 and CH_4 is of the same order. This indicates that at neutral pH, direct inhibition of MMO by NH_4^+/NH_3 is not the main reason for the often-observed steep decrease in methane oxidation. The decrease of methanotrophic activity observed above 2000 mg NH_4^+-N L^{-1} is possibly due to osmotic effects as stated by King & Schnell (1998). Indeed, 2250 mg NH_4^+-N L^{-1} correlates with 160 mM Cl^-, a concentration that resulted in decreased MOR for mixed methanotrophic communities under similar conditions (van der Ha et al., 2010).

While the methanotrophic community stayed relatively stable in controls with 125 mg NH_4^+-N L^{-1}, clear community shifts occurred at concentrations of 1000 mg NH_4^+-N L^{-1} and more, no matter how the NH_4^+ increase was applied. With concentrations of 1000 mg

NH_4^+-N L^{-1} and more, one specific MOB became dominant. This MOB was also present in the DGGE profiles of the starting inoculum and the control incubations. The latter indicates that this MOB is not restricted to high NH_4^+ environments, but rather exhibits a stronger adaptive capacity than the other MOB (Nyerges and Stein, 2009). The presence of this resilient MOB in the starting inoculum could explain why a lag phase occurred after the sudden NH_4^+ amendments with concentrations above 1000 mg NH_4^+-N L^{-1}, as it takes a few reproduction cycles before the community is able to shift to more NH_4^+ resilient species. Indeed, that particular band became also more dominant in the DGGE profiles of the incubations with a sudden increase of the NH_4^+ concentration to 1000 and - more distinct - 2000 mg NH_4^+-N L^{-1}.

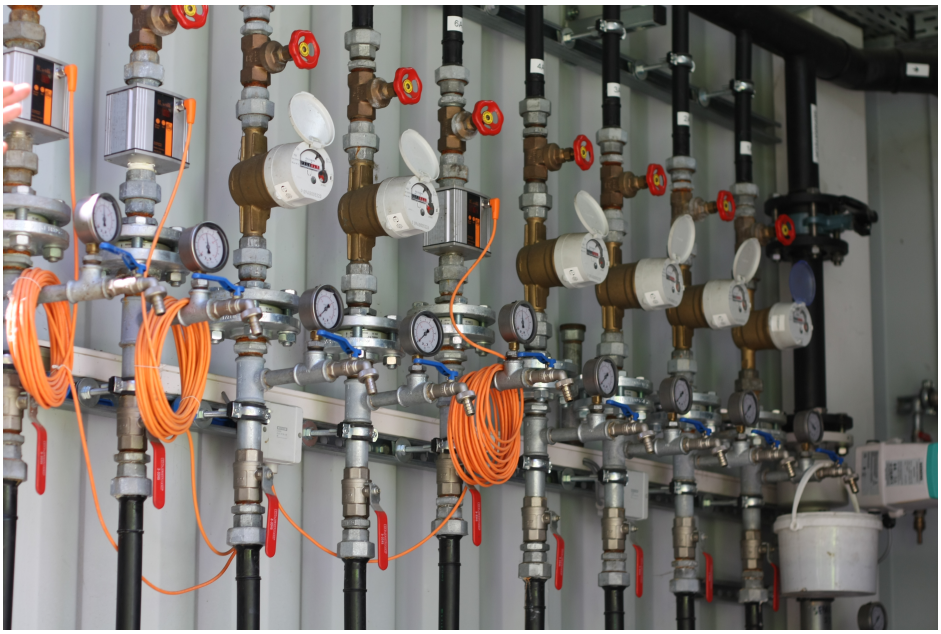
The microarray analysis showed that with high NH_4^+ concentrations, a *Methylosarcina*-like MOB became dominant, even though the relative abundance of type I MOB was very low in the starting inoculum. These results confirm observations of previous studies, where this subgroup also showed a higher tolerance for NH_4^+-N, and was even sometimes stimulated by it (Noll et al., 2008, Yang et al., 2011). Possibly, the different responses of MOB subgroups to ammonium loads reflect on their adaptable life strategies (Ho et al., 2012). Conclusively, these highly resilient MOB create opportunities for CH_4 mitigation in environments where NH_4^+ and CH_4 co-occur.

Acknowledgements

DVDHA, AH and NB designed the experiments. DVDHA and DG performed the experiments and analyzed the data. DVDHA wrote the manuscript. AH and BS performed and analyzed the micro-arrays. SH assisted with the experiments. SH, AH, NB and PV commented on the manuscript. This research was possible via financial support of the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005) and a PhD grant to David van der Ha from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen, SB-83259). We thank Tim Lacoere for excellent technical assistance and Frederiek-Maarten Kerckhof for practical help.

CHAPTER V

SELECTION OF ASSOCIATED HETEROTROPHS BY MOB AT DIFFERENT COPPER CONCENTRATIONS



Flow regulators, soil remediation plant, Geneva

Everybody knows that books are better than life. That's why they're books.

Pursy Will

Chapter V. Selection of associated heterotrophs by MOB at different copper concentrations

Abstract

Due to the increasing atmospheric methane concentration, more knowledge is needed on the management of methanotrophic communities. While most studies focused on the characteristics of the methane oxidizing bacteria (MOB), less is known about their interactions with associated heterotrophs. Interpretative tools based on DGGE allowed to evaluate the influence of copper – an important enzymatic regulator for MOB – on the activity and composition of the bacterial community. Over 30 days, enrichments with 0.1, 1.0 and 10 µM Cu^{2+} respectively, showed comparable methane oxidation activities. The different copper concentrations did not create major shifts in the methanotrophic communities, as a *Methylomonas* sp. was able to establish dominance at all different copper concentrations by switching between both known methane monooxygenases. The associated heterotrophic communities showed continuous shifts, but over time, all cultures evolved to a comparable composition, independent of the copper concentration. This indicates that the MOB selected for certain heterotrophs, possibly fulfilling vital processes like removal of toxic compounds. The presence of a large heterotrophic food web indirectly depending on methane as sole carbon and energy source was confirmed by a clone library wherein MOB only formed a minority of the identified species.

Redrafted after:

van der Ha, D.[*], Vanwonterghem, I.[*], Hoefman, S., De Vos, P., Boon, N. (2012) Selection of associated heterotrophs by methane-oxidizing bacteria at different copper concentrations. Antonie Van Leeuwenhoek, doi 10.1007/s10482-012-9835-7. ([*] contributed equally)

V.1 Introduction

Most of the aerobic methanotrophic diversity thus far obtained can be phylogenetically positioned within the classes of the *Gamma-proteobacteria* (often referred to as type Ia/Ib MOB) and the *Alpha-proteobacteria* (type II MOB) (Dedysh, 2009), although several non-proteobacterial MOB have been recently described (Ettwig et al., 2010, Op den Camp et al., 2009). Type Ia/Ib and type II MOB show distinct differences in their carbon assimilation pathway, cellular ultrastructures and other physiological and biochemical characteristics (Hanson and Hanson, 1996, Ward et al., 2004). Also the form of MMO that can be expressed differs: while many type Ia/Ib MOB can only express the particulate form (pMMO), most type II MOB are also capable of expressing the soluble form (sMMO) (Dalton, 2005). The pMMO has a copper active site and is only expressed when the copper-to-biomass ratio is high enough (Balasubramanian and Rosenzweig, 2008, Dalton, 2005). The sMMO on the other hand contains a non-heme diiron active site and starts to become inhibited typically at concentrations of about 0.8 µM Cu^{2+} in liquid enrichments of MOB (Hakemian and Rosenzweig, 2007, Koh et al., 1993). Therefore, the copper-to-biomass ratio is a major steering parameter towards the expression of both enzymes, hence indirectly influencing the methanotrophic activity and community structure (Semrau et al., 2010, van der Ha et al., 2010).

In nature, MOB are not only important in the global methane cycle, but they also form the base of a microbial food chain by supplying by-products and metabolites of the CH_4 oxidation processes to other microorganisms. Therefore, in the absence of other organic C sources, MOB perform a major ecological role as the accompanying heterotrophic bacteria completely rely on the activity of MOB for their carbon and energy needs (Hrsak and Begonja, 2000, Modin et al., 2010). The CH_4 oxidizing activity is likewise influenced by accompanying heterotrophic bacteria that either remove metabolites, which can inhibit MOB or produce metabolites that stimulate MOB activity and/or growth (Hesselsoe et al., 2005, Hrsak and Begonja, 2000, Jiang et al., 2010). Recent research suggested cobalamin produced by rhizobial strains strongly stimulated the growth and CH_4 oxidation of several

MOB (Iguchi et al., 2011). Other research groups also reported higher activity and/or growth of MOB in mixed cultures compared to monocultures (Hrsak and Begonja, 2000). It is however not elucidated if there is a selection of cooperative heterotrophs by the MOB or not since most research to date merely focussed solely on the MOB. Consequently, it is imperative to better understand the interrelationships between the MOB and the associated bacteria, and to investigate the influence of different environmental parameters on the microbial community composition and dynamics in order to gain more insights into the biogeochemical methane cycle and to steer methanotrophic communities in CH_4 mitigating strategies. Culture-independent techniques have been developed to explore the microbial community composition and Microbial Resource Management (MRM) comprehends necessary tools to better understand the diversity and dynamics as well as interrelationships of different microorganisms in order to adjust, control and/or steer microbial communities (Marzorati et al., 2008, Read et al., 2011, Wittebolle et al., 2008).

In this study, the activity and composition of a CH_4 oxidizing community was evaluated in relation to different copper concentrations (0.1, 1.0 and 10 µM Cu^{2+}). The mentioned parameters and a clone library created more insight on the interactions between this selective pressure, the MOB and the associated heterotrophic community.

V.2 Experimental section

V.2.1 Inoculation and cultivation of methanotrophic communities

All tests were performed in sterile, gastight Schott bottles (AG, Germany) sealed with butyl rubber stoppers (Ochs GmbH, Germany) and filled with 20 vol% CH_4 in air. The cultures were grown on dilute Nitrate Mineral Salts medium (dNMS) with 0.1% (v/v) of trace solution, according to Dunfield *et al.* (2003) and van der Ha *et al.* (2010). A methanotrophic community was assembled by mixing natural samples from a wetland (Bourgoyen, Ghent) and the top layer of an anaerobic tank of a wastewater treatment plant (WWTP Ossemeersen, Ghent, Belgium) and was enriched under 20% (v/v) CH_4 with a

copper concentration of 10 µM Cu^{2+} (2.5 mg L^{-1} $CuSO_4.5H_2O$). This enrichment was used as inoculum (10%) to create two subcultures only differing in their final copper concentration: 1.0 µM Cu^{2+} (0.25 mg L^{-1} $CuSO_4.5H_2O$) and 10 µM Cu^{2+} (2.5 mg L^{-1} $CuSO_4.5H_2O$), respectively. After three days of incubation, the resulting enrichments were used as inoculation cultures for the actual experiment.

V.2.2 Experimental design

Three types of sequence batch reactors (595 mL) were prepared in triplicate, each containing 95 mL dNMS and 20 % (v/v) CH_4 in air. The only difference between the reactors was the applied Cu^{2+}-concentration. Three copper concentrations commonly used in literature were tested, being 0.1 µM Cu^{2+} L^{-1}, 1.0 µM Cu^{2+} and 10 µM Cu^{2+}. The inoculation culture grown on 1 µM Cu^{2+} was used to inoculate the reactors with 0.1 and 1.0 µM Cu^{2+}, respectively (5% inoculation, *n*=3) while the inoculation culture grown on 10 µM Cu^{2+} was used as inoculant for the reactors with 10 µM Cu^{2+} (5% inoculation, *n*=3, Figure V-1). The reactors were placed on a shaker (100 rpm, INNOVA 4080, New Brunswick Scientific, Belgium) at 22°C (comparable to the initial inoculum temperature) for 10 cycles of 72h, and daily GC measurements were performed to observe the CH_4 oxidation activity during cycles 1, 2, 3, 4, 7 and 10. After each cycle of three days the reactors were opened, samples were taken for DNA-extraction and physico-chemical analyses, and 4/5th of the liquid phase was taken out and replaced with fresh medium. The reactors were left open to the atmosphere to allow equilibration with the air phase, after which 20 % (v/v) CH_4 was added again to the headspace. The hydraulic retention time and sludge retention time for each cycle were both 90 ± 2h and the reactors were maintained for 10 cycles (30 days).

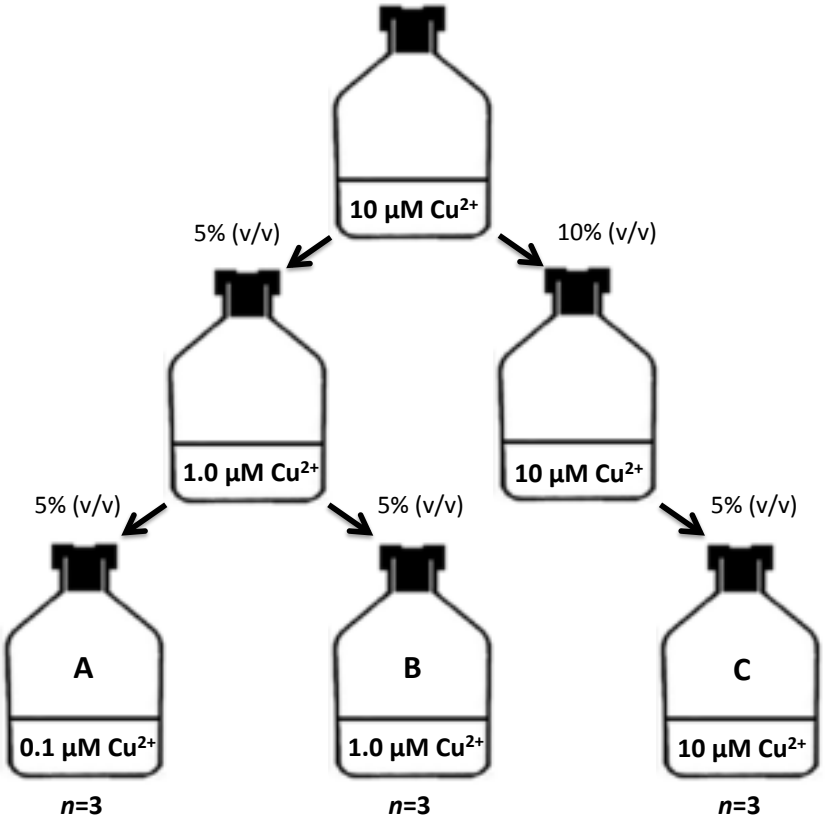


Figure V-1. Experimental set-up. A mother culture from the lab, grown on dNMS with 10 µM Cu^{2+}, was used to inoculate two inoculation cultures (10% inoculation) both grown on dNMS, one with 1.0 µM Cu^{2+} and one with 10 µM Cu^{2+}. These inoculation cultures were grown for three days and then used as starting point for the actual experiment. The culture grown on 1.0 µM Cu^{2+} was used to inoculate the reactors with 0.1 µM Cu^{2+} and 1.0 µM Cu^{2+}, and the culture grown on 10 µM Cu^{2+} was used to inoculate the reactor with 10 µM Cu^{2+} (5% inoculation, in triplicate). The reactors were refreshed every 3 days and were maintained for 10 cycles (30 days).

V.2.3 Growth and activity measurements

The CH_4, O_2 and CO_2 contents in the headspace were simultaneously determined with a Compact GC (Global Analyzer Solutions, Louvain-la-Neuve, Belgium), equipped with two channels, which were both connected to a thermal conductivity detector. The gas pressure in the bottles was measured with an Infield 7 pressure meter (UMS, München) to compensate for over- or under pressure. The methane oxidation rate (MOR) was

calculated and expressed as mg CH_4-COD L^{-1} d^{-1} by using a conversion factor of 4 mg COD (chemical oxygen demand) mg^{-1} CH_4. Results were statistically analyzed via an independent two-sample student t-test with equal variance and at a significance level of 99% (n results, p<0.01).

To verify sMMO expression, a modified version of the naphthalene oxidation assay of (Brusseau et al., 1990) was used. A crystal of naphthalene was added to 2 mL actively growing MOB culture and incubated on a shaker (120 rpm) at 28°C for 2h. A tetrazotized-o-dianisidine solution (2.68 mg L^{-1}) was freshly made and 200 µL was added to the MOB culture after incubation. The formation of a colored diazo-dye was monitored visually (Brusseau et al., 1990).

V.2.4 Physico-chemical analysis

The total chemical oxygen demand (COD) was measured after cycles 1, 2, 3, 4, 7 and 10 (n=3) using manual COD kits in the range of 15-160 mg L^{-1} or 100-1500 mg L^{-1} (Nanocolor® COD test kit, Macherey-Nagel, UK). The resulting color change was measured with a Nanocolor 500D Photometer (Macherey-Nagel, UK).

V.2.5 Molecular techniques

DNA extractions were performed using a FastDNA® Spin kit for Soil (Q-BIO gene, Belgium) and were executed according to a slightly modified version of the manufacturer's instructions: depending on the amount of bacterial growth, 2-10 mL of the cell culture was centrifuged (Micro 2416 microcentrifuge, VWR™, USA) at 13,000 x g to obtain a pellet which was then transferred to the lysis tube.

Polymerase Chain Reaction (PCR) amplification was performed by using the general bacterial primer set P63F/P1378r which targets the 16S rRNA gene (Ovreas et al., 1997) while the specific primer set A189f/mb661r, targeting the *pmoA* gene (Costello and Lidstrom, 1999), was chosen for MOB detection. The selected MOB specific primers exclude MOB only capable of sMMO expression, such as *Methylocella* spp. and

Methyloferula spp. (Dedysh, 2009), but clone library results showed that these species were not dominant in the reactors. PCR amplification was performed with a Gene Amp® PCR System 2700 or 2720 Thermal Cycler (Applied Biosystems,USA). Fermentas products (Germany) were used for the 16S rRNA gene PCR mixtures containing 19.3 µL PCR water, 2.5 µL dNTP's (10 nmol μL^{-1}), 1.5 µL 10x Taq buffer + KCl - $MgCl_2$, 0.5 µL $MgCl_2$, 0.5 µL of each primer (10 pmol μL^{-1}), 0.0625 µL Bovine serum albumin, 0.125 µL Taq Polymerase and 1 µL template DNA. The mixtures of the *pmoA* PCR contained 13.8 µL PCR water, 2.5 µL dNTP's (2 nmol μL^{-1}), 2.5 µL 10x Taq buffer + $MgCl_2$, 0.825 µL of each primer (10 pmol μL^{-1}), 2.075 Taq polymerase and 2.5 µL template DNA, and were made with products from Applied Biosystems (USA).

Denaturing gradient gel electrophoresis analysis (DGGE) was performed according to Ovreas *et al.* (1997) and Hoefman *et al.* (2012a) on one of the triplicates of all three reactor types during cycles 1, 2, 3, 4, 7 and 10. The resulting DGGE patterns were further processed using BioNumerics software version 5.10 (Applied Maths, Belgium). Analysis of the DGGE patterns was performed using the MRM approach, i.e. calculating the Gini coefficient (measure of evenness), community organization (indirect measure of evenness), dynamics and richness (measure of diversity), according to Boon *et al.* (2011), Marzorati *et al.* (2008) and Read *et al.* (2011). BioNumerics was also used to perform Principal Component Analysis (PCA), a mathematical procedure used to convert a set of possibly correlated variables into uncorrelated variables, called principal components. The different parameters (variables) were obtained by performing bandmatching on the DGGE profiles.

To identify the most important bands in all the different DGGE profiles, a bacterial 16S rRNA gene clone library was constructed based on a sample after 10 cycles of cultivation at 1.0 µM Cu^{2+} and a sample after 7 cycles of incubation at 10 µM Cu^{2+}. The combined DGGE profile of these two samples represented best the total number of visible bands for all samples. The clone library was constructed according to the manufacturer's instructions (Topo-TA cloning kit, Invitrogen, Carlsbad, CA). Bacterial 16S rRNA gene primers P63F and P1378r were used and the 16S rRNA gene PCR was performed according

to Ovreas *et al.* (1997) with the PCR mix (Fermentas products) mentioned previously. 16S rRNA-DGGE was performed on the different isolated clones together with the original samples allowing band matching for all samples based on the position on the gel and of the 49 positive clones, 20 unique band positions were found. Then, insert sequencing at AGOWA (Germany) was performed on these clones with primer 63F. The retrieved sequences were analyzed with 4Peaks software version 1.7.2 (Macintosh) and aligned with the SINA online aligner (SILVA, Belgium).

V.3 Results

V.3.1 Activity and growth characteristics of the methanotrophic community

Firstly, the influence of the three different tested copper concentrations (0.1, 1.0 and 10 µM Cu^{2+}) on the activity of the methanotrophic community was evaluated. The activity is represented in Figure V-2 by the methane oxidation rate (MOR). No significant differences in CH_4 oxidizing activity were observed (n=18, p<0.01).

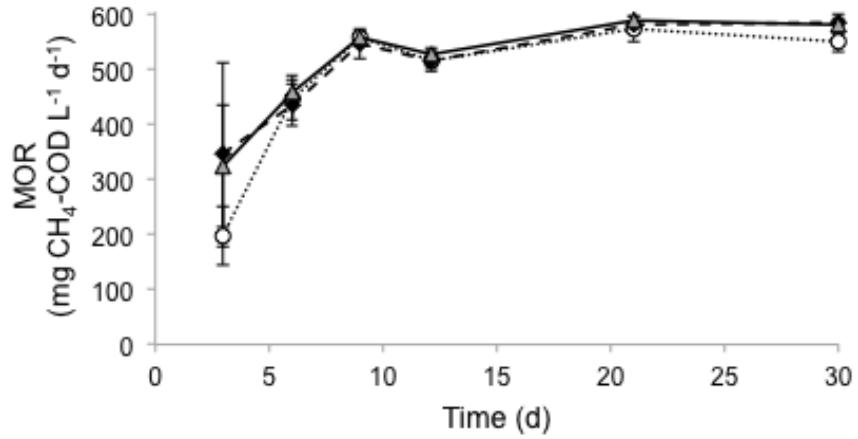


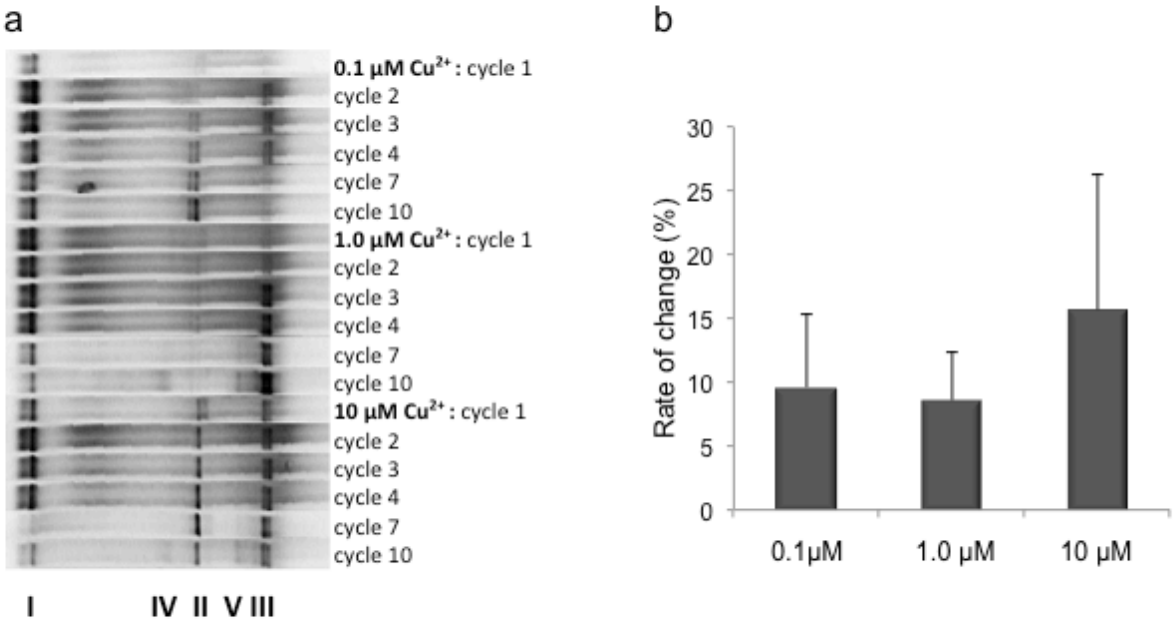
Figure V-2. Average methane oxidation rate (MOR) (n=3) in function of time for the reactors with 0.1 µM Cu^{2+} (open circle), 1.0 µM Cu^{2+} (diamond) and 10 µM Cu^{2+} (triangle).

As no other C source but CH_4 was added to the medium, chemical oxygen demand (COD) was a good proxy to measure the increase in biomass concentration. The COD content of the cultures amended with 0.1 µM Cu^{2+} increased from 135 ± 113 mg L^{-1} after the first cycle to 540 ± 50 mg L^{-1} at the end of the test period of ten cycles. The cultures with 1.0 µM Cu^{2+} showed an increase in COD content from 353 ± 235 mg L^{-1} to 485 ± 52 mg L^{-1} and the COD concentration of the cultures with 10 µM Cu^{2+} increased from 216 ± 116 mg L^{-1} to 499 ± 64 mg L^{-1} after cycle 10. The average COD concentration over the whole period was 383 ± 155 mg L^{-1} for the cultures with the lowest copper concentration, 429 ± 115 mg L^{-1} for the cultures with 1.0 µM Cu^{2+} and 438 ± 132 mg L^{-1} for the cultures with the highest copper concentration of 10 µM Cu^{2+}. Comparable to the MOR, the average COD values did not differ significantly between the reactor types (n=18; p<0.01).

The naphthalene oxidation assay indicated that sMMO expression only occurred in the reactors with the lowest copper concentrations of 0.1 µM Cu^{2+}.

V.3.2 Community organization and dynamics of the methanotrophic communities

The influence of the different copper concentrations on the methanotrophic community was evaluated with an MOB specific PCR reaction targeting the *pmoA* gene, followed by DGGE (Figure V-3). Three major bands (I, II and III) containing the dominant MOB could be detected in all reactor types. All three bands were present throughout the test period, but their dominance differed and fluctuated with time. From the seventh cycle on, two extra faint bands (IV & V) were observed in the DGGE profile of the reactors with 1.0 and 10 µM Cu^{2+}. The dynamics of the MOB communities were assessed by applying the similarity matrix for the densiometric curves of banding patterns. This matrix was used to perform moving window analysis, comparing the similarity (%) of the community fingerprints at two successive time points at an interval of 9 days (3 cycles). The average rate of change was respectively 10 ± 6% for the reactors with 0.1 µM Cu^{2+}, 9 ± 4% for the reactors with 1.0 µM Cu^{2+} and 16 ± 11% for the reactors with 10 µM Cu^{2+}, indicating relatively stable methanotrophic communities.



1.0 and 10 µM Cu^{2+}. (b) Graph representing the average rate of change (%) for the triplicates n° 3 of all reactor types, based on the matrix of similarities for the densiometric curves of the MOB specific DGGE band patterns.

V.3.3 Community organization and dynamics of the total bacterial communities

By pre-cultivating the cultures with 0.1 and 1 µM Cu^{2+} for three days on 1.0 µM Cu^{2+}, adaptation to lower copper concentrations could take place without important changes in the diversity, as almost similar DGGE profiles were observed for the start points of all three cultures (Figure V-4). Over the test period of 30 days, the major factors influencing the total bacterial community organization were evaluated via principle component analysis (PCA) based on band matching (Figure V-5) for all the reactor types, triplicates and cycles.

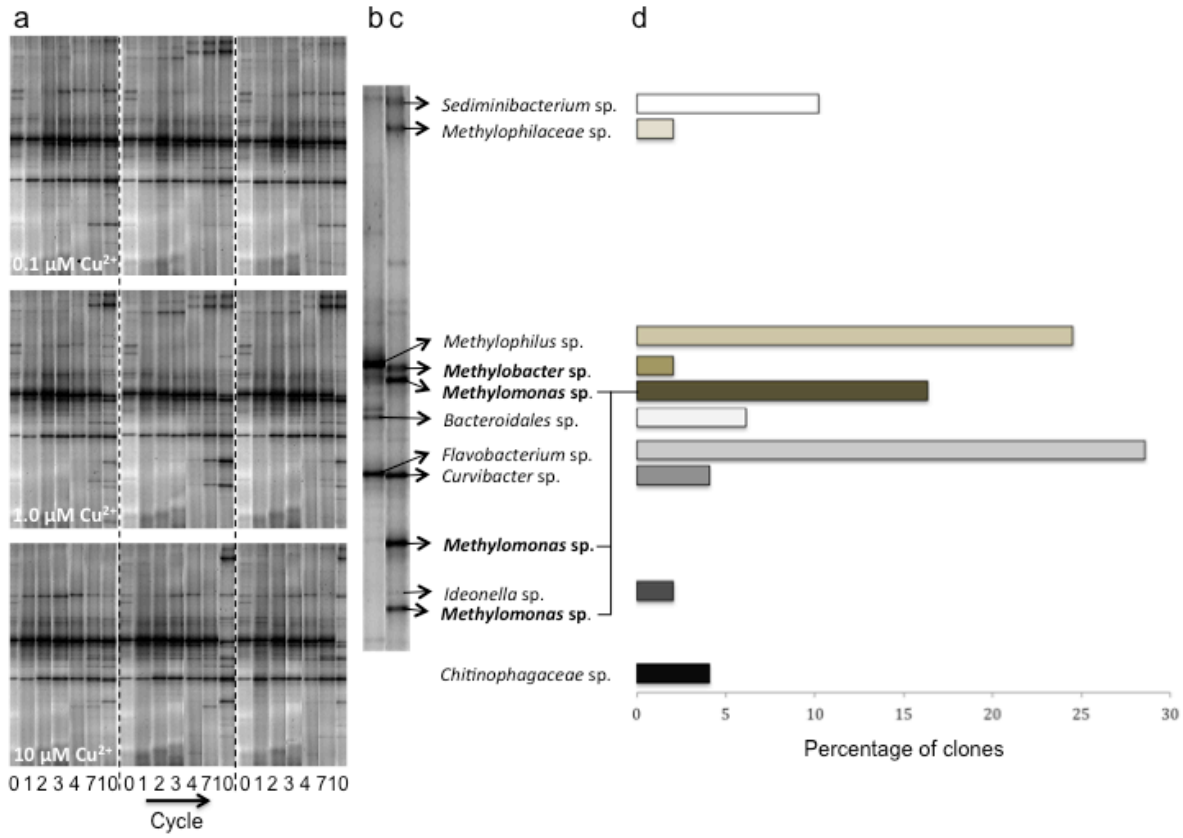
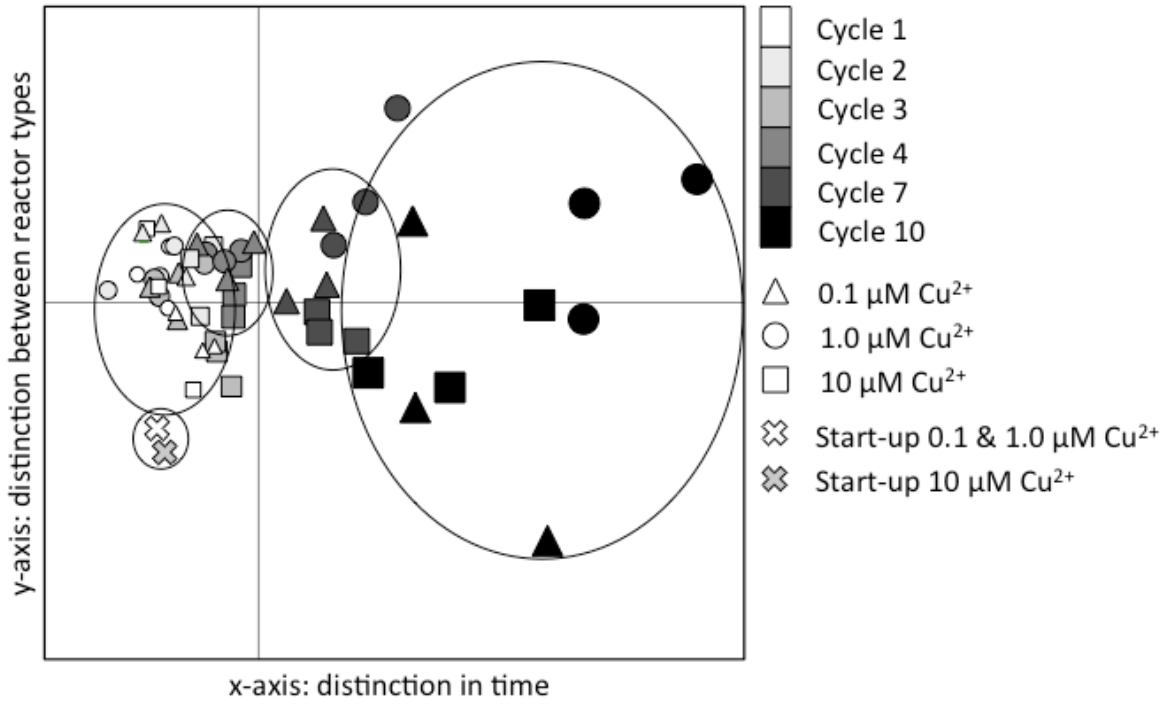


Figure V-4. 16S rRNA gene DGGE fingerprint pattern for all triplicates and cycles of the reactors with 0.1 µM Cu^{2+}, 1.0 µM Cu^{2+} and 10 µM Cu^{2+} (a). Clone library/sequencing results based on a sample taken from a reactor amended with 10 µM Cu^{2+} after cycle 7 (b) and a reactor with 1.0 µM Cu^{2+} after cycle 10 (c). The species in bold represent the MOB. The graph (d) shows the relative abundance of the genus, family or order to which the sequenced species belong, calculated by determining the percentage of clones in which a specific gene had been incorporated.

The largest variation for all three treatments is represented along the x-axis due to the influence of time, with the biggest shift occurring between cycles 7-10. Along the y-axis a smaller distinction can be made between reactor types, with the biggest difference occurring between the reactors with 0.1 and 1.0 µM Cu^{2+} and the reactors with 10 µM Cu^{2+}. The PCA also showed that the variability between reactor types increased in time, with maximal variability during the tenth cycle. This correlates to results obtained with cluster analysis, based on Pearson product-moment correlation coefficients, which

indicated that time (cycle) and not the Cu^{2+}-concentration was the most influencing factor on the total bacterial community composition, especially towards the end. At the same time, cluster analysis showed that the triplicates of each reactor type clustering together during each separate cycle.



10 µM Cu^{2+}) and the shades of color indicate the different cycles. The crosses represent the starting points and the overarching circles indicate the main trends.

The dynamics of the total bacterial community was assessed in the same way as with the MOB community above (Figure V-6). The similarity, based on the banding patterns, was high (80-100%) during all cycles for the reactors with 0.1 µM Cu^{2+}, but decreased sharply after cycle 7 from 92 ± 3% to 73 ± 6% for the reactors with 1.0 µM Cu^{2+} and from 95 ± 2% to 65 ± 26% for the reactors with 10 µM Cu^{2+}. The average rate of change was 9 ± 5%, 14 ± 11% and 16 ± 16% for the reactors with 0.1, 1 and 10 µM Cu^{2+}, respectively.

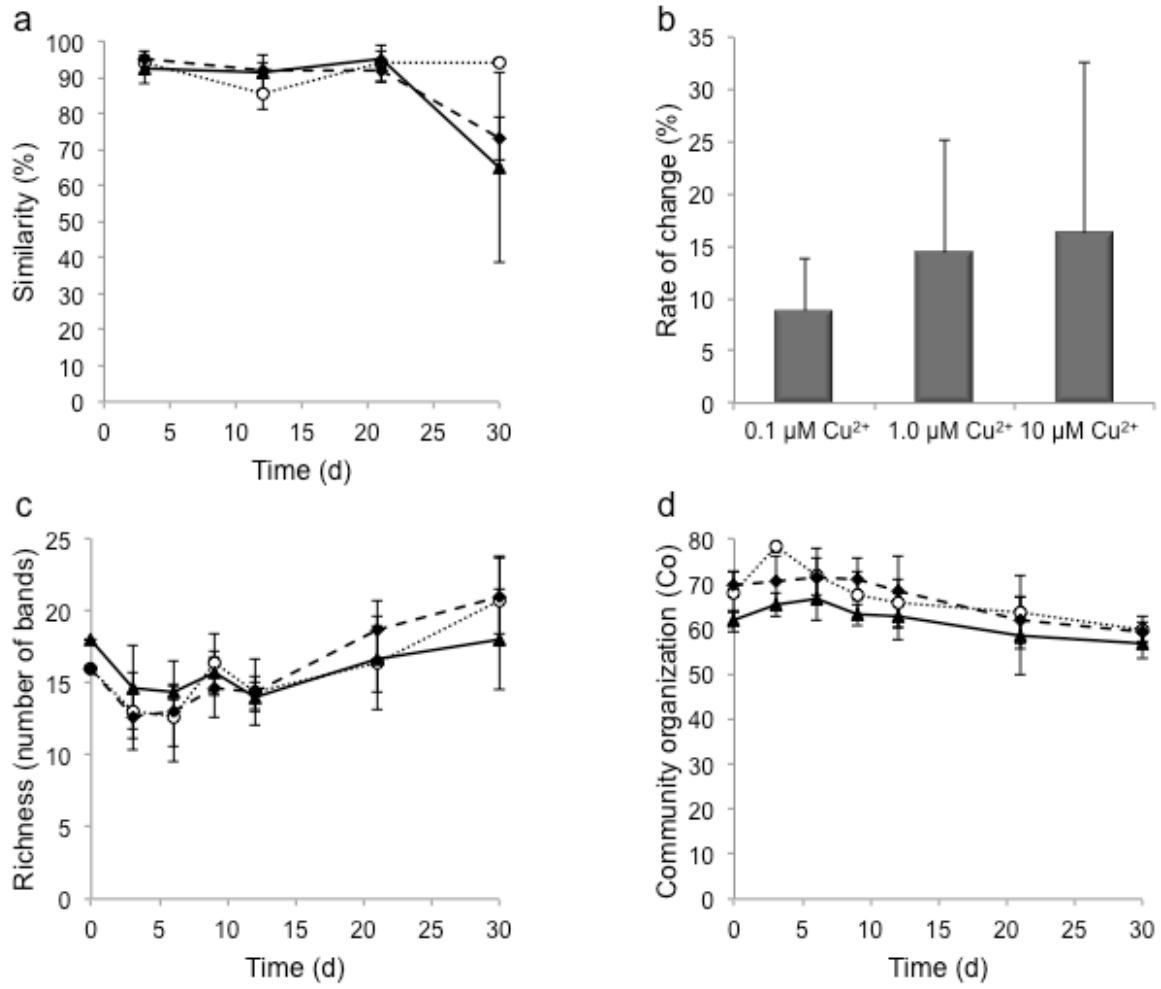


Figure V-6. Based on the matrix of similarities for the densiometric curves of the 16S rRNA gene DGGE band patterns for the reactors with 0.1 µM Cu^{2+} (open circle), 1.0 µM Cu^{2+} (diamond) and 10 µM Cu^{2+} (triangle), moving windows analysis was performed (a) which allowed to calculate the average rate of change (b). The amount of bands on the DGGE fingerprint allowed to determine the richness of the community (c). The community organization (d) was calculated using the evenness, which in turn is based on the Gini coefficient. These parameters were calculated for the 1st, 2nd, 3th, 4th, 7th and 10th cycle of each three days.

The evenness is quantified by the Gini coefficient and is based on the Pareto-Lorenz distribution curves which represent the cumulative proportion of abundance in relation to the cumulative proportion of species (Boon et al., 2011). The Gini coefficient was further

used to calculate the total bacterial community organization (Co) (Figure V-6). The communities showed high Co values (60-100) at the beginning, indicating low evenness. The Co-value of the cultures grown in dNMS with 0.1 µM Cu^{2+} increased from 68 ± 5 to 78 ± 1 after cycle 1 and then gradually decreased to 60 ± 3 after 30 days. The community organization of the cultures grown in the presence of 1.0 and 10 µM Cu^{2+} showed a slight increase respectively from 70 ± 3 to 72 ± 4 and from 62 ± 2 to 67 ± 5 between day 0 and day 6 (cycle 2). Then the Co gradually decreased to 59 ± 2 for the reactors with 1.0 µM Cu^{2+} and to 57 ± 3 for the reactors with 10 µM Cu^{2+} after cycle 10.

The richness (R) of the communities was determined by the number of bands on the fingerprint profiles (Figure V-6). The richness decreased between start-up and cycle 2 from 16 ± 0 to 13 ± 2 for the reactors with 0.1 µM Cu^{2+}, from 16 ± 0 to 13 ± 3 for the reactors with 1.0 µM Cu^{2+}, and from 18 ± 0 to 14 ± 1 for the reactors with 10 µM Cu^{2+}. From the seventh cycle on, the average number of bands increased again to 21 ± 3 for the reactors with 0.1 and 1 µM Cu^{2+}, and to 18 ± 3 for the reactors with 10 µM Cu^{2+}.

V.3.4 Changes in species composition of the total bacterial community

The obtained fingerprint patterns were combined with the results from a clone library, allowing identification of dominant bands in the DGGE profiles and visualization of community shifts (Figure V-4). All DGGE profiles contained a band of the methanotrophic *Methylomonas* sp., next to *Flavobacterium* sp. and the methylotrophic *Methylophilus* sp. Another identified MOB was *Methylobacter* sp., but due to the close position of *Methylophilus* sp. in the DGGE profile, no conclusive trend could be observed. A member of the *Bacteroidales* order was the single identified species that was correlated to a specific copper concentration, i.e. 10 µM Cu^{2+}. While all communities were relatively similar and stable up to the fourth cycle, important shifts were observed in the total bacterial community of all cultures from the seventh cycle on, as of then the heterotrophic species *Sediminibacterium* sp., *Flavobacterium* sp., *Ideonella* sp. and a new species from the *Methylophilaceae* family emerged in cultures of all three treatments. Moreover, two extra bands of *Methylomonas* sp. appeared in the reactors with 1.0 and 10

µM Cu^{2+}, coinciding with the appearance of two new faint bands for these samples in the MOB specific DGGE profiles.

Estimation of the relative abundance of the most dominant species was based on the percentage of clones in which a specific gene had been incorporated (Figure V-4). Although no organic C source was added, only 18% of clones were MOB (one *Methylobacter* sp. clone and seven *Methylomonas* sp. clones out of a total of 49). The *Methylomonas* clones were present at three different positions on the DGGE fingerprint pattern, indicating that it concerned different species. Two of them only appeared from the seventh cycle on, which coincided with the appearance of two extra bands in the MOB specific DGGE profiles for those samples. *Flavobacterium* sp. was the most dominant heterotroph with 29% of clones, while 26% of clones were member of the methylotrophic *Methylophilaceae* family. Furthermore, 10% of clones were related to *Sediminibacterium* sp., 6% were related to the order *Bacteroidales*, 4% were related to *Curvibacter* sp., another 4% were related to the family *Chitinophagaceae* and the remaining 2% were related to *Ideonella* sp.

V.4 Discussion

V.4.1 The methanotrophic communities evolved in the same direction

After adaptation during the first two cycles, the CH_4 oxidation rates increased during cycle 3 and were similar over time for all reactor types during the subsequent seven cycles. The observed MOR ranged between 150 and 620 mg CH_4-COD L^{-1} d^{-1} and corresponded to those found in previous reports, such as MOR ranging between 24 - 216 mg CH_4 L^{-1} d^{-1} (96 - 864 mg CH_4-COD L^{-1} d^{-1}) in a continuous reactor set-up (Melse and Van der Werf, 2005). The minor difference in MOR of the various reactors types was not significant ($p > 0.01$), showing that the copper concentration had no substantial influence on the CH_4 oxidizing activity of the MOB communities. Conversely, under comparable conditions, an increase in MOR of 50-70% was observed between additions of 0.1 and 10 µM Cu^{2+} (van der Ha et al., 2010), which was much higher than the non-significant 10% increase observed here. The

naphthalene oxidation assay showed that with the lowest copper concentrations of 0.1 µM Cu^{2+} sMMO expression occurred, while the presence of 1.0 µM Cu^{2+} was enough to favor pMMO expression over sMMO expression. This corresponds to previous enrichment reports saying copper concentrations of at least 50 µg Cu^{2+} L^{-1} are needed for pMMO expression (Hakemian and Rosenzweig, 2007).

Although cultures treated with different copper concentrations evolved independent from each other, the same three dominant MOB bands were always observed. Only from the seventh cycle on, when higher dynamics were observed, two new bands appeared in the MOB specific DGGE profiles of treatments with 1.0 and 10 µM Cu^{2+}, coinciding with the appearance of two identified bands of *Methylomonas* sp. in the 16S rRNA gene DGGE profiles of the same samples. These bands were additional to another identified *Methylomonas* sp., which was present in the samples of all treatments and was retrieved in the clone library. Depending on the imposed copper concentration, this omnipresent MOB expressed either sMMO or pMMO, a known characteristic of several members of the *Methylomonas* genus (Hoefman et al., 2012a). The sMMO/pMMO flexibility of this dominant MOB species may explain why the different copper concentrations did not drastically change the methanotrophic activity and community composition, indicating the importance of community characterization to understand the difference in behavior between mixed cultures.

V.4.2 Time was the dominant influence on the total bacterial community

Processing of the DGGE patterns was performed based on three levels of analysis, i.e. dynamics, community organization and richness (Marzorati et al., 2008). These parameters allow a visual ecological interpretation of raw fingerprinting patterns. Although DNA based, the presented approach allowed to evaluate shifts in the active part of the community since three cycles of growth and dilution occurred between subsequent sampling. That way only active and abundant species were detected by DGGE since non-active species were likely washed out or present in very low abundance, thus no longer visible on the DGGE profile. Both principle component analysis based on 16S rRNA gene

DGGE band matching and cluster analysis indicated that not the copper concentration but time influenced the community composition the most. For all three treatments, medium dynamics was observed, indicating that shifts in relative abundance of species were able to occur (Marzorati et al., 2008). This was confirmed by the cluster analysis where the communities after cycle 7 were scattered through the cluster, indicating that a shift in the community composition had taken place. Indeed, the increase in richness (R value) of the communities indicated that several species emerged, which also resulted in an increase of the community evenness. This shift turned the skewed communities (Marzorati et al., 2008) specialized in CH_4 oxidation into more even and ecological niche-oriented communities.

V.4.3 MOB provide metabolites for a specific associated heterotrophic community

Linking the clone library to the DGGE profiles allowed to identify the majority of dominant bands as 96% of the cloned species were also represented on the 16S rRNA gene DGGE gel. Towards the end of the cultivation period, only 18% of clones possessed a methanotrophic gene, with 16% of the sequenced species related to *Methylomonas* sp. and 2% to *Methylobacter* sp*.* Hoefman and colleagues previously found *Methylomonas* sp. to be the dominant isolate when enriching environmental samples in dNMS and 20% (v/v) CH_4, indicating that *Methylomonas* spp*.* can easily come to dominance under the applied cultivation conditions (Hoefman et al., 2012a). Interestingly, the majority of the bacterial community had a heterotrophic nature, although there was no direct organic C source present. Therefore, the abundant heterotrophs depended on the primary conversion of CH_4 to organic compounds by the MOB. The obtained DGGE fingerprint patterns in combination with the results from the clone library indicate that the bacterial community dynamics were not high and a selection for certain heterotrophs by the MOB occurred. The cultures with different copper concentrations also seemed to evolve in the same direction, although there was often a time lag. For example, members of *Sediminibacterium* sp., *Flavobacterium* sp. and *Ideonella* sp. became visible on different

DGGE profiles at different moments between the fourth and the tenth cycle. Only a single member of the *Bacteroidales* order was observed in reactors with just one specific copper concentration. Others like *Curvibacter* sp. appeared in the DGGE profiles of the reactors with the lowest and highest copper concentration, emphasizing that the copper concentration only had a minor effect on the heterotrophic community. It could be hypothesized that this was due to the presence of a shared dominant *Methylomonas* sp. closely interacting with these heterotrophs.

Based on the clone library results, the most abundant non-methanotrophic species were organisms that metabolize small organic molecules, i.e. *Flavobacterium* sp., aerobic bacteria capable of growing on several polysaccharides such as glucose, lactose and sucrose (Bernardet and Bowman, 2007) and *Methylophilus* sp., aerobic obligatory methylotrophic bacteria capable of growing on C1-compounds such as methanol (Schrader et al., 2009). The latter shows that methanol as first intermediate of CH_4 oxidation could be an important C source for methylotrophic heterotrophs (Bowman, 2006). These results support the hypothesis that a large heterotrophic community is supported by MOB as in this study CH_4 was the sole externally provided carbon and energy source. Moreover, the previously mentioned non-methanotrophic species were dominantly present throughout the entire 30-day cultivation period, indicating that these species were best capable of utilizing the metabolites produced by the MOB and may fulfil a crucial role within the CH_4 oxidizing community. The removal of toxic intermediates such as formaldehyde could be an important biological process for the stabilisation of CH_4 oxidizing communities. A selection for associated heterotrophs by the MOB can be assumed as all three bacterial communities evolved over time to a similar composition despite the observed dynamics and meaningful community shifts. In the investigated methanotrophic communities wherein an MOB was dominantly present with the ability to switch between sMMO and pMMO, no major shifts in the methanotrophic communities occurred and therefore no indirect effect of the copper concentrations on the total bacterial community was observed.

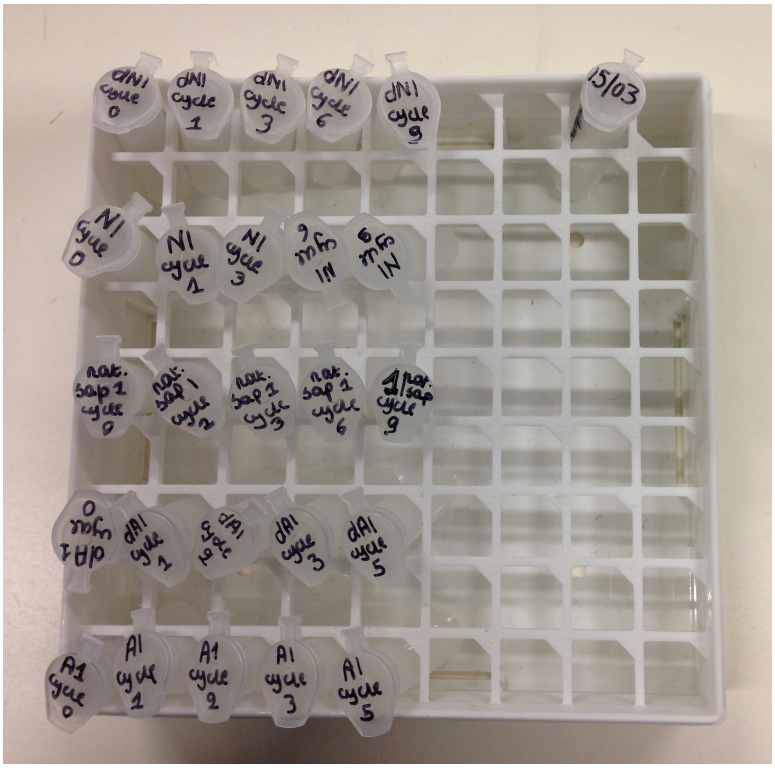
Considering the large amount and diversity of heterotrophs in such cultures, a better understanding of the interactions between MOB and these heterotrophs is required to get an idea of the importance of these interactions and to allow management of these communities for eventual applications.

Acknowledgements

DVDHA, IV and NB designed the experiment. DVDHA and IV performed the experiments, analyzed the data and wrote the manuscript. SH gave support for the experiments. SH, PDV and NB commented on the manuscript. The authors like to thank Tim Lacoere for his assistance during the analyses. This research was funded by a PhD grant for David van der Ha from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen, SB-83259) and research grants from the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005) and from the Flemish Fund for Scientific Research (FWO-Vlaanderen, 3G070010).

CHAPTER VI

METABOLIC NETWORKING WITHIN THE METHANOTROPHIC MICROBIOME



DNA samples, LabMET

Weggaan. En terugkomen.

Dromen. En niet meer dromen.

En niet meer weggaan.

En echte weemoed, niet om hoe het vroeger was,

Maar om hoe het ook vroeger nooit is geweest.

Een ziekte die je nooit had,

Waarvan je nooit geneest,

Of zo. En alles wordt bedachtzamer.

Je bent in mij als schemer in een kamer

Herman De Coninck

Chapter VI. Metabolic networking within the methanotrophic microbiome

Abstract

Methane oxidizing bacteria (MOB) form the base of certain microbial food webs, as carbon fluxes occur between MOB and associated microorganisms. In this work, an assessment was made of the metabolite production of MOB when CH_4 is the sole carbon source present. It was observed that about 25% of the produced organic matter was present in the culture medium. Stable isotope probing showed that after 96h, up to 100% of bands observed in the 16S rRNA gene DGGE profiles of three different methanotrophic communities were also present in the ^{13}C gradient fractions. This provided evidence that the associated community rapidly incorporated ^{13}C-compounds, originating from CH_4 oxidation processes by MOB. A possible application of CH_4 based food chains was investigated, whereby CH_4 was provided as sole energy and carbon source to cocultures of the MOB *Methylocystis parvus* and the methylotrophic yeast *Pichia pastoris* GS115, capable of producing valuable products. The maximum growth rate of *Pichia pastoris* GS115 was significantly higher when cocultured with an active MOB strain, compared to cases with an MOB monoculture or in coculture with a low-active MOB. These results demonstrate that metabolic networking takes place within the methanotrophic microbiome, creating CH_4 derived food webs of which some may have possible industrial potential.

Redrafted after:

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VI.1 Introduction

Methane oxidizing bacteria (MOB) are aerobic bacteria able to acquire their energy and carbon needs solely from CH_4 (Hanson and Hanson, 1996, Semrau et al., 2010). Consequently, they are able to be part of ecosystems lacking organic C sources. Moreover, MOB are known to initiate interactions with other micro- and macroorganisms. Several examples are known, such as the symbiotic association between MOB and marine invertebrates, thereby allowing higher organisms to live indirectly from CH_4 (Dalton, 2005, Petersen and Dubilier, 2009, Semrau et al., 2010). The host hereby provides the MOB of an adequate position in the water column while the MOB provide the host with carbon-rich metabolites. Another example is the interaction with mosses. Peat bogs store up to one third of all terrestrial carbon on Earth and large quantities of CH_4 can be produced by anaerobic digestion of organic matter. However, a large fraction of this CH_4 is consumed through symbiosis of *Sphagnum* mosses with partly endophytic methanotrophs, which is why only a relatively small part of CH_4 is released into the atmosphere (Kip et al., 2012, Kip et al., 2010, Raghoebarsing et al., 2005).

Methane oxidizing bacteria also form the base of microbial food webs driven by CH_4 derived carbon. Hereby, they metabolize CH_4 and supply metabolites to associated bacteria present in the methanotrophic community (Murrell and Jetten, 2009, Schrader et al., 2009). This indicates that the methanotrophic microbiome is rich in opportunities for interaction and cooperation, resulting in the emergence of new trophic strategies by combining metabolic and behavioural capabilities (Petersen and Dubilier, 2009, Semrau et al., 2010, Wintermute and Silver, 2010). However, there are still many questions to be answered about the metabolism of CH_4 and its derived compounds. Also, more knowledge is needed about the interactions within methanotrophic microbiomes, in order to further elucidate the consequences of metabolic networking within these microbial microbiomes. For example, It has been observed that MOB seem to grow better when cocultured with heterotrophs (Hrsak and Begonja, 2000). This finding gave rise to the hypothesis that cooperative bacteria either remove metabolites, which can inhibit MOB, and/or produce

metabolites, which can stimulate methanotrophic activity and growth (Hesselsoe et al., 2005, Hrsak and Begonja, 1998, Iguchi et al., 2011, Modin et al., 2007).

Therefore, this study focussed on metabolic networking between MOB and the associated community, capable of utilizing CH_4 derived metabolites. Firstly, an estimation was made of the amount and identity of organic compounds available to the associated members. Thereafter, stable isotope probing (SIP) was used to evaluate the rate whereby the whole bacterial community receives and incorporates CH_4 derived carbon. Lastly, a possible application of this CH_4 based food chain was explored in which a methylotrophic yeast, capable of producing valuable products, was grown in coculture with an axenic MOB strain.

VI.2 Experimental section

VI.2.1 Assessment of metabolite production

To assess the metabolites produced by MOB, 11 MOB isolates (Hoefman et al., 2012a) and 2 MOB reference strains (*Methylosinus* sp.; DSM 17706^T and *Methylomonas* sp.; NCIMB 11130^T) were incubated in sterile, gastight Schott bottles (AG, Germany) filled for 20% with dilute Nitrate Mineral Salts medium (dNMS; 10 µM Cu^{2+}) (Dunfield et al., 2003). After sealing the reactors, 20% (v/v) CH_4 was added to the headspace. The bottles were then placed on an incubator shaker (120 rpm, INNOVA 4080, New Brunswick Scientific, Belgium). After one week of incubation at 20°C, the metabolites of the isolates and reference strains were characterized by measuring the concentration (mg L^{-1}) of cell dry weight, (soluble) chemical oxygen demand, (soluble) total organic carbon, soluble sugars, short chain fatty acids and formaldehyde, respectively (see further).

VI.2.2 Carbon flux assessment with Stable Isotope Probing

To evaluate carbon fluxes in the methanotrophic microbiome, three methanotrophic consortia were used. Samples were taken from the denitrification tank of a WWTP (Ossemeersen, Ghent) and from a swamp (Bourgoyen, Ghent). Reactors (500 mL) were prepared containing 95 mL dNMS medium, 5 mL sample (5% inoculation) and 20% (v/v)

^{12}C-CH_4 in the headspace. Daily GC measurements were performed to observe methane oxidation activity, while the samples were enriched for 8 days. Thereafter, SIP reactors (1 L) were prepared containing 160 mL dNMS and 20% (v/v) ^{13}C-CH_4 (Figure VI-1) in order to investigate the metabolic networking between MOB and the associated heterotrophic community. One reactor was inoculated with 20% WWTP sample (A), one with 25% swamp sample (B) (due to the lower CH_4 oxidation activity during enrichment) and one with 20% sample of a lab reactor (C) containing an active methanotrophic community growing on dNMS. For each SIP-reactor, a control reactor was prepared containing the same amounts of medium and sample, but with unlabelled ^{12}C-CH_4. A time course of the metabolic networking was made by sampling for further DNA-extraction after the following time intervals: t_0 (before inoculation), t_1 = 1h, t_2 = 4h, t_3 = 24h, t_4 = 48h, t_5 = 72h and t_6 = 96h. However, since the reactor containing swamp sample showed lower activity, no sample was taken at t_1 = 1h but an extra sampling time was included at the end (120h).

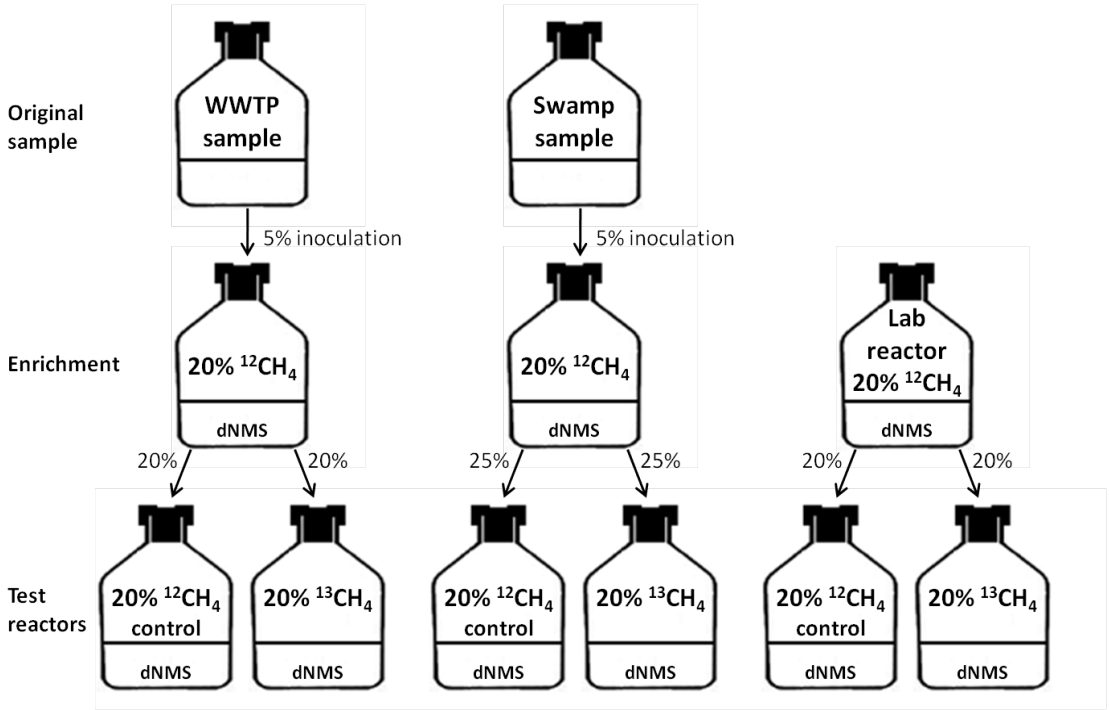


Figure VI-1. Enrichment of two natural samples and the subsequent Stable Isotope Probing experiment. Samples for DNA extraction and SIP analysis were taken at different time intervals to monitor the distribution of ^{13}C metabolites within the community by means of a CH_4 based food chain.

VI.2.3 Metabolic networking

An axenic MOB culture of *Methylocystis parvus* NCIMB 11129^T was grown in combination with the heterotrophic bacterium *Escherichia coli* K12 and the methylotrophic yeast *Pichia pastoris* GS115. The latter were inoculated from plates into sterile 12 mL tubes (Greiner Bio-One, Belgium) filled with nutrient broth (NB) medium containing 3.0 g L^{-1} beef extract and 5.0 g L^{-1} peptone. The tubes were placed on an incubator shaker (120 rpm) at 37°C under sterile conditions. After one or two days of growth, the bacterial and yeast cultures were centrifuged at 4000 g for 5 min after which the supernatant was removed and the pellet was washed with dNMS medium. This process was repeated and the resuspended cells were then added to the methanotrophic monocultures.

The *Escherichia coli* or *Pichia pastoris* strains were added to separate MOB monocultures (1% inoculation) at a 1/1000 lower cell density, to prevent heterotrophic dominance. Daily, GC measurements were performed to determine the methane oxidation rate (MOR). In parallel, the MOB were grown in monoculture to monitor the possibly inhibitory effect of coculturing with *Escherichia coli* or *Pichia pastoris* on the CH_4 oxidation activity. Mono- and cocultures with *Methylocystis parvus* were incubated for three days. In parallel, the non-methanotrophic strains were grown in monoculture to evaluate whether they were capable of growing on dNMS with CH_4 as sole carbon source. After the cultivation period, MPN series were conducted on NB medium at 28°C to determine the amount of viable *Escherichia coli* and *Pichia pastoris* cells as described by Hoefman et al. (2012).

Since the initial cell concentration of these cultures was unknown, the increase in cell density following cultivation could not be determined. Therefore, an additional test was conducted with *Pichia pastoris* GS115 in monoculture and in coculture with *Methylocystis parvus* (in triplicate), while flow cytometry with live/dead staining was used to quantify *Pichia pastoris* growth.

VI.2.4 PCR-DGGE

DNA extractions were performed using a FastDNA® Spin kit for Soil (MP Biomedicals, USA) in accordance with van der Ha et al. (2012). Polymerase Chain Reaction (PCR) amplification was performed with general bacterial primers (P63F/P1378r), which target the 16S rRNA gene (Ovreas et al., 1997) as described by van der Ha et al. (2012). Thereafter, Denaturing Gradient Gel Electrophoresis (DGGE) was performed using an INGENY phorU2x2 System (Goes, The Netherlands) and the resulting DGGE patterns were further processed using BioNumerics software version 5.10 (Applied Maths, Belgium).

VI.2.5 Flow cytometry

Cell densities were measured by means of flow cytometry (Cyan™ ADP Analyzer with Summit 4.3 software, DakoCytomation, The Netherlands). Samples were initially diluted at a dilution ratio of 1:2, 1:5 or 1:100, respectively, depending on the turbidity of the sample. For the dilution ratio of 1:2, 500 µL sample was added to 470 µL filtered Evian mineral water, which was used because of its stable chemical composition and microbial community, and low background noise (Berney et al., 2008). Ten µL of CountBright absolute counting beads (Dako Cytocount Catn° S2366) were added to each sample to quantify the amount of cells detected. Live/Dead staining was performed to measure the percentage of living cells. Therefore, 10 µL Na_2EDTA (500 mM, pH 8) and 10 µL Live/Dead stain (1mL Live/Dead stain contained 10 µL SYBR green, 20 µL propidium iodide and 990 µL filtered dimethyl sulfoxide) was added to each sample and the solutions were then placed in the dark for ± 15 min. Data are shown as two parameter correlated plots, called cytograms, and are presented as a dot plot in which each cell is represented by a single dot.

VI.2.6 Stable Isotope Probing (SIP)

DNA based stable isotope probing (DNA-SIP) was performed according to a slightly modified version of Neufeld *et al.* (2007). The different steps of the DNA-SIP protocol are

presented in (Figure VI-2). Samples were first incubated with a ^{13}C labelled substrate (^{13}C-CH_4, 99 atom% ^{13}C, Campro Scientific GmbH, Belgium) and DNA was extracted by using a FastDNA® Spin kit as mentioned previously. The ultracentrifugation protocol used a gradient medium without EtBr and the DNA was subsequently retrieved by fractionation. Ultracentrifugation and subsequent fractionation was performed to separate the 'heavy' ^{13}C labelled DNA from the 'light' ^{12}C labelled DNA. In a sterile 12 mL tube, 4.5 mL of the CsCl stock solution (density 1.88 g mL^{-1}) was added to 80 µL of DNA solution and 980 µL Gradient Buffer to obtain a desired final CsCl density of 1.725 g mL^{-1} (Neufeld et al., 2007). The tubes were mixed by gentle inversion and the solution was transferred to a ultracentrifugation tube (4.9 mL tubes, Beckman Optiseal, USA) with a syringe and needle. Ultracentrifugation conditions were 43,000 rpm in a Vti 65 rotor (Beckmann L7-55 Ultracentrifuge, USA) with vacuum at 20°C for 40-44h. After the spin, the DNA was retrieved by gradient fractionation (12 fractions of ± 400 µL each) and the subsequent DNA precipitation was performed according to Neufeld *et al.* (2007). The DNA was stored at -20°C and 16S rRNA gene PCR/DGGE was performed as mentioned previously.

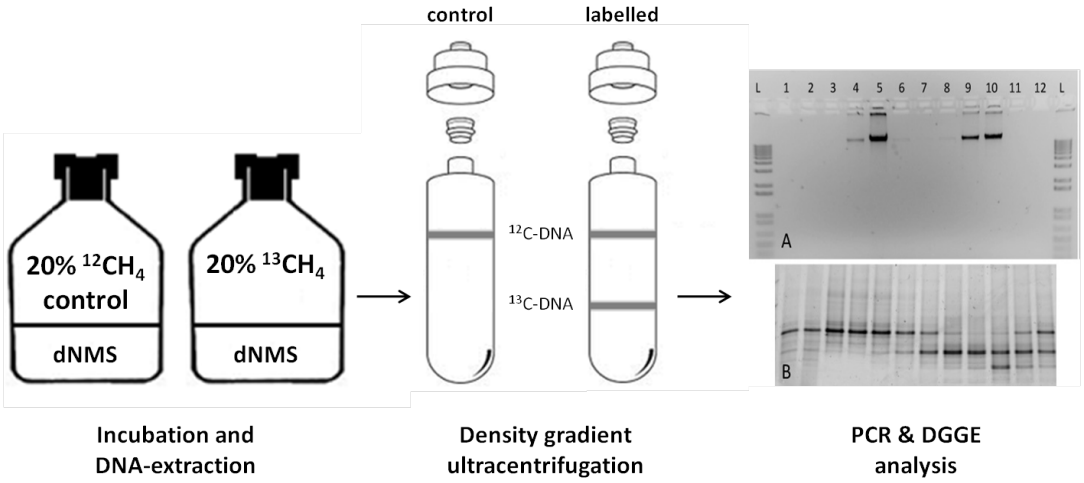


Figure VI-2. Stable Isotope Probing analysis. Two reactors were set up, one with ^{13}C labelled CH_4 and one control reactor with ^{12}C-CH_4. After incubation, DNA was extracted and density gradient ultracentrifugation using CsCl was performed to separate ^{13}C labelled DNA from ^{12}C labelled DNA. Fractionation and subsequent precipitation was performed to retrieve the DNA, which was then subject to PCR and DGGE analysis.

VI.2.7 Physico-chemical analyses

Microbiological growth resulted in visual turbidity, measured by the optical density (OD) at 600 nm using an ISIS 9000 MDA Photometer (DR Lange, Belgium). The total chemical oxygen demand (COD) was measured using manual COD kits (Nanocolor® COD test kit, Macherey-Nagel, UK). The chemical oxidation reaction resulted in a color change, which was measured with a Nanocolor 500D Photometer (Macherey-Nagel, UK. To determine soluble COD (sCOD), samples were first centrifuged (SORVALL RC 5C plus, Sysmex, The Netherlands) for 10 min at 10,000 g and the supernatant analyzed. Total organic carbon (TOC) analysis was performed by means of the Hach Lange difference method (cuvette test LCK381, Hach Lange, Germany) with a measuring range of 60-735 mg TOC L^{-1}. The concentration was assessed after 2h of digestion using a Xion 500 model LPG-385 spectrophotometer (Hach Lange, Germany). To determine the amount of soluble TOC (sTOC), the samples were also centrifuged. Then, the supernatant was analyzed with the Hach Lange difference method (cuvette test LCK380) with a measuring range of 2-65 mg TOC L^{-1}. The amount of total suspended solids (TSS) was determined according to Greenberg et al. (1992). The SO_4^{2-}-, PO_4^{3-}-, $CHOO^-$-, Cl^--, NO_3^-- and NO_2^--concentrations were measured with a Dionex Ion chromatograph (IC 761 Compact, Metrohm, Switzerland) described by van der Ha et al. (2011), while short chain fatty acids (SCFA) extractions and analyses were performed according to Rabaey et al. (2005). The amount of formaldehyde in the supernatant was measured with a semi-quantitative colorimetric method using a color card and sliding comparator (Aquamerck®, Merck chemicals, Germany). Soluble sugar analysis of the supernatant was done by means of a phenol-sulphuric acid method based on absorbance measurements (Masuko et al., 2004).

GC analysis was used to determine the CH_4, O_2 and CO_2 content (% v/v) in the headspace by means of a Compact GC (Global Analyzer Solutions, Louvain-la-Neuve, Belgium), equipped with two channels, both with a thermal conductivity detector (van der Ha et al., 2011). Gas pressure measurements were performed using an Infield 7 pressure meter (UMS, München) indicating the relative pressure in hPa. The methane oxidation rate

(MOR) was calculated and expressed as mg CH_4-COD L^{-1} d^{-1} using a conversion factor of 4 mg COD mg^{-1} CH_4. Results were statistically analyzed with an independent two-sample student t-test with equal variance and a significance level of 99% (*n* results, $p<0.01$).

VI.3 Results

VI.3.1 Metabolite production by MOB

In order to evaluate possible carbon fluxes from MOB towards the associated community, biomass related parameters were analyzed (Table VI-1). The results obtained in these tests indicated that 24.83 ± 7.95% (*n*=11) of the organic matter was present in the culture liquid phase. No formaldehyde or SCFA were detected; yet, soluble sugars were detected with on average 0.72±0.33 mg sugars mg^{-1} sCOD. Values and ratios of the measured parameters differed considerably, even between different *Methylomonas* sp. strains.

VI.3.2 Carbon fluxes in the methanotrophic microbiome

Three methanotrophic communities were incubated with ^{13}C-CH_4, allowing to observe fluxes of labelled carbon from the metabolized CH_4 to the MOB and the associated heterotrophic community by means of SIP. During the 8-day enrichment step, an average daily MOR of 199 mg CH_4-COD L^{-1} d^{-1} was observed for the culture taken from a WWTP and 42 mg CH_4-COD L^{-1} d^{-1} for the culture taken from the swamp. Although GC measurements were not performed during the SIP experiment to avoid disturbance of the communities, the decrease in pressure from 1.25 atm to less than 0.1 atm indicated that a methanotrophic community was active.

Table VI-1. The concentration (mg L^{-1}) of CDW, (s)COD, (s)TOC and soluble sugars for the medium, 11 isolates and 2 reference strains. Not determined values are indicated by "-".

Strain	Genus ID	CDW	COD (mg L^{-1})		TOC (mg L^{-1})		Soluble sugars
		(mg L^{-1})	total	soluble	total	soluble	(mg L^{-1})
dNMS	-	15	< 15	< 15	< 10	< 5	-
R - 45362	*Methylomonas sp.*	255	190	59	60	10	35
R - 45363	*Methylomonas sp.*	248	212	44	59	26	28
R - 45364	*Methylomonas sp.*	273	200	39	77	25	42
R - 45370	*Methylomonas sp.*	265	216	57	83	11	47
R - 45371	*Methylomonas sp.*	270	261	42	51	14	59
R - 45372	*Methylomonas sp.*	250	271	53	75	32	22
R - 45374	*Methylomonas sp.*	233	183	39	69	9,2	32
R - 45377	*Methylomonas sp.*	109	29	< 15	< 5	< 10	< 10
R - 45378	*Methylomonas sp.*	237	298	90	111	19	66
R - 45379	*Methylosinus sp.*	146	54	25	16	< 10	19
R - 45383	*Methylomonas sp.*	240	251	60	70	18	25
DSM 17706^{T}	*Methylosinus sp.*	232	198	40	71	24	34
NCIMB 11130^{T}	*Methylomonas sp.*	229	251	58	101	21	12

The flux of ^{13}C-labeled methanotrophic metabolites towards the whole methanotrophic community was followed up by means of DGGE. The 16S rRNA gene DGGE profiles were analyzed over time, thereby dividing the number of bands in the 'heavy' ^{13}C labelled DNA fraction (fraction 4) of each sample by the maximum total number of bands visible in the same sample. The latter varied between 9 and 12 for SIP run A - WWTP, between 15 and 17 for SIP run B - swamp, and between 6 and 14 for SIP run C - lab reactor. The percentage of bands in the ^{13}C gradient fractions increased from 22% after 1 hour to 100% after 96 hours for the WWTP sample (Figure VI-3), from 38% after 4 hours to 59% after 120 hours for the sample taken from a swamp (Figure VI-4), and from 0% after 1 hour to 50% after 96 hours for the sample taken from a lab reactor (Figure VI-4). These percentages increased to values over 70% in case fraction 5 was also included.

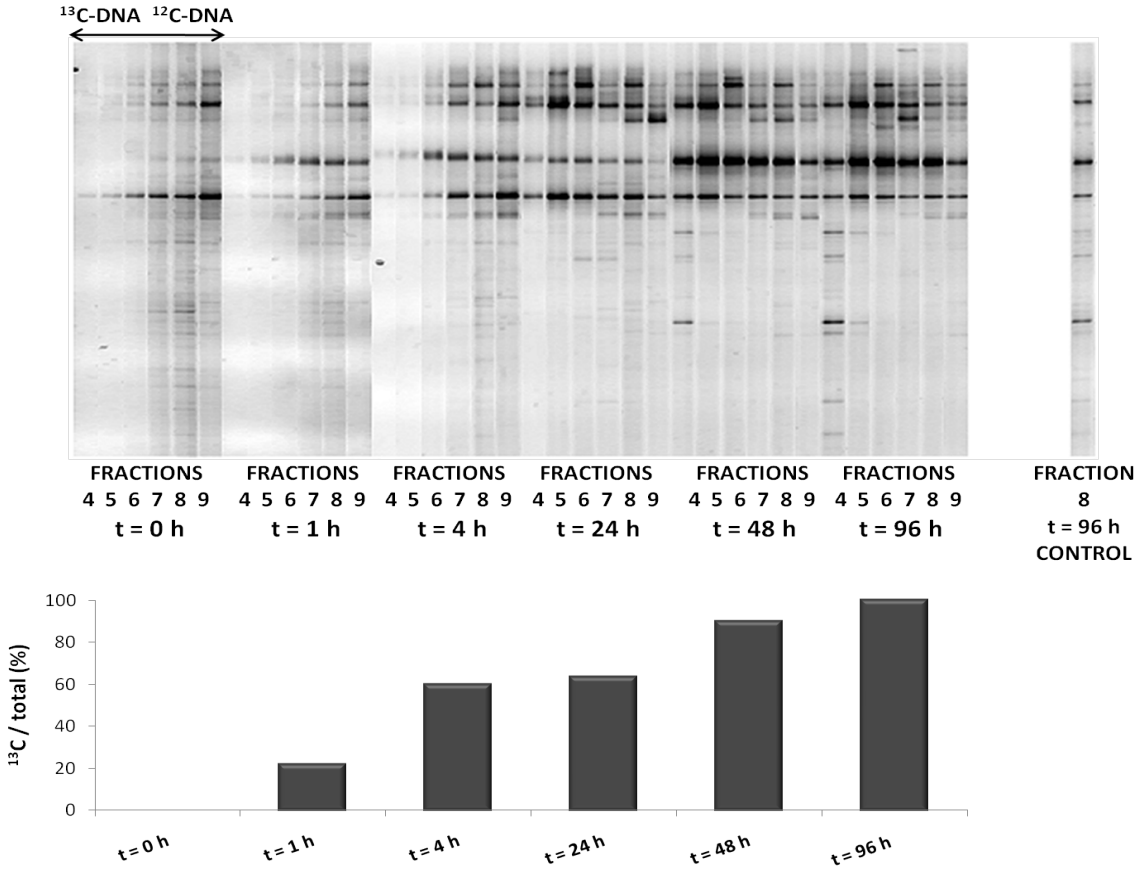


Figure VI-3. DGGE fingerprint pattern (top) of SIP run A (WWTP) representing gradient fractions 4 ('heavy' ^{13}C-DNA) to 9 ('light' ^{12}C-DNA) at different time points, and gradient fraction 8 (^{12}C-DNA) of the control. The bottom graph shows the % bands in the ^{13}C-DNA fraction (fraction 4) relative to the maximum total number of bands for that time point.

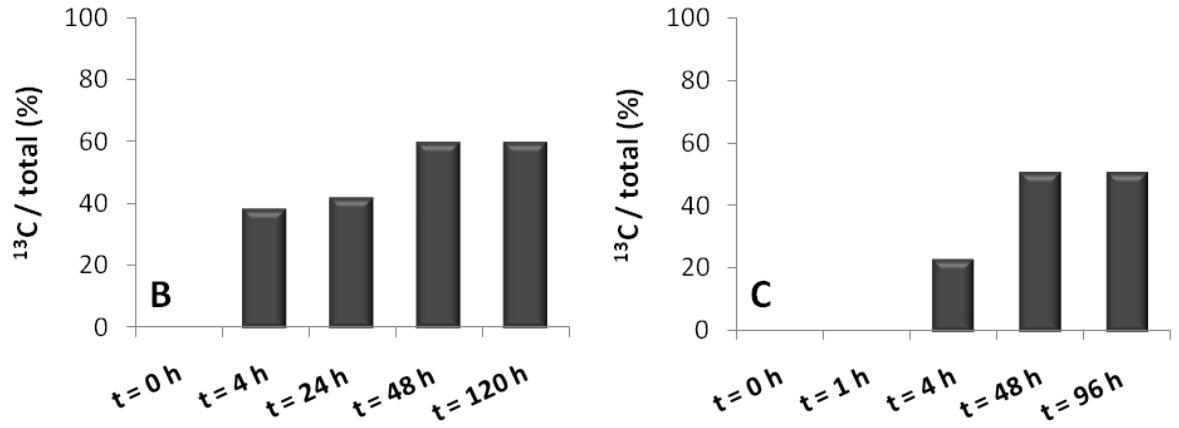


Figure VI-4. Percentage of bands in the 'heavy' ^{13}C-DNA fraction (fraction 4) of each time sample relative to the maximum total number of bands visible in the same time sample. B: swamp; C: lab reactor.

The number of bands in the ^{13}C DNA fraction (fraction 4) of each sample was also divided by the number of bands in the light ^{12}C DNA fraction (fraction 8) of the same sample. These results showed an analogous pattern and the percentages increased from 22% after 1 hour to 125% after 96 hours for the WWTP sample, from 40% after 4 hours to 59% after 120 hours for the swamp sample, and from 0% after 1 hour to 67% after 96 hours for the sample taken from a lab reactor. Percentages of more than 100% were obtained as progressively less ^{12}C and more ^{13}C was incorporated into the DNA. This caused the bands on the fingerprint pattern to gradually disappear from the ^{12}C-fraction whilst emerging in the ^{13}C-fraction during incubation. This occurred within the monitored time frame for SIP run A (fractions 4&5, after 48 h) and SIP run C (fraction 5, after 96 h). Considering increasingly more ^{13}C was incorporated into the bacterial DNA, DGGE bands in the ^{13}C fraction did not merely appear but also increased in intensity (expressed as height of the densiometric curve) with time. In addition, the maximum band intensity gradually shifted from fractions 8-9 (^{12}C) to fractions 4-5 (^{13}C). To investigate this effect, the band intensity was determined for one of the most dominating bands of each SIP run (Figure VI-5). For one of the most dominating bands of SIP run A, the band height in fraction 5 increased from 2.7 x 10^3 after 1h to 45.0 x 10^3 after 48 hours, and its maximum intensity shifted gradually from fraction 9 to fraction 5. The band height in fraction 4 increased from 3.3 x

10^3 after 4h to 44.6 x 10^3 after 120h for the most dominating band of SIP run B, and its maximum intensity gradually shifted from fraction 8 to fraction 5. For the most dominating band of SIP run C, the band height in fraction 5 increased from 3.8 * 10^3 after 1h to 56.4 x 10^3 after 96h, and its maximum intensity shifted from fraction 8 to fraction 5.

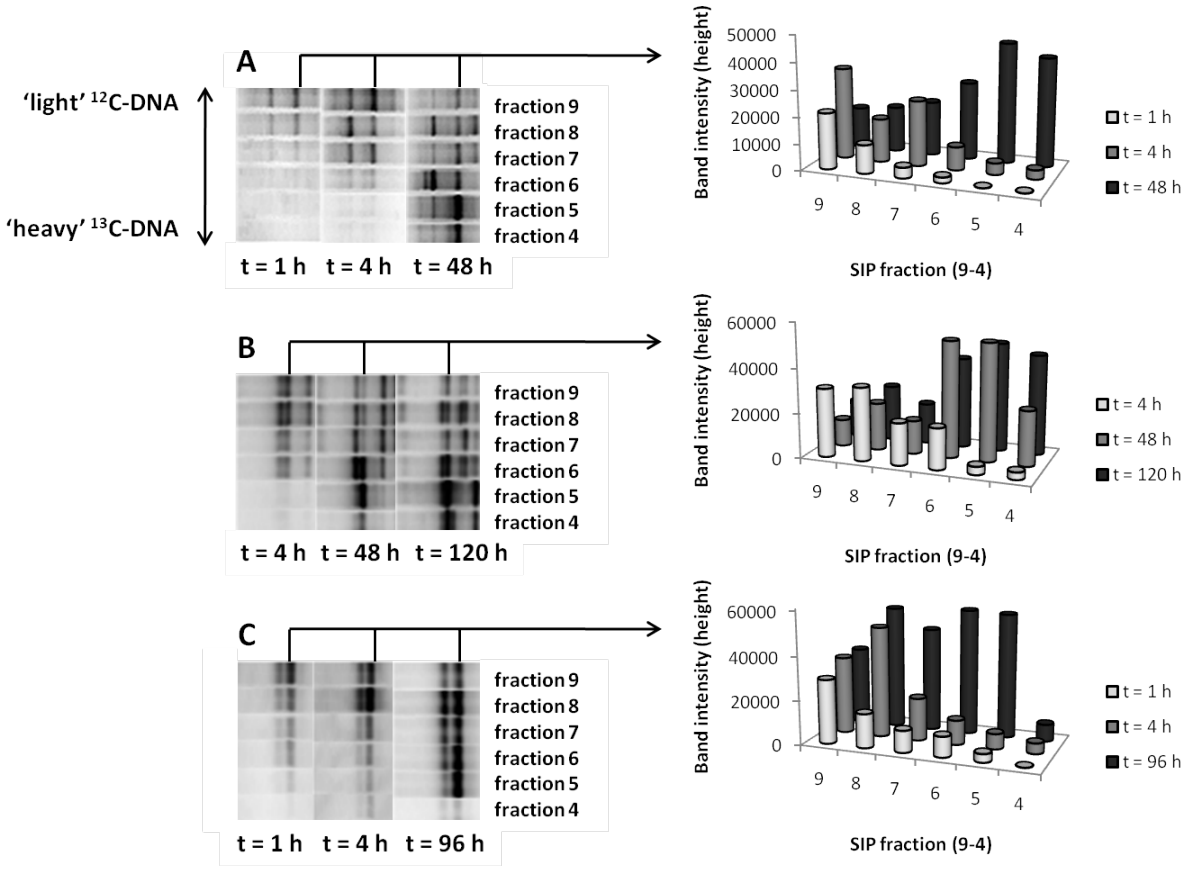


Figure VI-5. Part of the DGGE fingerprint pattern visualizing some of the most dominating bands, for gradient fractions 9 ('light' ^{12}C-DNA) to 4 ('heavy' ^{13}C-DNA) at different points in time (left). Per SIP run (A: WWTP, B: swamp, C: lab reactors), one of the most dominating bands, as indicated by the arrows, was selected for further analysis of the band intensity. The graphs on the right show the evolution of the investigated band intensity, represented by the height of the densiometric curve, for gradient fractions 9 (^{12}C) to 4 (^{13}C) at different points in time.

VI.3.3 Metabolic networking

In order to evaluate potential applications based on metabolic networking, the mutual influence of an MOB and a heterotrophic *Escherichia coli* or a methylotrophic *Pichia pastoris* yeast, respectively, on each others growth and activity was investigated. To evaluate the influence of the non-methanotrophs on the methanotrophic activity, the MOR was compared. This was 468 mg CH_4-COD L^{-1} d^{-1} for the *Methylocystis parvus* monoculture, 496 mg CH_4-COD L^{-1} d^{-1} in coculture with *Pichia pastoris* and 507 mg CH_4-COD L^{-1} d^{-1} in coculture with *Escherichia coli*. These results indicated that coculturing did not have a significant effect (p<0.05) on the methanotrophic activity. There was no CH_4 removal when *Escherichia coli* or *Pichia pastoris* was grown in monoculture on dNMS.

A second experiment was conducted in order to better quantify the carbon flux processes. Three cocultures of *Methylocystis parvus* and *Pichia pastoris* and one *Methylocystis parvus* monoculture were incubated with 20% CH_4 (v/v). The average MOR was 45 mg CH_4-COD L^{-1} d^{-1} for coculture n° 1, 440 mg CH_4-COD L^{-1} d^{-1} for coculture n° 2 and 443 mg CH_4-COD L^{-1} d^{-1} for coculture n° 3. These results indicate that cocultures n° 2 and 3 were active and showed growth, unlike coculture n° 1 which showed low activity and little growth. The *Pichia pastoris* monoculture showed no CH_4 removal activity and hardly any growth. These results were confirmed by the OD and COD increments over the whole incubation period (Table VI-2), as the OD and COD increments are significantly higher (p<0.01) for the two active cocultures compared to the monoculture and the low-active coculture.

Table VI-2. OD and COD values of the *Pichia pastoris* mono- and cocultures at time t = 0d and t = 3d. The low-active coculture n° 1 is indicated in grey.

	OD_{600nm}		COD (mg L^{-1})	
	t = 0 d	**t = 3 d**	**t = 0 d**	**t = 3 d**
monoculture	0.024	0.056	34	49
coculture n° 1	0.049	0.119	40	93
coculture n° 2	0.049	0.759	35	626
coculture n° 3	0.049	0.803	40	402

At the beginning and end of the incubation period, cell densities of *Pichia pastoris* were measured with flow cytometry, as this allows appropriate quantification (Figure VI-6). Due to the larger size of the yeasts, differentiation with the MOB was possible, while at the same time Life/Dead staining indicated the active state of the majority of the yeast cells. The cell density of the yeast monoculture increased with a factor 1.15, the cell density of *Pichia pastoris* in coculture n° 1 increased with a factor 1.24, in coculture n° 2 with a factor 1.68 and in coculture n° 3 there was an increase in cell density with a factor 2.24 (Figure VI-7). These results are a slight underestimation of the actual increase in cell density since the flow cytometer could not separate MOB and yeast cells perfectly, and therefore there was a small overlap, which was not included.

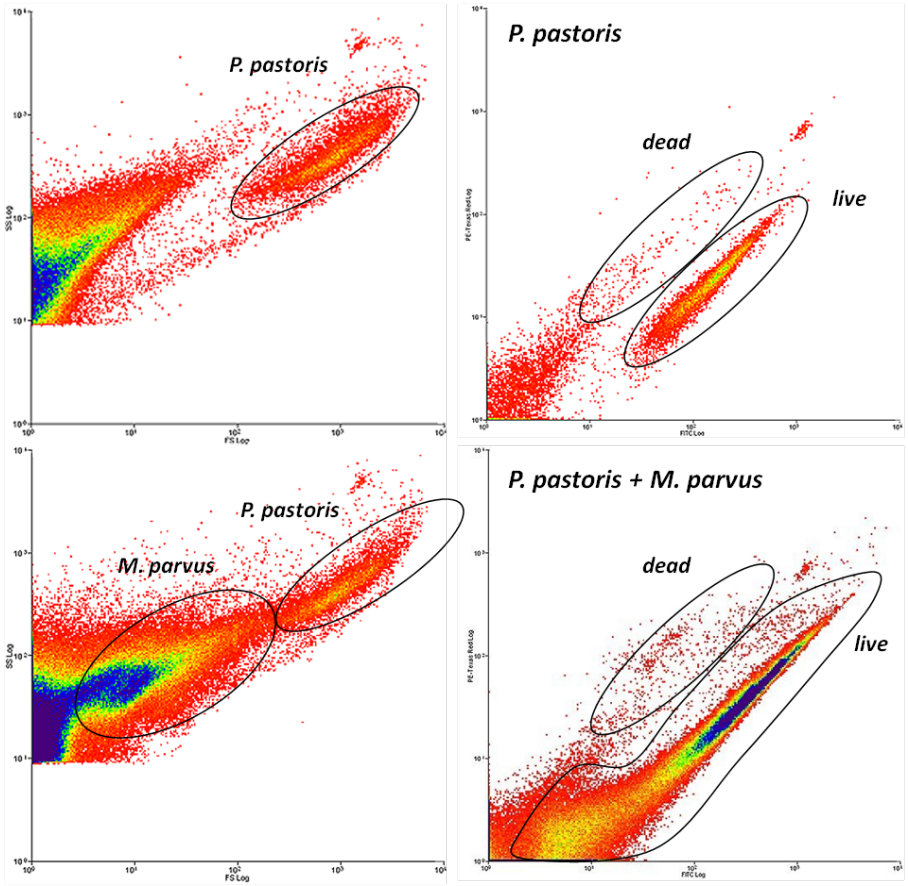


Figure VI-6. Flow cytometry data plotting sideward scatter (SS log) in function of forward scatter (FS log) (left) and PE-Texas Red log in function of FITC log (right) for Live/Dead staining. The top graphs are dot plots of a *Pichia pastoris* monoculture and the bottom graphs are dot plots of a coculture containing *Pichia pastoris* and *Methylocystis parvus*.

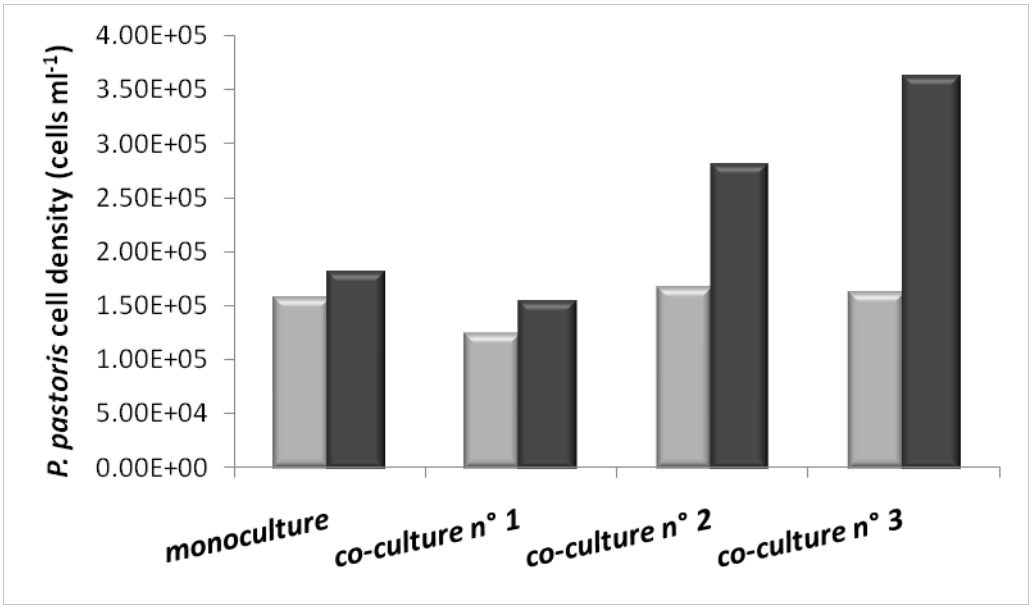


Figure VI-7. Cell densities of the *Pichia pastoris* mono- and cocultures at time t= 0 d (light grey) and t = 3 d (dark grey) measured using flow cytometry.

Live/Dead staining was performed in combination with the cell density measurements to determine the percentage of viable *Pichia pastoris* cells (Table VI-3). At the end of the incubation period, the percentage of living cells was highest for the cocultures n° 2 and 3, and the percentage of dead cells increased for the yeast monoculture and low-active coculture n° 1.

Table VI-3. Live/Dead staining results of the *Pichia pastoris* mono- and cocultures at the beginning and end of the incubation period. The low-active coculture n° 1 is indicated in grey.

Sample	Living cells		Dead cells	
	t = 0 d	t = 3 d	t = 0 d	t = 3 d
monoculture	99%	65%	1%	35%
coculture n° 1	99%	74%	1%	26%
coculture n° 2	99%	98%	1%	2%
coculture n° 3	98%	97%	2%	3%

VI.4 Discussion

VI.4.1 MOB release considerable amounts of organic carbon to the environment

In order for associated community members to obtain and metabolize CH_4 derived organic C, MOB first need to release suitable compounds. Exploratory experiments were conducted to evaluate the amount and composition of the metabolites produced by MOB and leaking into the culture medium. It appeared that clear differences occurred between the various MOB strains investigated, but on average 25% of the organic matter was present in the culture medium, probably as sugar-containing compounds, extrapolymeric substances and/or methanol, since formaldehyde and short chain fatty acids tests proved to be negative. Although decayed MOB cells could be a source of organic C for heterotrophs, a methylotrophic yeast strain only showed significant growth when an active MOB was present. This indicates that active C fluxes took place and that the metabolic networking is more than just phagotrophy.

VI.4.2 CH_4 derived carbon is distributed rapidly over the methanotrophic community

A time course labelling experiment allowed to monitor the distribution of ^{13}C labelled metabolites and the subsequent incorporation of ^{13}C into the DNA of the community members. Based on the DGGE fingerprint pattern, the percentage of bands in the 'heavy' ^{13}C labelled DNA fraction was determined relative to the maximum amount of bands in all fractions, at that specific point in time. For the WWTP sample, the percentage of bands present in the ^{13}C gradient fraction relative to the maximum amount of bands reached 100% after 96 hours of incubation indicating that the ^{13}C had progressively spread over the entire community. This percentage was however lower, but above 50%, for the other two samples. The methane oxidation activity of the reactor containing the swamp sample was lower than that of the WWTP sample, and this could be the reason why the metabolic networking was also slower in the swamp community compared to the WWTP community.

The obtained results indicated that metabolic networking occurred, since ^{13}C labelled compounds were used by a considerable amount of detected and therefore prominently present bacteria. Although the bands were not identified, it is clear that heterotrophs were involved, as in analogous incubations with comparable inocula, more than half of the methanotrophic community members observed with the same 16S rRNA gene based DGGE had a heterotrophic nature (van der Ha et al., 2012). In conclusion, the conducted experiments provide proof of the existence of a microbial food chain driven by CH_4 derived organic C. The obtained results are in agreement with research performed at the Max Planck Institute (Dumont et al., 2011). DNA- and mRNA-based SIP experiments showed that cross-feeding of methanotrophic metabolites by other microorganisms occurred and this cross-feeding of the ^{13}C was primarily detected by DNA-SIP.

Molecular techniques like high throughput sequencing methods could be useful to analyze the composition of the heavy fractions, in order to determine how many of the bands in the 16S rRNA DGGE represent MOB and how many represent associated members. Moreover, it would be of interest to know which organisms are first in line to use which ^{13}C-labelled metabolites produced by the MOB. If the network is very specific, then the same type of species are expected to be the first to appear in the ^{13}C fraction of the DGGE fingerprint pattern, independent of the original sample. However, if the network is rather random, the metabolites produced by the MOB will be used by a variety of species. Experiments could also be conducted in which an extra non-labelled carbon source is provided to the methane oxidizing community. This could result in less intensive networking and consequently, the incorporation of ^{13}C into the DNA of the entire community will progress more gradually. In order for all these tests to work, a large amount of samples should be taken and both the substrate affinity and growth characteristics, i.e. doubling time, of all species should be known.

VI.4.3 Towards applications

The previously mentioned results indicate that metabolic networking occurs within methanotrophic communities and therefore a possible application of this networking was

explored. While coculturing experiments with *Escherichia coli* were not successful, *Pichia pastoris* GS115 was capable of growing in coculture with the MOB, without any readily available carbon source. Moreover, active methane oxidation was necessary for the survival of *Pichia pastoris* GS115, indicating metabolites specifically produced by the methanotrophs (e.g. extrapolymeric substances and/or methanol) were used by *Pichia pastoris* GS115 instead of dead biomass. The cell density of *Pichia pastoris* GS115 grown in coculture with *Methylocystis parvus* increased with a factor 2.24 compared to *Pichia pastoris* GS115 grown in monoculture on dNMS with CH_4 as sole carbon source. The percentage of living yeast cells after a 3-day cultivation period was 97% in a coculture showing high methane oxidation rates, 75% in a coculture showing very low methane oxidation rates, and merely 65% in monoculture.

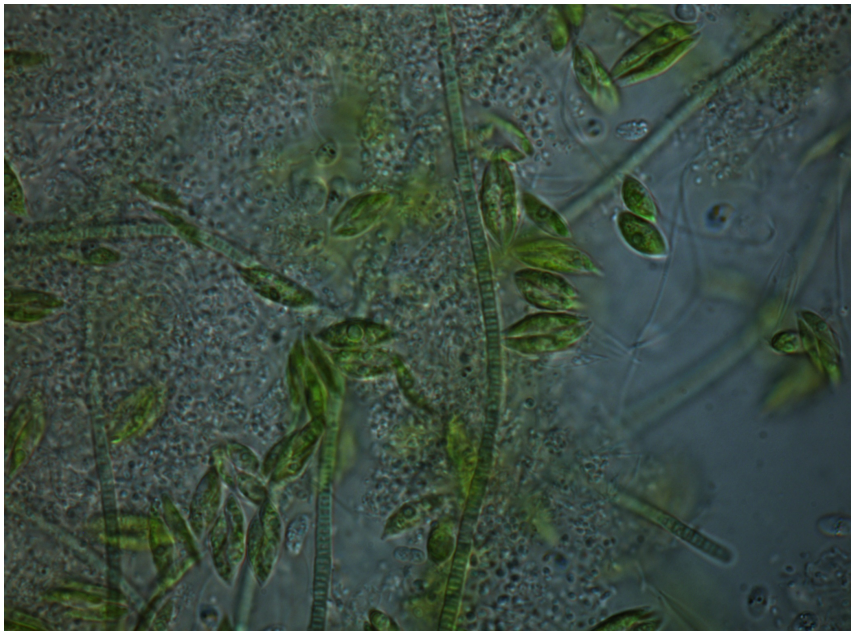
The conducted experiments deliver proof of principle that the metabolic network can exist between MOB and other associated microorganisms. This can have potential applications in the biotechnological industry. Further research is necessary to determine the long-term effects and upscaling possibilities. In addition, tests should be conducted with other MOB species, since *Methylocystis parvus* is known to produce few extracellular substances. Therefore, superior results could be obtained with MOB species that produce more metabolites or extrapolymeric substances, respectively. Additionally, methylotrophic bacteria can also be tested in coculture with MOB, since this diverse group of organisms is capable of growing on C1-substrates such as methanol. It is important to further elucidate the metabolic networking and to fully characterize the metabolites produced by MOB in order to optimize this concept and select the best possible methanotrophic-heterotrophic cocultures.

Acknowledgements

DVDHA, IV, WV and NB designed the experiments. IV and DVDHA performed the experiments, analyzed the data and wrote the manuscript. SH maintained the MOB cultures, analyzed the soluble sugar content and gave support for the analyses. PDV, SH, WV and NB commented on the manuscript. This work would not have been possible without the support of Tim Lacoere. Moreover, our gratitude for the assistance we got from Sue Read, Prof. dr. C. Murrell, Geert Meesen and the department of molecular biotechnology (UGent). This research was funded by a PhD grant for David van der Ha from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen, SB-83259) and a research grant from the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005).

CHAPTER VII

CARBON NEUTRAL METHANE OXIDATION BY A PARTNERSHIP OF MOB AND MICROALGAE



Light microscopic image of a methalgae floc, LabMET

Don’t take any wooden nickels

When you sell your soul.

Mr. E

Chapter VII. Carbon neutral methane oxidation by a partnership of MOB and microalgae

Abstract

Effluents of anaerobic wastewater treatment plants are saturated with methane. A novel approach is presented to treat such effluents using a coculture of methane oxidizing communities and microalgae, allowing microbial methane oxidation with minimal CO_2 emissions. Coculturing such *methalgae* communities in sequence batch reactors under continuous illumination yielded a factor of about 1.6 more biomass relative to the control reactors without microalgae. Moreover, 55% less external O_2 supply was needed to maintain the methane oxidation, as O_2 was produced *in situ* by the microalgae. An overall methane oxidation rate of 171 ± 27 mg CH_4 L^{-1} liquid phase d^{-1} was accomplished in a semi-batch set-up, while the excess CO_2 production was lower than 1 mg CO_2 L^{-1} d^{-1}. Moreover, *methalgae* cultures adapted easily to an industrial digestate. Even under anoxic conditions, considerable methanotrophic activity was observed due to the fact that MOB could rely on *in situ* O_2 production by algal photosynthetic processes. These results show that a coculture of microalgae and methane oxidizing communities could lead to a novel treatment for dissolved CH_4 in anaerobic effluents.

Redrafted after:

van der Ha, D., Bundervoet, B., Verstraete, W., Boon, N. (2011) A sustainable, carbon neutral methane oxidation by a partnership of methane oxidizing communities and microalgae. Water Research 45: 2845-2854.

VII.1 Introduction

There is an increasing interest for anaerobic treatment technology, such as upflow anaerobic sludge blanket digestion, which allows recovery of energy and nutrients (van Lier et al., 2001, Verstraete et al., 2009). However, during such processes, part of the generated biogas dissolves in the liquid effluent, resulting in a considerable release of methane into the atmosphere and consequently also a loss of energy. Although under typical conditions of an anaerobic digestion process (35°C, 60% CH_4, 40% CO_2 (v/v)), only 11 mg CH_4 L^{-1} is dissolved in the effluent, CH_4 losses can be up to 25% of the produced CH_4, especially when treating low strength wastewater (Cakir and Stenstrom, 2005, Hartley and Lant, 2006). When the loading rate is lower than 0.7 g biological oxygen demand L^{-1}, anaerobic treatment can emit even more CO_2-equivalents than treatment with traditional activated sludge processes (Cakir and Stenstrom, 2005). The reason for these high emissions is twofold: firstly, there is a continuous outflow of relatively large volumes of CH_4 saturated effluent. Secondly oversaturation up to a factor of 4 has been observed in digester effluents (Hartley and Lant, 2006). Only few studies have described reactors to treat dissolved CH_4 in such effluents (Hatamoto et al., 2010, Matsuura et al., 2010). The introduction of an economically feasible CH_4 oxidizing unit in the typical polishing step of anaerobic digester effluents, could lower the greenhouse gas emissions of anaerobic treatment installations (Verstraete et al., 2009). Moreover, if dissolved CH_4 and CO_2 could be transformed into products with added value, treatment of low-strength wastewater would effectively be seen as a valuable service.

Methane is particularly unreactive: it requires 435 kJ mol^{-1} to break the chemical bond between the carbon and hydrogen atom (Dalton, 2005). As a result it is not easily removed with physico-chemical techniques. Aerobic methane oxidizing bacteria (MOB) are able to break this strong C-H bound by means of the enzyme methane monooxygenase, which exists in a soluble and particulate form (Dalton, 2005, Hanson and Hanson, 1996). A drawback of applying such MOB in anaerobic effluents is that oxygen needs to be added externally under such conditions that the CH_4 is not stripped from the liquid.

To avoid external aeration and stripping, O_2 can be provided through the addition of microalgae in the system. Both prokaryotic cyanobacteria, eukaryotic microalgae and diatoms produce O_2 *in situ* during the photosynthetic reactions, when sufficient light energy is provided for CO_2 fixation (Mata et al., 2010, Melis, 2009, Thajuddin and Subramanian, 2005). The synergistic relationship whereby microalgae provide O_2 for bacterial processes has been successfully applied for tertiary treatment of different industrial wastewaters, with the focus on removal of organic carbon, nitrogen and phosphorus (Molinuevo-Salces et al., 2010, Munoz and Guieysse, 2006). Algal-bacterial processes have also been used for treatment of hazardous contaminants. For example, different algal-bacterial cocultures were able to degrade phenanthrene, acetonitrile and salicylate by pollutant-specific bacteria without an external O_2 supply (Borde et al., 2003, Guieysse et al., 2002, Munoz et al., 2005). As far as the authors know, no research has been performed on cocultures of microalgae and CH_4 oxidizing communities for CH_4 mitigation. In nature however, synergistic relationships are ubiquitous and have been observed between MOB and photosynthetically active mosses (Kip et al., 2010, Raghoebarsing et al., 2005). While the MOB provide the C source for the eukaryotes, O_2 is provided in return by the photosynthetically active species. This type of relationship has also been observed previously between MOB and microalgae in fresh water lakes (Kankaala et al., 2006, Trimmer et al., 2010).

In this study, a coculture of MOB and microalgae, further indicated as *methalgae*, was evaluated in the scope of a greenhouse gas free CH_4 oxidation. The aim of this study was to achieve a sustainable CH_4 oxidation with a lower need for externally supplied O_2, while observing the influence of the N source. Lastly, it was tested if *methalgae* cultures are able to sustain activity in an industrial digestate without external aeration. The outcomes of this study provided a first successful attempt towards the development of a treatment unit for dissolved CH_4 in O_2-depleted effluents.

VII.2 Experimental section

VII.2.1 Sampling and inoculation

Nine identical gastight bottles, each with a total volume of 1150 mL served as reactor. Six of the latter were filled with 200 mL dNMS-medium (per liter: 1 g KNO_3; 0.2 g $MgSO_4.7H_2O$; 3.59 g $Na_2HPO_4.2H_2O$; 1.36 g KH_2PO_4; 45 mg $CaCl_2.6H_2O$; 4.5 mg Na_2-EDTA; 25 µg $CuSO_4.5H_2O$ (0.1 µM); 3.5 mg $FeCl_3.6H_2O$) according to Whittenbury et al. (1970b). The three other gastight bottles were filled with 200 mL dAMS-medium, which has the same composition as dNMS-medium, except that 140 mg NO_3^--N was replaced with 140 mg NH_4^+-N, in the form of NH_4Cl. Additionally, 1 mL trace solution L^{-1} was added to the dNMS or dAMS medium, according to van der Ha et al. (2010). The pH of both media was 6.9. An active CH_4 oxidizing community, previously enriched at the laboratory, was used as inoculum (van der Ha et al., 2010). Equal volumes (20% v/v liquid phase) of this mixed inoculum were added to each reactor. The gas phase (950 mL) contained 20% (v/v) CH_4 in air.

VII.2.2 Experimental set-up

Three different treatments were set-up over a period of 30 days: reactors with CH_4 oxidizing communities, referred as “MOC-reactors” and two sets of reactors containing a coculture of methane oxidizing bacteria and algae (MAC), with nitrate (N-MAC) or ammonium (A-MAC) as respective N sources. Firstly, three reactors with a methane oxidizing community (MOC), not inoculated with microalgae, were shielded from the light and had nitrate as N source. Secondly, six reactors with a *methalgae* community were inoculated with a mixed microalgae culture (5% v/v liquid phase), originating from an open pond type photobioreactor, which was used to grow a mixed algal culture in mineral medium under continuous bubbling of CO_2 (De Schamphelaire and Verstraete, 2009). Three of these reactors contained 140 mg NO_3-N as N source (N-MAC), while three contained 140 mg NH_4^+-N as N source (A-MAC). The initial VSS concentration was 61±1 mg

L^{-1} for the reactors with a MOC and 76±1 mg L^{-1} for the N-MAC and A-MAC reactors. All reactor where placed on a shaker at 120 rpm.

All six MAC-reactors were exposed to four TL-lamps (36W, Master TL-D 90 Deluxe, Philips, Eindhoven, The Netherlands), which posses a light spectrum comparable with the solar radiation spectrum. The light intensity (photosynthetic active range; PAR) at the level of the liquid/gas interphase (inside the gastight bottles) was 80 μmol photons-PAR m^{-2} s^{-1}. The illuminated area and illuminated area/volume ratio were 195 cm^2 and 0.97 cm^2 cm^{-3} respectively. The temperature of the water phase in the reactors was measured after each cycle and was 22±2°C.

After each cycle of 72h, the liquid phases of the triplicates were merged together and four fifths of the well-mixed liquid phase was replenished with freshly made medium. Hence, the hydraulic retention time and sludge retention time were both 90 h. To maintain the microbial diversity, 2% (v/v) tertiary effluent of a WWTP (Ossemeersen, Gent, Belgium), wherein microalgae were visibly present, was added in between all cycles. The reactors were open for at least 30 minutes, which allowed the gas phase to equilibrate with the outside air. A volume of 200 mL of CH_4 (99.95% pure, Air Liquide, Liège, Belgium) was then added to the closed reactors. The gas phase of a control reactor after 15 min of equilibration with the water phase contained respectively 193 mL of O_2, 235 mL of CH_4, 793 mL of N_2 and 0.07 mL of CO_2 per L of gas phase. After 10 cycles, the reactors with MOC and N-MAC were discontinued.

VII.2.3 Effect of the N source, ethyn addition and autoclavation

Additional tests were performed, all with an active A-MAC. In all cases, three A-MAC reactors were set up in parallel, as mentioned above, and applied as controls.

- **Nitrogen source** To test the influence of the N source, three reactors with an A-MAC were given 140 mg NO_3-N L^{-1} instead of NH_4^+-N L^{-1} as N source.

- **Ethyn addition** The influence of the CH_4 oxidation inhibitor ethyn (C_2H_2) was analyzed by adding 0.2% (v/v) C_2H_2 to three reactors with an A-MAC. In another three reactors, the composition of the gas phase was altered to 13% (v/v) CH_4, 8% (v/v) CO_2 and 0.2% (v/v) C_2H_2 in air, to investigate the effect of C_2H_2 on the algal CO_2-fixating activity.
- **Autoclavation** When the tests were completed, the reactors were autoclaved (20 min, 121°C, 1 bar) to estimate the influence of losses and chemical adsorption processes. The gastight bottles were cooled down to room temperature and placed on a shaker, with new CH_4 added.

VII.2.4 Growth in industrial digestate

Methalgae cultures were incubated under anoxic conditions in the digestate of an upflow anaerobic sludge blanket (UASB) reactor, treating potato waste of Agristo NV (Harelbeke, Belgium). The digestate was pumped on site into gastight alumina bags at the outflow of the anaerobic digester, while passing a filter with a mesh of 2 mm. The bags were transported to the lab and conserved at 4°C until application. Temperature and DO were measured on site, while DO, pH, anion concentrations, dissolved H_2S concentration, VSS, TSS, TIC, TOC, TC, COD and sCOD were analyzed in samples of three different sampling campaigns. The BOD was measured once. Before starting the test, six Schott bottles (V=595 mL) were flushed for 30 min with N_2 in order to create an anoxic atmosphere, whereafter 100 mL digestate and 100 ml CH_4 were added. Inoculation occurred with 5 mL of an active methanotrophic community, originating from a sequence batch reactor and pre-cultivated on the digestate under an aerobic atmosphere. Algal inoculation occurred by means of 5 mL of active non-axenic algae cultures, i.e. *Chlorella emersonii* (*n*=3) or *Scenedesmus* sp. (*n*=3), respectively. The bottles were placed on a shaker (90 rpm) at a light intensity of 92 µmol m^{-2} s^{-1} and a temperature of 32°C. The gas phase composition of the bottles was daily analyzed by means of GC analyzses.

VII.2.5 Chemical analyses

At the end of each cycle, samples were taken for further analysis. Filtered samples (0.45 µm filter, Millipore, Brussels, Belgium) were analyzed for Cl^-, NO_3^-, NO_2^-, SO_4^{2-}, PO_4^{3-} and $CHOO^-$ by means of an 761 Compact Ion Chromatograph, equipped with a conductivity detector (Metrohm, Zofingen, Switzerland). The ammonium concentration was determined by steam distillation, according to Greenberg et al. (1992). Kjeldahl nitrogen was analyzed by standard methods (Greenberg et al., 1992) and pH was determined with a SP10B pH electrode, connected to a Consort C532 multimeter analyzer (Turnhout, Belgium). A specific color reaction for aldehydes (Schiff's reagent, Merck, Belgium; detection limit: 2 mg L^{-1}) was used to monitor formaldehyde formation, while quantification of the soluble methane monooxygenase activity, by means of a naphthalene oxidation assay, was performed according to van der Ha et al. (2010): a crystal of naphthalene is oxidized by the soluble methane monooxygenase to naphtanol, which reacts with a tetrazotized-o-dianisidine solution. The reaction product was measured with a spectrophotometer at a wavelength of 525 nm. Volatile suspended solids (VSS) were assessed according to Greenberg et al. (1992). The dissolved total carbon (TC), total inorganic carbon (TIC) and total organic carbon (TOC) content was determined with a LCK 381 TC/TOC cuvette test (difference method). This method has a detection range between 60 and 735 mg TOC L^{-1} and was assessed using a Xion 500 model LPG-385 photospectrometer (Hach Lange GMBH, Düsseldorf, Germany). The total and soluble chemical (*n*=3) oxygen demand (COD and sCOD) were analyzed by means of manual COD kits in the range of 100-1,500 mg COD L^{-1} (Nanocolor COD test kit, Macherey-Nagel, UK). For sCOD measurements, samples were filtered with a 0.45 µm Milex filter. The biological oxygen demand (BOD) was also measured by means of the Winkler method (5d, 20°C) according to Greenberg et al (1992). Lastly, sulphide concentrations were analyzed by means of LCK 653 sulphide kits (Hach Lange) with a measuring range between 0.1 and 2.0 mg S^- L^{-1}. The architecture of the *methalgae* flocs was examined with a Zeiss Axioskop 2 Plus epifluorescence microscope (Carl Zeiss, Jena, Germany).

VII.2.6 Gas composition analyses

For each cycle, a gas sample (1 mL) was taken immediately after the addition of CH_4, after 24 h, 48 h and at the end of each cycle (72 h), with a gastight syringe (Hamilton, Sigma Aldrich, Bornem, Belgium). The gas phase composition was analyzed with a Compact GC (Global Analyzer Solutions, Breda, The Netherlands), equipped with a Porabond pre-column and a Molsieve SA column. Concentrations of CH_4, O_2, CO_2, N_2O and N_2 were determined by means of a thermal conductivity detector with a detection limit of 1 ppmv for each gas component. Significant changes in the N_2 concentration of the gas phase were never observed.

VII.2.7 Estimation of the relative algal abundance

Standard methods to quantify chlorophyll content failed due to partial extraction of the chlorophyll. Therefore, a semi-quantitative analysis was developed that allowed to measure differences in chlorophyll content within the *methalgae* flocs: well mixed samples were sonicated (Labsonic M, B. Braun Biotechnology Enterprise GmbH, Melsungen, Germany) for 5 min and transferred to a cuvette, followed by measurement of the chlorophyll peak. The latter was detected at an absorbance wavelength of 685 nm whereas the cultures without microalgae showed a linear pattern between 600 and 800 nm. Thus, the relative chlorophyll peak height was calculated as $absorbance_{685\ nm}$ – ($absorbance_{635\ nm}$ + $absorbance_{735\ nm}$)/2. To validate this parameter, different mixtures of a MOC with the algal inoculum were tested, which resulted in a linear relationship between the algal concentration and the measured ratio, with a coefficient of determination (R^2) value of 0.98 (n=28). At the end of the 10^{th} cycle, the relative peak height was also measured before sonication. This allowed the estimation of the partition of the algal biomass over the liquid phase and the flocs, respectively.

VII.2.8 Statistical analysis

Throughout this study, the standard deviation is given for the triplicates, with average values being calculated over the last five cycles (cycle 6 – cycle 10), unless otherwise mentioned. Likewise, concentrations and rates are expressed per liter liquid phase, unless otherwise mentioned. Statistical analysis was performed with SPSS for Windows, version 15 (SPSS Inc., Chicago, Illinois, USA). Homogeneity of variances and normality of the data were determined with a Levene's test and a Kolmogorov Smirnov test, respectively. When a normal distribution and homogeneity of variances were observed, significant differences between mean values were analyzed by a One-way ANOVA test, with a significance level of 0.05 (LSD). If no normal distribution was observed, the differences in the means were statistically analyzed by Kruskal-Wallis, followed by a Mann-Whitney post hoc test including a Bonferroni correction.

VII.3 Results

VII.3.1 Methane oxidation rates

The overall methane oxidation rates (MOR) of the CH_4 oxidizing communities with NO_3^- as N source (MOC), the *methalgae* communities with NO_3^- as N source (N-MAC), and the *methalgae* communities with NH_4^+ as N source (A-MAC) were measured over ten cycles. During the first two cycles, a significantly lower MOR was found for the A-MAC in comparison with the MOC and N-MAC (Figure VII-1). Thereafter, no consistent differences ($p>0.05$) in MOR were found. During each cycle, the daily observed MOR differed for the MOC and MAC, respectively. During the first 24h, the MOC had a significantly higher MOR than both N-MAC and A-MAC (Table VII-1). However, during the second day of a cycle, the MOR were very similar. During the last day of the cycle, the MOR decreased in the MOC to 54 ± 16 mg CH_4 L^{-1} d^{-1}, while an almost identical MOR was found for the N-MAC (169 ± 27 mg CH_4 L^{-1} d^{-1}) and A-MAC (161 ± 35 mg CH_4 L^{-1} d^{-1}) (Table VII-1).

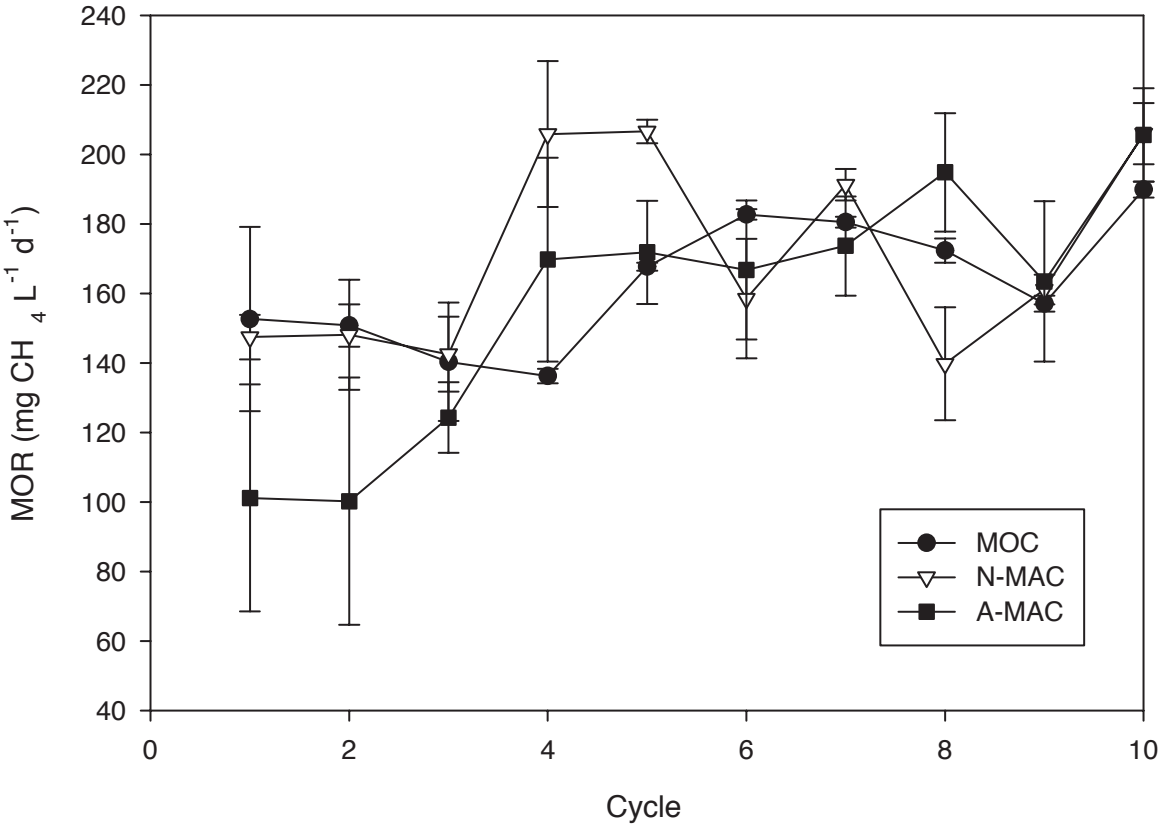


Figure VII-1. The overall methane oxidation rate (mg CH_4 L^{-1} d^{-1} ; average over 72h) for the methane oxidizing communities (MOC; circle), *methalgae* communities with NO_3^--N (N-MAC; triangle) and *methalgae* communities with NH_4^+-N (A-MAC; square), showing a stable trend in time for all communities.

Table VII-1. The average daily and overall methane oxidation rate (MOR; mg CH_4 L^{-1} d^{-1}) over the whole cycle (72h) for the methane oxidizing community (MOC), the *methalgae* community with nitrate as N source (N-MAC) and the *methalgae* community with ammonium as N source (A-MAC) from cycle 6 to 10.

	MOC	N-MAC	A-MAC
Day 1	266 ± 57	135 ± 39	173 ± 53
Day 2	210 ± 46	210 ± 44	208 ± 37
Day 3	54 ± 16	169 ± 27	161 ± 35
Overall	177 ± 12	171 ± 27	181 ± 23

VII.3.2 Carbon balances

On average, 386 ± 60 (N-MAC) and 407 ± 51 (A-MAC) mg CH_4-C L^{-1} $cycle^{-1}$ were metabolized. A comparable value of 394 ± 27 mg CH_4-C L^{-1} $cycle^{-1}$ was found for the MOC (Table VII-2). After 72h, an average CO_2 production of 138 ± 10 mg CO_2-C L^{-1} $cycle^{-1}$ was detected in the MOC-reactors, due to dissimilatory CH_4 oxidation and microbial respiration. This concentration corresponded with a CO_2-$C_{produced}$:CH_4-$C_{consumed}$ ratio of 0.35 ± 0.03. In the reactors with MAC however, the overall CO_2 production was in all MAC-reactors lower than 2 mg CO_2-C L^{-1} $cycle^{-1}$ from the second cycle on (Table VII-2).

Table VII-2. Overview of the CH_4 oxidation (mg CH_4-C L^{-1} $cycle^{-1}$), the CO_2 production (mg CO_2-C L^{-1} $cycle^{-1}$), the ratio of total organic carbon over CH_4-C (mg TOC mg^{-1} CH_4-$C_{consumed}$), the accumulation of volatile suspended solids (mg VSS L^{-1} $cycle^{-1}$), the nitrogen consumption (mg N L^{-1} $cycle^{-1}$), the ratio of total organic nitrogen over total organic carbon (mg TON mg^{-1} TOC), the average pH change and the molar $O_{2\ consumed}$:$CH_{4\ oxidized}$ ratio over a cycle for the reactors with a methane oxidizing community (MOC), a *methalgae* community with nitrate as N source (N-MAC) and a *methalgae* community with ammonium as N source (A-MAC) respectively.

	MOC	N-MAC	A-MAC
CH_4 oxidation	394 ± 27	386 ± 60	407 ± 51
CO_2 production	138 ± 10	< 2	< 2
TOC ratio:CH_4-C*	0.40 ± 0.03	0.85 ± 0.13	0.91 ± 0.13
VSS accumulation	411 ± 80	641 ± 83	688 ± 106
N consumption	38 ± 6	76 ± 15	61 ± 9
TON:TOC ratio*	0.18 ± 0.01	0.22 ± 0.03	0.19 ± 0.02
pH change	-0.2	+0.6	-0.3
O_2:CH_4 ratio	1.14 ± 0.09	0.50 ± 0.11	0.69 ± 0.12

* *average value for the 10^{th} cycle*

The concentration of formaldehyde, a metabolite of the CH_4 oxidation reactions, was always lower than the detection limit (2 mg L^{-1}), while the concentration of formic acid never exceeded a concentration of 2 mg HCOOH L^{-1}. When applying a ratio of 1.49 mg COD (chemical oxygen demand) mg^{-1} VSS as observed in comparable reactors (van der Ha et al., 2010), an average growth yield of 0.29±0.05 g CDW-COD g^{-1} CH_4-COD was observed in the MOC.

Analysis of the total organic carbon (TOC) and inorganic carbon (TIC) content of the liquid phase resulted in a $TOC_{accumulated}$:CH_4 -$C_{consumed}$ ratio of 0.85 ± 0.13 and 0.91 ± 0.13 for the N-MAC and A-MAC respectively, where a ratio of 0.40 ± 0.03 was observed for the MOC (Table VII-2). Based on the TOC measurements, the N-MAC assimilated a factor 2.3 more $CH_{4\ oxidized}$-C into biomass, compared to the MOC (Figure VII-2). The TIC content was with 10 ± 4 (N-MAC) and 5 ± 1 (A-MAC) mg C L^{-1} significantly lower than the TIC content of the MOC (35 ± 8 mg C L^{-1}) (Figure VII-2). Although it was impossible to quantify the separate VSS accumulation of the microalgae and bacteria respectively, analysis of the total VSS content confirmed the higher biomass accumulation when microalgae were present. The observed VSS accumulation of 641 ± 83 (N-MAC) and 688 ± 106 (A-MAC) mg VSS L^{-1} $cycle^{-1}$ was a factor 1.6 and 1.7 higher than in the MOC-reactors, respectively (Table VII-2).

VII.3.3 Apparent oxygen consumption

The apparent O_2 consumption was always significantly lower for the MAC than for the MOC. The average $O_{2\ consumed}$:$CH_{4\ consumed}$ ratio (mol/mol) was 0.50 ± 0.11 (N-MAC) and 0.69 ± 0.12 (A-MAC) respectively, i.e. 55 an 43% lower than in the MOC-reactors (1.14 ± 0.08).

The $O_{2\ consumed}$:$CH_{4\ oxidized}$ ratio was significantly higher for the A-MAC compared to the N-MAC. This difference was also observed when the N source of the N-MAC was changed to ammonium, as the $O_{2\ consumed}$:$CH_{4\ consumed}$ ratio rose from 0.48 ± 0.04 to 0.72 ± 0.18. There was also a change in the ratio when the N source of an A-MAC was changed to nitrate. The $O_{2\ consumed}$:$CH_{4\ oxidized}$ ratio dropped to 0.59 ± 0.08 with nitrate as N source, while with

ammonium as N source it was still 0.75 ± 0.05 mol. In contrast, no change in the ratio was found when the N source of the MOC was altered to NH_4^+ (data not shown). Also, no ammonium oxidizing activity was observed when A-MAC were grown in the absence of CH_4 (data not shown).

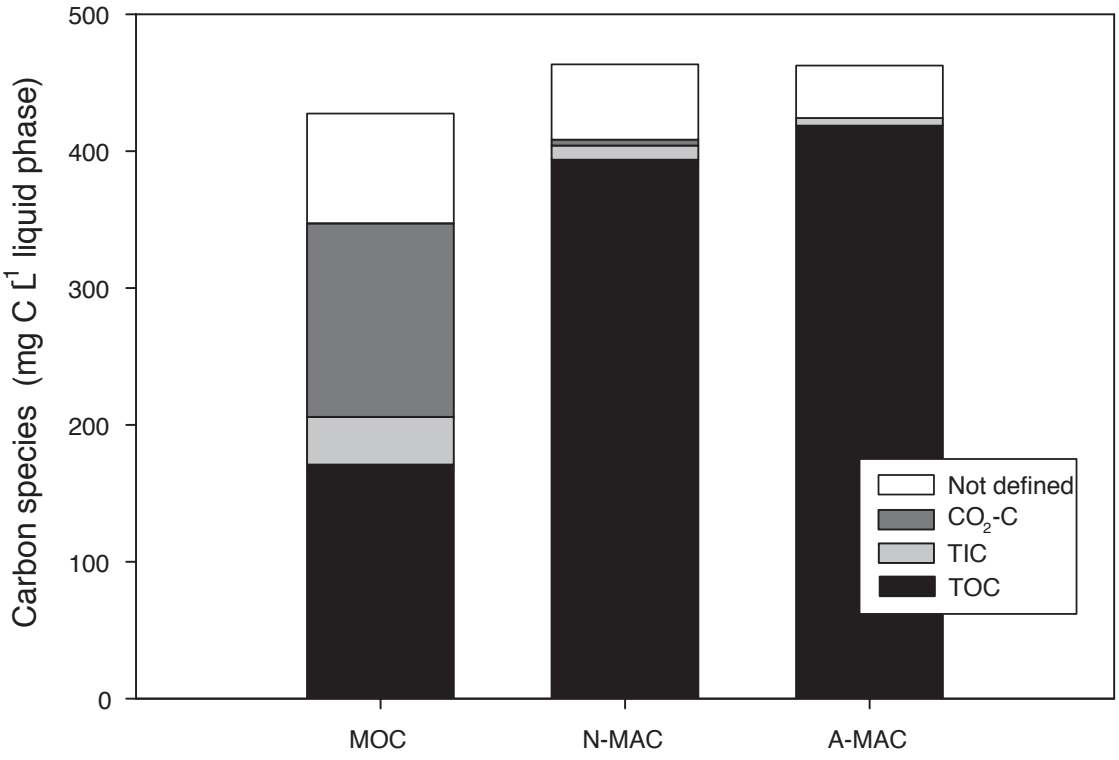


Figure VII-2. Overview of the average carbon balances for the methane oxidizing community (MOC), *methalgae* community with nitrate as N source (N-MAC) and the *methalgae* community with ammonium as N source (A-MAC) during the 10th cycle. The height of the columns shows the total amount of oxidized CH_4-C (mg CH_4-C L^{-1} liquid phase) during one cycle The total organic carbon (TOC), total inorganic carbon (TIC) and CO_2 production in the gas phase (CO_2-C) are expressed as mg C L^{-1} liquid phase. The difference between the measured CH_4-C removal and the total production of TIC, TOC and CO_2-C is shown as 'not defined'.

VII.3.4 Macro- and microscopic community organization

In the algal inoculum, the microalgae *Chlorella* sp., *Scenedesmus* sp., *Closterium* sp., *Euglena* sp. and *Cosmarium* sp. and the cyanobacterium *Phormidium* sp. were classified, based on their cell morphology. Over time, there was a clear selection for *Scenedesmus* sp., which became dominant in all reactors.

The addition of microalgae had an influence on the macroscopic, as well as the microscopic organization of the community. While most bacteria of the MOC were in suspension or formed smaller flocs, larger flocs (up to 2 mm) were formed by the MAC (Figure VII-3A). On a microscopic scale, randomly organized microalgae and bacteria were observed in the *methalgae* flocs (Figure VII-3B). Until the sixth cycle, the relative chlorophyll content increased for the MAC, which indicated an accumulation of algal biomass (Figure VII-4). Until the 8th cycle, the total amount of chlorophyll was significantly higher in the N-MAC-reactors compared to the A-MAC-reactors (Figure VII-4).

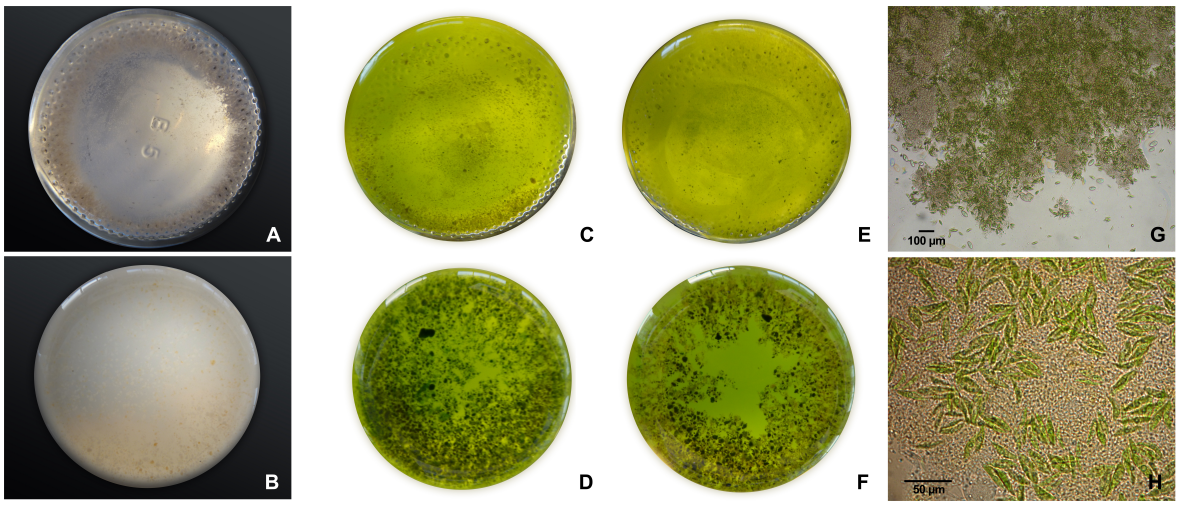


Figure VII-3. Images of the reactors with a methane oxidizing community (A-B), a *methalgae* community with NO_3^- as N source (C-D) and a *methalgae* community with NH_4^+ as N source (E-F), after the first (upper row) and fifth (lower row) cycle. Microscopic images of the bioflocs from a *methalgae* community with NO_3^- as N source (cycle 5) are shown with a 100x enlargement (G) and 1000x enlargement (H), respectively.

Based on the relative chlorophyll peak height before and after sonication, 51 ± 3 (N-MAC) and 47 ± 2% (A-MAC) of the algal biomass was present in the flocs. To test the settleability of the cultures, the VSS content of both the decanted liquid phase (90% of total volume) and the sludge phase were measured after 5 minutes of sedimentation. Respectively 65 ± 6% (N-MAC) and 61 ± 5% (A-MAC) of the VSS were present in the sludge phase (10% of total volume), where this was only 23 ± 4% for the MOC.

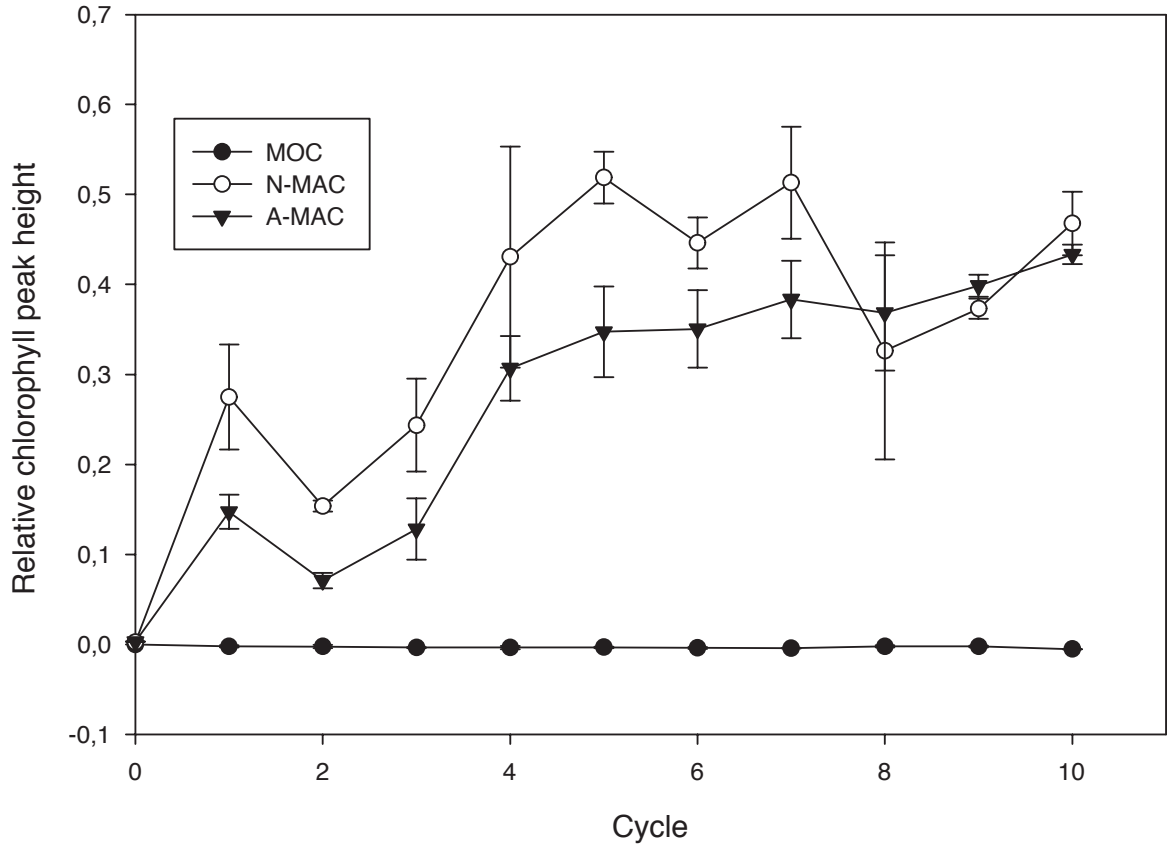


Figure VII-4. The relative chlorophyll peak height for the three types of communities. No chlorophyll was found back in the methane oxidizing community (MOC). For both the *methalgae* community with nitrate as N source (N-MAC) and ammonium as N source (A-MAC), an upward trend in algal growth was observed.

VII.3.5 The influence of heat treatment, ethyn addition and incubation in the dark

The microbial nature of the CH_4 oxidation activity was confirmed by the fact that the CH_4 oxidizing rate declined to 0.2 ± 2.1 mg CH_4 L^{-1} d^{-1} after heat treatment (20 min, 121°C, 1 bar). Addition of 0.2% (v/v) C_2H_2, a known inhibitor of MOB, also led to a complete inhibition of the CH_4 oxidation activity. To verify the CH_4 oxidation capacity of the microalgae, the A-MAC reactors were incubated with a gas phase consisting out of 13% (v/v) CH_4, 8% (v/v) CO_2 and 0.2% (v/v) C_2H_2 in air. After 72h, all CO_2 was incorporated into biomass by the microalgae, while no significant CH_4 oxidizing rate could be observed.

The presence of the soluble methane monooxygenase was detected in the three communities. A naphthalene oxidation rate of 155 ± 33 nmol mg^{-1} protein h^{-1} was found for the MOC (Koh et al., 1993). Although naphthalene oxidation was observed visually for the MAC, no quantification of the soluble methane monooxygenase was possible, since the microalgae interfered with the color measurement. The A-MAC was also incubated under dark conditions. The overall CH_4 oxidizing rate of 131 ± 1 mg CH_4 L^{-1} d^{-1} under dark conditions was 29% lower than under lighted conditions (182 ± 10 mg CH_4 L^{-1} d^{-1}). This difference was the largest (44%) during the first 24h of the cycle.

VII.3.6 The influence of the N source

The N source influenced the CH_4 oxidizing rate during the first two cycles (Figure VII-1). During those cycles, the overall CH_4 oxidizing rate of the A-MAC was 101 ± 34 mg CH_4 L^{-1} d^{-1}, significantly lower than the CH_4 oxidizing rate of 148 ± 11 mg CH_4 L^{-1} d^{-1} observed for the N-MAC. From the third cycle on, no consistent differences in the overall CH_4 oxidizing rate or in the VSS accumulation could be observed between the MOC, N-MAC and A-MAC (Table VII-2). Although the absolute nitrogen consumption of the N-MAC and A-MAC was a factor 2 and 1.6 higher than for the MOC (Table VII-3), respectively, the $TON_{accumulated}$:$TOC_{accumulated}$ ratios were comparable (Table VII-2).

Table VII-3. Overview of the consumed and produced nitrogen species for the methane oxidizing community (MOC), the *methalgae* community with nitrate as N source (N-MAC) and the *methalgae* community with ammonium as N source (A-MAC), during the 10th cycle. The N-consumption is expressed as mg NO_3-N L^{-1} liquid phase cycle^{-1} for the MOC and N-MAC, and as NH_4^+-N for the A-MAC. The TON-, NO_3^--, NH_4^+- and NO_2^--concentrations are expressed as mg N L^{-1} liquid phase cycle^{-1}. The N_2O is expressed as mg N_2O-N in the gas phase L^{-1} liquid phase. The difference between the observed nitrogen consumption and production, respectively, is shown as 'not defined'.

	MOC	N-MAC	A-MAC
N-consumption			
NO_3^--N	41.7 ± 3.7	73.6 ± 1.9	n.a.
NH_4^+-N	n.a.	n.a.	63.0 ± 2.3
N-production			
NO_3^--N	n.a.	n.a.	0.1 ± 0.2
NH_4^+-N	1.2 ± 0.1	1.8 ± 0.9	n.a.
NO_2^--N	0.5 ± 0.1	1.0 ± 0.2	0.4 ± 0.4
N_2O-N	5.2 ± 0.8	u.d.l.	u.d.l.
TON	25.4 ± 1.9	67.6 ± 1.7	64.6 ± 8.3
Not defined	9.4 ± 0.8	3.2 ± 0.4	2.1 ± 0.1

u.d.l.: under detection limit

n.a.: not applicable

An evaluation of the nitrogen balances was performed for the three communities (Table VII-3). The nitrite production was in all cases relatively low, with maximum values of respectively 0.5 ± 0.1 (MOC), 1.0 ± 0.2 (N-MAC) and 0.4 ± 0.4 (A-MAC) mg NO_2-N L^{-1} cycle^{-1}. Note that in the MOC-reactors, N_2O production was observed when O_2 concentrations decreased below 10% (v/v) (Table VII-3). The N source also influenced the pH evolution. The average pH increased with 0.6 units per cycle in the N-MAC reactors. The A-MAC on

their turn lowered the pH with 0.3 units per cycle. The average pH of the MOC-reactors, with nitrate as N source, decreased on average with 0.2 during each cycle (Table VII-2).

VII.3.7 *Methalgae* activity in anoxic non-synthetic effluents

The activity of a *methalgae* culture was evaluated in an anoxic effluent originating from an anaerobic digester, fed with potato waste. Essential physico-chemical parameters were analyzed and are represented in Table VII-4.

Table VII-4. Overview of physico-chemical parameters of the anoxic effluent. Where possible, average values (*n*=3) are given together with the standard deviation (SD).

Parameter	Average±SD	Unit
Temperature	34	°C
DO	0.25	mg O_2 L^{-1}
pH	7.45±0.33	-
NH_4^+-N (TAN)	291±6	mg NH_4^+-N L^{-1}
NO_2^--N	<0.5	mg NO_2^--N L^{-1}
NO_3^--N	0.84±0.84	mg NO_3^--N L^{-1}
PO_4^{3-}-P	147±33	mg PO_4^{3-}-P L^{-1}
Cl^-	908±459	mg L^{-1}
SO_4^{2-}	3.65±0.23	mg L^{-1}
H_2S	7.26±3.68	mg L^{-1}
CZV_{totaal}	462±152	mg O_2 L^{-1}
$CZV_{opgelost}$	315±13	mg O_2 L^{-1}
VSS	484±170	mg L^{-1}
TC	838±75	mg C L^{-1}
TIC	525±199	mg C L^{-1}
TOC	269±187	mg C L^{-1}
BZV	380	mg O_2 L^{-1}

Thereafter, six bottles were inoculated with a pre-incubated methanotrophic culture and algae cultures of *Scenedesmus* sp. or *Chlorella emersonii*, respectively, while the evolution of the O_2 concentration and the methane oxidizing activity were followed up (Figures VII-5 and VII 6, respectively)

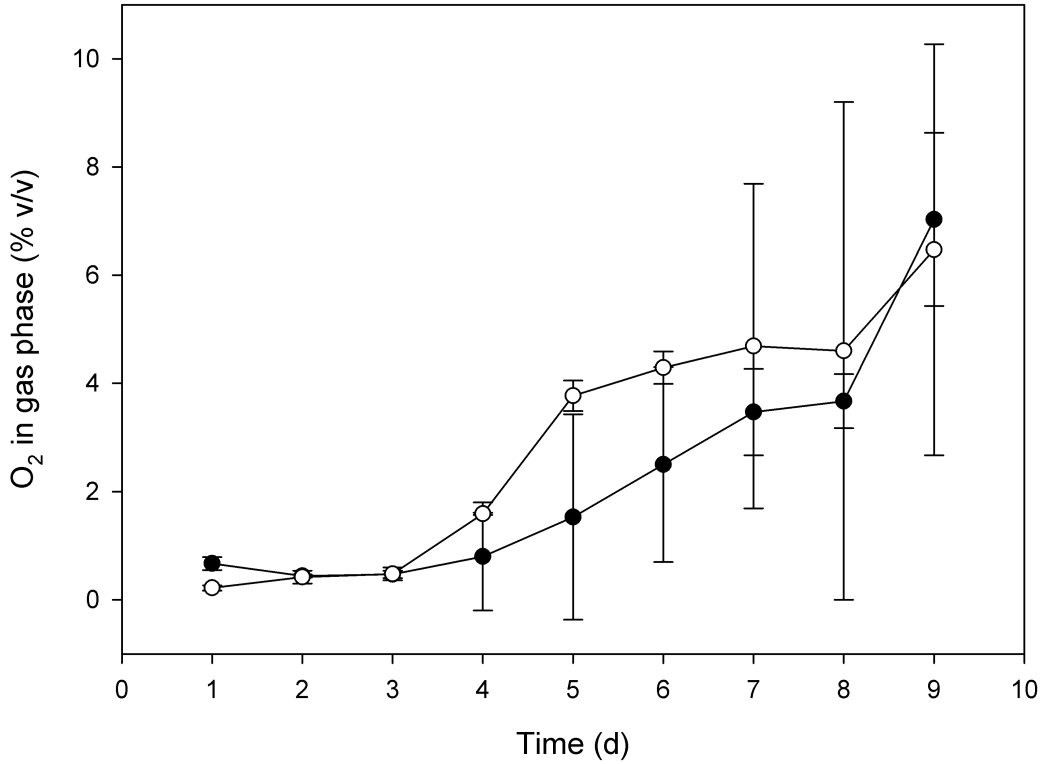


Figure VII-5. Overview of the O_2 concentration in the gas phase of bottles wherein *methalgae* cultures were cultivated in anoxic effluent. Tests were performed in triplicate with two different algae species, i.e. *Scenedesmus* sp. (filled circles) and *Chlorella emersonii* (open circles). The error bars indicate the standard deviation. Significant apparent O_2 production was observed from day 4 on (p<0.05).

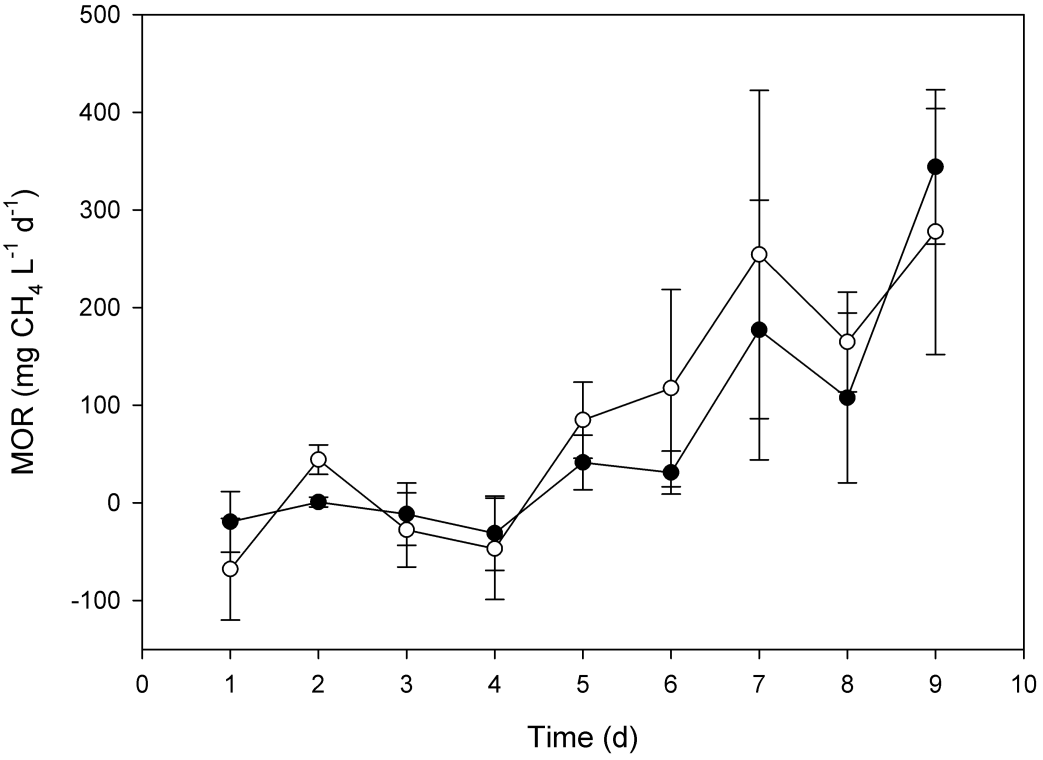


Figure VII-6. Representation of the measured methane oxidation rates (mg CH_4 L^{-1} d^{-1}) of *methalgae* cultures cultivated with anoxic effluent. Tests were performed in triplicate with two different algae species, i.e. *Scenedesmus* sp. (filled circles) and *Chlorella emersonii* (open circles). The error bars indicate the standard deviation. Significant apparent O_2 production was observed from day 5 on (p<0.05).

During the first three days, no apparent O_2 production was observed. This was in agreement with the fact that no algal growth was visually observed. From the fourth day on, a significant O_2 production occurred and thereafter, the O_2 concentrations kept on increasing. No significant differences were observed between the activity of both tested algae species (p<0.05). Yet, substantial differences were observed within the triplicates, as in both cases, one of the bottles hardly showed algal activity. The MOR profiles followed the same pattern. During the first four days, CH_4 was rather produced than consumed. However, significant CH_4 oxidation was observed from the fifth day on, when the effluent became oxygenated. Thereafter, the MOR showed an increasing trend, with a maximum of 344±166 mg CH_4 L^{-1} d^{-1} for the *Scenedesmus* sp. culture after nine days of incubation.

VII.4 Discussion

VII.4.1 Methane oxidation rates in the presence of microalgae

To accomplish a technically feasible and environmentally sustainable CH_4 oxidation, a high methane oxidation rate (MOR) is needed. The presence of microalgae did not lead to a significant decrease in the MOR, which shows that no significant inhibitory effect from the microalgae on the MOB was present. Only during the first 2 cycles, a less performing A-MAC was observed, which may have been due to the inoculant being precultured with NO_3^- as N source. The overall CH_4 oxidizing rates over one cycle of 177 ± 12 (MOC) and 171 ± 27 (N-MAC) mg CH_4 L^{-1} d^{-1} were a factor of 1.5 higher than previously achieved with the same type of reactor (van der Ha et al., 2010). The higher sludge retention time (90h versus 140h) and the higher ratio of gas phase over liquid phase (5 versus 2) probably led to a longer period without CH_4 and O_2 limitations. These CH_4 oxidizing rates are in the range of continuous CH_4 oxidizing reactors reported in the literature, where CH_4 oxidizing rates were achieved in a range of 24 to 696 mg CH_4 L^{-1} d^{-1} (Melse and Van der Werf, 2005, Nikiema et al., 2005).

The significantly lower CH_4 oxidizing rate of the MAC compared to the MOC during the start-up phase of a cycle could be due to the observed floc formation. In the MAC, more bacteria and microalgae were observed living closely together in *methalgae* flocs (Figure VII-3). Therefore, competition for available O_2, CH_4 and nutrients must take place inside the flocs (Hamdi, 1995, Modin et al., 2008). Additionally, a longer lag time may have been due to CH_4 and O_2 diffusion through the *methalgae* flocs (Hamdi, 1995, Xavier et al., 2005). During a cycle, newly formed flocs were observed with a smaller diameter, thus encountering less diffusion limitations, which may explain the higher CH_4 oxidizing rate during the second day. It was also observed that the CH_4 oxidizing rate of the MOC decreased significantly during the third day of each cycle, concomitant with the limiting concentration of available O_2. In contrast, the CH_4 oxidizing rates of the N-MAC and A-MAC was 4 times higher, as the microalgae produced enough O_2 to maintain the CH_4 oxidation. With the experimental set-up used in this work, the gas composition varied

during each cycle, which explains the variation in the MOR. However, the MOR during the second day was the most representative value, as both the effect of the start-up adaptation and the limiting CH_4/O_2-concentrations were negligible.

VII.4.2 CO_2 fixation supported an increased biomass production

The dissimilatory CH_4 oxidation processes, in combination with the heterotrophic respiration, led to a CO_2-$C_{produced}$:CH_4-$C_{consumed}$ ratio of 0.35 for the MOC. This has also been mentioned in previous studies, where a ratio between 0.17 and 0.40 was observed for methanotrophic communities (Börjesson et al., 1998, Whittenbury et al., 1970b). In the MAC reactors, almost no CO_2 was measured in the gas phase at the end of each cycle. This shows that there was enough light energy present for the CO_2 fixation of the algae, so light intensity was not a limiting factor for the photosynthetic processes. No (bi)carbonate was added to the medium, which means that algal growth was completely dependent on the microbially produced CO_2. This indicated that the CH_4-C was dissimilated to CO_2-C by the MOB, and was to a large extent transformed to TOC, as the microalgae were able to readily use the microbial produced CO_2 for their metabolism. This algal CO_2 fixation led to a 130 ± 20% higher TOC accumulation, compared to the MOC.

Based on the average MOR of 210±44 mg CH_4 L^{-1} d^{-1} in the N-MAC reactors (Table VII-1) and the ratio of 0.85±0.13 mg total organic $carbon_{produced}$ for every mg of CH_4-$C_{consumed}$ (Table VII-2), an average carbon biofixation of about 135 mg CH_4-C L^{-1} d^{-1} can be calculated. This value is similar to the carbon removal rate of 127±26 mg TOC-C L^{-1} d^{-1} that was observed with a similar reactor type for an algal-bacterial coculture treating four times diluted swine slurry under anaerobic conditions (Gonzalez et al., 2008).

VII.4.3 Algae lowered the need for an external oxygen supply

In the MOC, a molar $O_{2\ consumed}$:$CH_{4\ consumed}$ ratio of 1.14 ± 0.09 was observed, which is comparable to the ratio of 1.2 to 1.4, observed with a comparable previous study (Modin et al., 2010). The algal O_2 production, due to photosynthetic processes, lowered the

overall O_2 need in the N-MAC with 55%. The presence of O_2 producing algae allowed the MOB in the MAC-reactors to sustain CH_4 oxidation during the third day of each cycle, when the bacteria were deprived from O_2 originally present in the gas phase. When additional (bi)carbonate or CO_2 would be supplied, microalgae could provide enough O_2 for the bacterial community to make external O_2 supply obsolete. This could lower the cost for a CH_4 oxidizing biofilter drastically if an efficient transfer of light energy could be achieved. This CO_2 supplementation is easily achieved, as CO_2 is, due to the biogas production, already present in the effluent of the anaerobic digestion process. The O_2 consumption was dependent of the applied N source: with NH_4^+ as N source, the O_2 consumption of the *methalgae* was a factor 1.4 higher than with NO_3^- as N source. The fact that this difference was not observed with the MOC, indicates that the difference in apparent O_2 consumption is caused by the algal metabolism. The underlying reason is unknown, although it seems to be related to nitrogen assimilation processes.

VII.4.4 The coculture of bacteria and microalgae has the tendency to form flocs

The coculture of bacteria and microalgae tends to form flocs, where about half of the algal and bacterial biomass accumulates. This floc formation may be initiated through the attractive forces of surface hydrophobicity (van Loosdrecht et al., 1987) or production of algal or/and bacterial EPS (Fuentes et al., 1999, Grossart et al., 2006, Wilshusen et al., 2004a). Moreover, it could be a mutually beneficial syntrophic relationship, as they are interdependent for their nutrient sources. This floc formation did not seem to lead to a differentiation in the floc configuration. However, the tendency of the *methalgae* to form flocs, made it easier to separate the biomass from the liquid phase.

VII.4.5 The bacterial methane oxidation activity

The CH_4 oxidation activity was clearly of microbial origin as a heat treatment stopped the CH_4 oxidation activity almost completely. The CH_4 oxidation activity of the MOB was also completely stopped by the addition of C_2H_2, a known inhibitor of MOB (Chan and Parkin,

2000, Prior and Dalton, 1985). This indicates that MOB were responsible for the CH_4 oxidation activity. Their presence was confirmed by the detection of the soluble methane monooxygenase, an enzyme that is only present in MOB (Dalton, 2005).

Moreover, microalgae did not show any CH_4 oxidizing capacity. After addition of C_2H_2 and CO_2 in the gas phase of the *methalgae* culture, no CH_4 oxidation activity was observed, although all the readily available CO_2 was removed, due to algal carbon fixation. This shows that C_2H_2 is a good selective inhibitor for MOB versus microalgae. The lack of CH_4 oxidizing activity of the microalgae was also observed under dark incubation conditions: a distinctive CH_4 oxidation activity was observed, although no light energy was present, necessary for the algal photosynthetic processes. However, the measured MOR was significantly lower than the MOR of the control reactors, incubated in the light. This implies that the O_2 production of the microalgae inside the *methalgae* flocs positively influenced the CH_4 oxidation activity.

VII.4.6 The influence of the nitrogen source

The nitrogen need of both microalgae and MOB is higher than 0.15 mg N mg^{-1} incorporated C (Klausmeier et al., 2004, Scheutz et al., 2009a). Microalgae, as well as MOB, can use NO_3^- and NH_4^+ as N source (Bodelier and Laanbroek, 2004, Li et al., 2010b, Lourenco et al., 1998). However, it has been observed that ammonium can have an inhibitory effect on MOB (Begonja and Hrsak, 2001, Nyerges and Stein, 2009). Microalgae on the other hand are sensitive for both ammonium as ammonia: 28 mg NH_3-N L^{-1} inhibited the growth of *Scenedesmus obliquus*, at a pH of 8 (Azov and Goldman, 1982, Kallqvist and Svenson, 2003). In the current study, no inhibition was observed related to the N source, as no significant differences in MOR or VSS accumulation were found between N-MAC and A-MAC. Due to a higher VSS accumulation, the nitrogen consumption of the N-MAC was a factor 2 higher than the N-consumption of the MOC. The $TON_{accumulated}$:$TOC_{accumulated}$ ratio was however not significantly different for the three communities. The relatively low nitrite concentrations did not induce inhibitory effects (King and Schnell, 1994b, Nyerges and Stein, 2009). The N_2O production of the MOC was

probably caused by aerobic CH_4 oxidation coupled to denitrification (AME-D), a known process when O_2 levels are low (Modin et al., 2010). It was however unexpected that this process could occur when 4 mg O_2 L^{-1} was still present in the liquid phase.

The N source also influenced the pH changes in the MAC-reactors. The pH drop in the MOC reactors was caused by the acidifying effect of the produced CO_2 and (bi)carbonates that dissolved in the water phase. In the MAC-reactors, the acidifying effect of the bacterially produced CO_2 was neutralized by the algal fixation. This however cannot explain the pH rise, as no other C source was present in considerable amounts. The rise of the pH in the N-MAC probably was due to the alkalizing effect of assimilatory nitrate reductase (Eisele and Ullrich, 1975, Fuggi et al., 1981). In the A-MAC however, the pH decreased as the assimilation of ammonium into microbial biomass produces protons (Fuggi et al., 1981, Li et al., 2008).

VII.4.7 Methalgae activity in anoxic industrial effluents

There is a considerable difference between microbial activity in optimal growth medium under aerobic conditions and activity in anoxic effluents of anaerobic digesters. Still, conditions in the digestate allowed microbial activity as at least a close to neutral pH occurred, while substantial amounts of NH_4^+-N, PO_4^{3-}-P and SO_4^{2-}-S were available. Moreover, a large amount of TIC was present, allowing algal photosynthesis. During the first four days of incubation, no apparent O_2 production was observed as the algae probably had to adapt to the new environment. Moreover, other processes like H_2S oxidation and heterotrophic activity form alternative O_2 sinks. Still, from the fourth day on, O_2 concentrations increased considerably, resulting in methanotrophic activity. The methane oxidation rates were in the same order as with the A-MAC and N-MAC communities from the former tests. In conclusion, *methalgae* communities can easily adapt to at least some industrial anoxic wastestreams without loss of activity. Moreover, no external O_2 addition was necessary, as the microalgae supplied the latter *in situ*.

VII.4.8 Conclusions

Coculturing CH_4 oxidizing communities and microalgae allowed a greenhouse gas neutral oxidation of CH_4 to microbial biomass. Microalgae converted almost all CO_2, produced by methane oxidizing bacteria, to biomass. In this configuration, the presence of photosynthetic active microalgae lowered the need of externally supplied O_2 for the CH_4 oxidizing community with 55%. Moreover, *methalgae* cultures adapted easily to an industrial digestate. Even under anoxic conditions, considerable methanotrophic activity was observed as the MOB could rely on *in situ* O_2 production by algal photosynthesis. This *methalgae* concept therefore creates perspectives for a sustainable treatment of CH_4 saturated effluents. The low light intensity needed, the observed floc formation and the interchangeability of the N source support this conceptual approach. However, implementation of a continuous system under anoxic conditions with a high methane oxidation rate per unit reactor volume, an efficient transfer of light energy and a low spatial footprint are prerequisites for application in practice.

Acknowledgements

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CHAPTER VIII

FACULTATIVE PONDS HARBOR METHANE OXIDIZING COMMUNITIES SUPPORTED BY ALGAE

Facultative pond under the South African sky, EBRU, Grahamstown

I am not apt to follow blindly the lead of other men. I have steadily endeavoured to keep my mind free.

Charles Darwin

Chapter VIII. Facultative ponds harbor methane oxidizing communities supported by algae

Abstract

Waste stabilization ponds are key units of many low-cost wastewater treatment plants. Typically, anaerobic processes taking place in the sludge blanket result in emissions of the greenhouse gas CH_4. A South African pilot facultative waste stabilization pond was used to evaluate the fate of CH_4 in the oxygenated pond surface layer. On site emission analyses showed that such facultative ponds are substantial CH_4 sources. Yet, part of the produced CH_4 is consumed by aerobic methane oxidizing bacteria (MOB) before it can reach the atmosphere. Both type Ia, Ib and II MOB were isolated out of enriched samples originating from the surface layer of 25 cm, wherein at least 10^4 cultivable MOB mL^{-1} were observed. These enrichments also showed a high methane oxidizing activity of 190±5 mg CH_4 L^{-1}_{liquid} d^{-1}. The algal community had an important indirect effect on the methanotrophic activity as the algae provided the MOB with O_2. In laboratory simulations with a stagnant water column, methane oxidation rates were significantly higher when algae were able to produce O_2, which was subsequently consumed by MOB. As such, algae have an essential supportive role in the CH_4 oxidation of facultative ponds and could therefore influence their carbon footprint to a substantial extent.

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VIII.1 Introduction

All over the world, treatment of wastewater is a necessity to obtain a sustainable way of living for an ever-increasing population (Verstraete et al., 2009). While industrialized countries have the possibility to invest in state-of-the art technologies like activated sludge systems or anaerobic digesters, sewage treatment with a low capital and operational cost is often the only possibility in many developing countries (Aiyuk et al., 2004, De Almeida et al., 2009, Mara, 2004). Therefore, waste stabilization ponds (WSP) have been successfully implemented for wastewater treatment, largely due to the pioneering work of Oswald and Marais (Caldwell, 1946, Marais, 1966, Oswald, 1976). WSP are characterized by long retention times to allow for the gradual breakdown of organic waste by microorganisms (Mara, 2005). In addition to anaerobic and aerobic ponds, facultative versions exist and these are the most common WSP types in the USA (Reed, 1995). They differ from anaerobic ponds as there is an aerobic surface layer present. Aside from wind effects, this layer is mainly oxygenated by algae and cyanobacteria, which produce oxygen as a product of photosynthesis (Mara, 2005). Often, these algae enter the system naturally and a clear selection frequently occurs for motile algae that can optimize their vertical position, therefore having an advantage over non-motile algae (Mara, 2005, Pearson et al., 1987). At the bottom of the pond however, an almost permanent anaerobic sludge blanket results in the release of large amounts of biogas comprising H_2S, NH_3, CO_2 and CH_4 (Craggs et al., 2008). Due to the nature and construction of these ponds, large emissions of CO_2 and CH_4 from the surface area can be expected and CH_4 production rates of 0.17 kg CH_4 kg^{-1} BOD_{waste} were found in models of anaerobic ponds fed with municipal waste (DeGarie et al., 2000, van der Steen et al., 2003). Picot et al. (2003) estimated that about 3.3 m^3 $capita^{-1}$ y^{-1} of CH_4 is produced in real scale ponds, when a daily sewage production of 100 g chemical oxygen demand (COD) $capita^{-1}$ d^{-1} is assumed. Taking into account that CH_4 is a 25 times stronger greenhouse gas than CO_2 (Forster et al., 2007), a production of 0.25 ton CO_2-equivalents of CH_4 y^{-1} per household of four persons can be estimated.

Due to these high emissions and the presence of O_2 in the surface layer of facultative ponds, aerobic methane oxidizing bacteria (MOB) might be expected to be part of the resident microbial community. Although van der Steen et al. (2003) did not find evidence of significant microbial CH_4 oxidation activity in simulated WSP bottles, MOB were identified in the root zone of *Lemna minor*, a common aquatic plant regularly floating on the surface of such ponds (Hanson et al., 1993). It has been observed at laboratory scale that algae can provide the MOB with molecular O_2, but this interaction is still poorly described under natural conditions (Bahr et al., 2011, van der Ha et al., 2011).

The present study focuses on a pilot scale Primary Facultative Pond (PFP), receiving domestic wastewater. In an effort to evaluate the influence of the algal photosynthetic activity on the methanotrophic activity, pond samples were enriched with CH_4 under illuminated and dark conditions. Furthermore, the amount of cultivable MOB in the pond was estimated by means of a most probable number approach. Finally, an isolation campaign on the enriched pond samples allowed to positively identify cultivable MOB.

VIII.2 Experimental section

VIII.2.1 Study site

The facultative pond used in this study is a component of an Integrated Algae Pond System located at the Institute for Environmental Biotechnology Rhodes University (EBRU), adjacent to the Grahamstown Wastewater Treatment Plant (33° 19’ 07” South, 26° 33’ 25” East), which operates uninterrupted and treats 80 m^3 d^{-1} of domestic effluent. The complete system comprises a 840 m^2 PFP, a single fermentation pit, two 500 m^2 high rate algal ponds and two algal settling ponds. Upflow velocity in the fermentation pit is maintained at 1-1.5 m d^{-1} while the Hydraulic Retention Times (HRT) in the fermentation pit and PFP are 3 and 20 d, respectively. All experiments and sampling took place in the period from February 2nd until April 25th, 2012.

VIII.2.2 Estimation of methane emissions

Emission of CH_4 from the facultative pond was estimated by means of a free-floating gas capture device positioned in an area equidistant from the centre and the border of the pond. The device was constructed of polystyrene (2 dm^2) with an attached gastight polyethylene terephthalate bottle (420 mL) and an open cross section of 20 cm^2. Biogas produced from the PFP expelled the water from the bottle allowing quantification of produced CH_4 over time. Extrapolated quantification of CH_4 production was based on measurements during six different periods of 24 to 120h.

VIII.2.3 Influence of algae on the physico-chemical conditions in the pond

In order to estimate the physic-chemical conditions in the pond, depth profiles of temperature, dissolved oxygen (DO) and pH were constructed at different time intervals at a predefined spot approximately 6 m from the centre of the pond, thereby not disturbing the water column. The mentioned parameters were measured every 1 cm from the surface to a depth of 50-60 cm. DO and temperature were measured using a handheld dissolved oxygen meter (Model 85, YSI Inc., OH, USA), while pH was measured with a WTW330 (WTW, Weilheim, Germany) instrument.

On March 13th 2012, three samples (1 L) were harvested aseptically from the PFP using a pump. Two samples ($sample_{5cm,\ no\ visible\ algae}$ and $sample_{25cm,\ no\ algae}$), from a depth of 5 cm and 25 cm respectively, were taken 6 m from the centre of the pond where no algae were visibly present. A third sample ($sample_{5\ cm,\ visable\ algae)}$ was taken at 5 cm depth, 50 cm away from the edge of the pond, where a large amount of motile algae were visibly present. At the time of sampling, the light intensity at the water surface was 1604 µmol m^{-2} s^{-1} (PAR photosynthetic active range)**,** while ambient air temperature was 28.4°C. The pond was at that time largely anaerobic, with the exception of some zones where motile algae appeared to gather. The water temperature, pH and DO were measured *in situ.* Chlorophyll content, VSS and COD analyses were performed within 1h of sampling. Subsamples were immediately filtered using a 0.22 µm filter (Millipore, Belgium) and

stored at 4°C until analyses of anion concentration, soluble COD (sCOD) and NH_4^+-N, respectively.

VIII.2.4 Amount and activity of MOB

Based on physico-chemical analyses of the pond water, diluted Ammonium Mineral Salts medium (dAMS) was chosen as medium for enrichment of the pond samples, as it had a similar NH_4^+-content and pH value as the pond water, without the presence of organic C sources, which would make the enrichment and isolation of MOB more difficult. Sterile dAMS medium (pH 6.9) was prepared by adding 0.1 g NH_4Cl; 0.2 g $MgSO_4.7H_2O$; 7.17 g $Na_2HPO_4.2H_2O$; 2.72 g KH_2PO_4; 30 mg $CaCl_2.6H_2O$; 4.5 mg Na_2-EDTA; 3.5 mg $FeCl_3.6H_2O$; 2.5 mg $CuSO_4.5H_2O$ (10 µM) and other trace elements to one liter of demineralized water up to a volume of 1 L, in accordance to Bowman (2006).

Quantification of living MOB in the three mentioned pond samples was done by dilution to extinction (*n*=3), up to a 10^7 fold dilution. Therefore, 900 µL of sterile dAMS medium and 100 µL sample from the lower dilution were pooled into 12 mL vacutainers (BD, South Africa) under an atmosphere of 20% (v/v) CH_4 in air. After 12 days, presence of MOB was evaluated based on measurements of the absorbance (A_{600nm}), supported by gas phase analyses. Therefore, control vials were used that were treated similarly, but without addition of CH_4 to the gas phase. Dilutions were considered positive for methanotrophic activity when the absorbance increased with a factor 10 and the CH_4 concentration dropped below the average initial value, substracted by five times the standard deviation. This information was used to estimate the abundance of cultivable MOB under the given growth conditions using most probable number tables and is expressed together with its 95% confidence interval (Anonymous, 2008).

The methanotrophic activity was evaluated by measuring the CH_4 oxidation rate of enrichments from the three mentioned pond samples. Therefore, six Schott bottles with a total volume of 590 mL were sterilized and sealed with butyl stoppers. To three bottles, 100 mL sterile dAMS medium was added while the three remaining bottles were filled

with 100 mL filtered (0.22 µm) supernatant (pH 7.1), obtained after centrifugation of pond water (15 min, 10,000 × g, Beckman Coulter Avanti J-E Centrifuge). All bottles were inoculated with 5% (v/v) of one of three fresh pond samples, filled with 20% (v/v) CH_4 in air and placed on a shaker (80 rpm) at 28°C in the dark. The CH_4 oxidation rate and apparent O_2 consumption were measured daily until the O_2 concentration was below 3% (v/v). To indicate the microbial nature of the CH_4 oxidation, the same approach was used after autoclaving the cultures (20 min, 121°C, 1 bar). No significant CH_4 oxidation was observed in this bottle during a period of 96h.

VIII.2.5 Isolation and identification of cultivable MOB

After four days of incubation, the enriched samples with dAMS were subsampled (25 mL) and MOB were isolated according to Hoefman et al. (2012a). Briefly, samples were serially diluted in 12-fold to the point of extinction (10^{-3} up to 10^{-10}) in 96-well microtiter plates with dAMS. After incubation for two weeks at 28°C under an atmosphere of 50 % (v/v) in air in gastight jars (Oxoid), the highest dilution of each series that was visually positive for growth, was plated onto dAMS agar and sub-cultured to purity. Pure cultures were confirmed for methane oxidation by CH_4 and O_2 consumption and CO_2 production through GC analysis (Compact GC; Global Analyzer Solutions, Belgium) of batch cultures. Pure cultures positive for CH_4 oxidation were identified via 16S rRNA gene sequence analysis and dereplicated via $(GTG)_5$ rep-PCR fingerprinting. Purity of the MOB isolates was confirmed by (i) colony morphology, (ii) phase-contrast microscopy, (iii) absence of heterotrophic growth on 1/10 trypticase soy agar under air and (iv) absence of growth on dAMS under air.

VIII.2.6 Algae assisted methane oxidation

To evaluate the influence of algal photosynthetic activity on the CH_4 oxidation rate, pond samples were taken 5 cm below the surface (DO = 0.4 mg O_2 L^{-1}). Into three darkened bottles and three illuminated bottles with a volume of 590 mL, 80 mL of filtered pond water was added. These bottles were incubated with 10 mL of an active MOB enrichment

on filtered pond water and 10 mL of a native mixed algae culture, both originating from the pond. All bottles were sealed, filled with 20% (v/v) CH_4 in air and placed in an incubation room at 28±2°C without shaking. While the dark bottles were shielded from the light, the illuminated bottles were placed continuously under fluorescent lamps for 72h, (71 µmol m^{-2} s^{-1}, PAR) and the CH_4 and O_2 concentrations in the gas phase measured daily. Control bottles without algal inoculation indicated that the supplied light intensity had no inhibitory effect on the methanotrophic activity as such.

VIII.2.7 Physico-chemical analyses of the liquid and gas phase

The concentration of volatile suspended solids (VSS) was assessed according to Greenberg et al. (1992). For chlorophyll extraction, a volume of 20-200 mL was filtered using a Whatman GF/C filter and the filter cake extracted in darkness with 90% acetone for 24h according to Eaton et al. (1998). The absorbance was measured using an AquaMate Plus Spectrophotometer (Thermo Scientific, Waltham, MA). The concentrations of NH_4^+, NO_3^-, NO_2^- and PO_4^{3-} and the concentration of chemical oxygen demand (COD) were analyzed by means of the respectable Merck Spectroquant® test kit (Merck, Whitehouse Station, NJ) and analyzed with the same spectrophotometer. The soluble COD (sCOD) content was measured with a Nanocolor COD test kit 160 (Macherey-Nagel, Germany) with a measuring range between 0 and 160 mg COD L^{-1}. Absorbance was measured with a Nanocolor vario 4 (Macherey-Nagel, Germany). Identification of algae species was done by means of a Carl Zeiss Axiostar Plus light microscope (400× and 1000× magnification).

Analyses of the O_2 and CH_4 concentrations in the gas phase was by injection of 250 µL into an Agilent 6820 gas chromatograph (Agilent Technologies, Santa Clara CA) equipped with a flame ionization and thermal conductivity detector and fitted with either a Molsieve 5A packed column (6 ft × 2.1 mm i.d., Restek Corporation, Bellefonte, PA) or a GS-GasPRO capillary column (60 m × 0.32 mm i.d., Agilent Technologies, Santa Clara, CA). The thermal conductivity detector was set at 300°C with helium as the carrier gas at a flow rate of 0.9 mL min^{-1}. The gas pressure in the bottles was measured with an Infield 7 pressure meter (UMS, Germany) to compensate for over- or under pressure.

VIII.2.8 Statistical analysis

Results were statistically analyzed by performing a Welch modified two-sample t-test with equal variance and at a significance level of 95% (*n* replicates, $p<0.05$). Significant CH_4 removal was defined as a difference of 5% compared to the former sampling point. Analyses were performed with SPSS (Duncan, version 19.0).

VIII.2.9 Nucleotide sequence accession numbers

The 16S rRNA gene sequence data generated in this study has been deposited in GenBank/EMBL/DDBJ with accession numbers HF558987 to HF558991.

VIII.3 Results

VIII.3.1 Influence of algae on the physico-chemical conditions in the pond

Depth profiles over time showed that the physico-chemical conditions in the pond fluctuated constantly: temperature, DO and pH shifted drastically over short periods, depending on the preceding weather conditions (Figure VIII-1 and VIII-2). The maximal depth where O_2 saturation occurred depended strongly on the algal activity and was positioned between 55 and less than 1 cm below the pond surface. On sunny days, O_2 levels in the surface layer regularly reached oversaturated values above 25 mg O_2 L^{-1}, though the pond became completely anaerobic during shaded days. Microscopic determination showed that most algae present belonged to the motile *Pyrobotris* species while small amounts of *Chlorella* sp., *Scenedesmus* sp., *Pediastrum* sp. and cyanobacterial species were also observed.

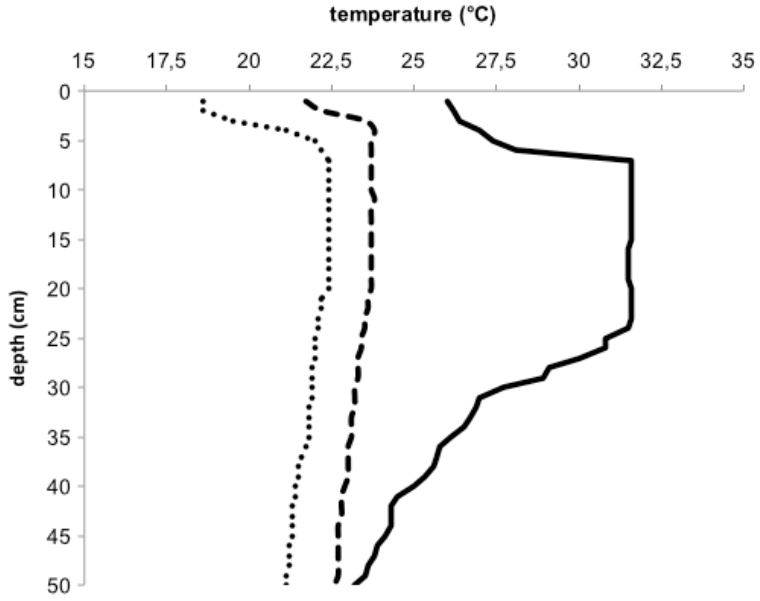

Figure VIII-1. Temperature profiles of the pond (6 m away from the centre of the pond) in steps of 10 mm, at three different times (February, 7 : solid line, February, 9th: dashed line, February,

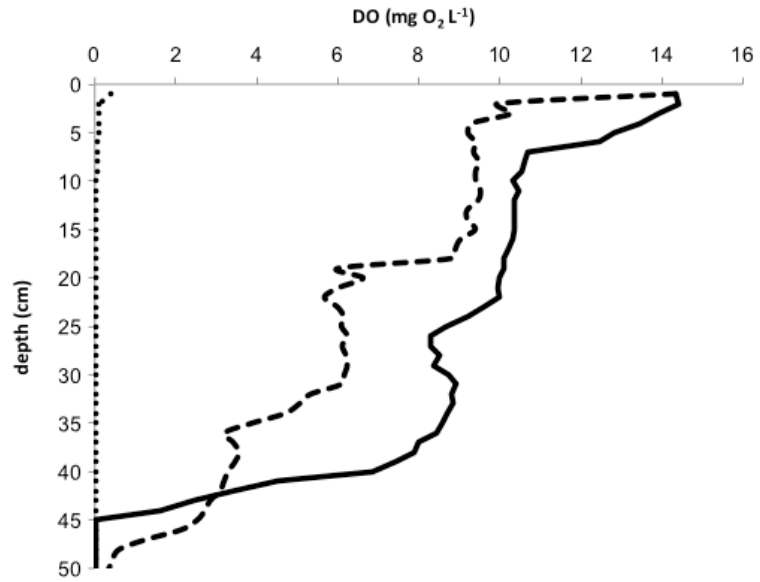

Figure VIII-2. Dissolved oxygen (DO, mg O) profiles of the pond (6 m away from the centre of the pond) in steps of 10 mm at three different times (February, 7th: solid line, February, 9th: dashed line, February, 10

To evaluate the influence of algal presence on the physico-chemical conditions in the pond, three samples were taken at a time when the pond was largely anaerobic: $sample_{5cm,visible\ algae}$ was taken at 5 cm depth and algae were visibly observed. $Sample_{5cm,\ no\ visible\ algae}$ was also taken at 5 cm depth but without visibly observable algae while $sample_{25cm,\ no\ algae}$ was taken at 25 cm depth and also without visibly observable algae. In the latter, no chlorophyll was detected and the DO concentration in that sample was below the detection limit of 0.05 mg O_2 L^{-1} (Table VIII-1). In the other two samples, taken at 5 cm depth, algae were present and concentrations of 0.1±0.1 ($sample_{5cm,\ no\ visible\ algae}$) and 4.4±0.7 ($sample_{5cm,visible\ algae}$) mg chlorophyll L^{-1} were measured. The DO concentrations of these samples were 3.5 and 13.6 mg O_2 L^{-1} respectively, demonstrating the correlation between algal presence and DO. Higher amounts of algae also resulted into increased COD and VSS concentrations (Table VIII-1).

Table VIII-1. Overview of physico-chemical parameters for the sample taken at 25 cm depth and samples taken at 5 cm depth with and without visible algae presence, respectively.

	$Sample_{5,\ no\ visible\ algae}$	**$Sample_{5,\ visible\ algae}$**	**$Sample_{25,\ no\ algae}$**	**Units**
Water temp.	27.1	26.4	23.7	°C
pH	7.4	8.4	7.1	-
DO	3.5	13.6	<0.05	mg O_2 L^{-1}
VSS	282±52	584±64	248±26	mg VSS L^{-1}
COD	186±13	430±17	180±6	mg COD L^{-1}
sCOD	99	122	84	mg sCOD L^{-1}
Chlorophyll	0.12±0.02	4.38±0.71	<0.02	mg Chl L^{-1}
NO_3^--N	<0.05	<0.05	<0.05	mg NO_3^--N L^{-1}
NO_2^--N	<0.05	<0.05	<0.05	mg NO_2^--N L^{-1}
NH_4^+-N	17.0	18.5	21.0	mg NH_4^+-N L^{-1}
PO_4^{3-}	4.8	5.2	4.8	mg PO_4^{3-} L^{-1}

VIII.3.2 Amount and activity of MOB

To assess the amount of cultivable MOB in the pond, most probable number analysis was performed on the three pond samples. 9.3 x 10^4 MOB mL^{-1} [1.8 x 10^4 to 4.2 x 10^5 MOB mL^{-1}] were found for $sample_{5cm,\ visible\ algae}$ and $sample_{5cm,\ no\ visible\ algae}$, while $sample_{25cm,\ no\ algae}$ had a concentration of 2.3 x 10^4 MOB mL^{-1} [4.6 x 10^3 to 9.4 x 10^4 MOB mL^{-1}].

The CH_4 emissions of the primary facultative pond (PFP) were measured during late summer/early autumn with a floating gas capturing device. An average CH_4 emission of 8.6±1.9 m^3 CH_4 d^{-1} was found for the whole pond, indicating the presence of dissolved CH_4, a necessity for methanotrophic activity. dAMS was chosen as enrichment medium since this typical growth medium for MOB has a similar pH of 6.9 and a similar ammonium content of 25 mg NH_4^+-N L^{-1} as the pond water. Presence of MOB was confirmed by enrichment in dAMS as well as in filtered pond water. No significant differences in CH_4 oxidation rates were observed between both growth media, indicating that the use of dAMS had no influence on the methanotrophic activity of the enriched community. After a lag-phase of about 24h, significant CH_4 oxidation rates were observed for both media and the maximal daily CH_4 oxidation rates of 190±5 and (dAMS) and 179±24 mg CH_4 L^{-1}_{liquid} d^{-1} (pond water) were found during the third day of enrichment. At the same time, respectively 441±48 and 412±90 mg O_2 L^{-1}_{medium} d^{-1} were consumed, indicating that there was no significant difference in apparent O_2 consumption. This led to apparent molar ratios of 1.16±0.13 (dAMS) and 1.15±0.19 (pond water) $O_{2\ consumed}$ over $CH_{4\ oxidized}$ respectively, for the whole microbial community.

VIII.3.3 Isolation and identification of cultivable MOB

In total, 16 MOB pure cultures were isolated out of the three pond samples, enriched on dAMS. Five type II MOB were isolated, identified as *Methylocystis* sp. (closest related type strain of *Methylocystis parvus* with 99.4-99.5% 16S rRNA gene sequence similarity) and dereplicated into at least three different strains based on rep-PCR fingerprinting. Seven type Ib MOB were isolated; these had identical rep-PCR profiles and were identified as

Methylococcaceae sp., very distantly related to any of the known type Ib MOB (closest related type strain of *Methylocaldum gracile* with 94.2% 16S rRNA gene sequence similarity). Four type Ia MOB were isolated and identified as *Methylomonas* sp. (closest related type strain of *Methylomonas koyamae* with 100% 16S rRNA gene sequence similarity). The rep-PCR profile of these four isolates were identical but distinct from the type strain of *Methylomonas koyamae* (Figure VIII-3).

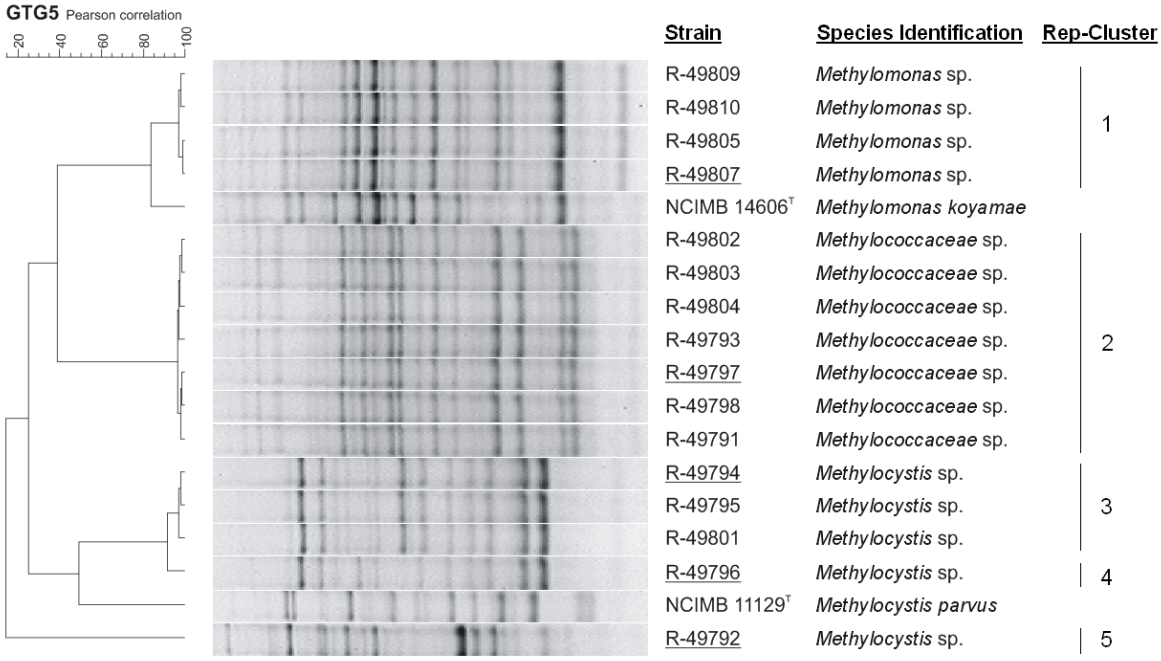

Figure VIII-3. GTG_5 rep-PCR fingerprinting using Pearson product moment correlation coefficient and UPGMA. MOB isolates were divided into five rep-clusters. Four isolates identified as *Methylomonas* sp. by 16S rRNA gene sequencing had a similar rep-profile (cluster 1), distinct from the most closely related type strain *Methylomonas koyamae* NCIMB 14606^T. Seven *Methylococcaceae* isolates shared a similar rep-profile (cluster 2). The five *Methylocystis* isolates could be divided into three groups (cluster 3, 4 and 5) based on their rep-PCR fingerprint, distinct from the most closely related type strain *Methylocystis parvus* NCIMB 11129^T. A representative of each cluster was selected randomly (underlined) for accession number assignment.

VIII.3.4 Algal assisted methane oxidation

To evaluate how the *in situ* algal O_2 production influenced the CH_4 oxidizing activity, fresh pond water with a DO concentration of only 0.6 mg O_2 L^{-1} was added to bottles and inoculation occurred with enrichments of MOB and microalgae originating from the same facultative pond. Under an atmosphere of 20% CH_4 (v/v) in air, the CH_4 oxidation rate was evaluated under stagnant conditions, simulating a water column open to the air. Three dark bottles were shielded from the light, while three illuminated bottles allowed the indigenous algae to produce O_2. During the first day of incubation, a lag-phase without significant CH_4 oxidation was observed in both test series. From the second day on, CH_4 oxidation started in the darkened as well as in the illuminated bottles. However, a significantly higher CH_4 oxidation rate was observed in the illuminated bottles (Table VIII-2).

Table VIII-2. Comparison of activity parameters between three illuminated bottles and three darkened bottles: the total CH_4 oxidation (mg CH_4 L^{-1}_{liquid}), methane oxidation rate (MOR, mg CH_4 L^{-1}_{liquid} d^{-1}) for the second and third day, total O_2 consumption (mg O_2 L^{-1}_{liquid}), molar ratio of apparent consumption of O_2 over CH_4 and concentration of dissolved O_2 in the liquid phase after the test period of 72h. Significant differences between darkened and illuminated bottles are marked with an asterisk (*) (p<0.05).

	Illuminated bottles	Darkened bottles
Total CH_4 oxidation	318±27*	202±25*
MOR 24-48h	135±33*	64±28*
MOR 48-72h	172±25	141±29
Total O_2 consumption	616±49	596±25
Molar O_2:CH_4 ratio	0.97±0.14*	1.48±0.26*
Dissolved oxygen	7.3±0.6*	3.1±0.4*

Also during the third day, a higher CH_4 oxidation rate was observed in the illuminated bottles. This led to a 1.6 times higher amount of CH_4 removed over the whole test period. At the same time, a similar apparent O_2 consumption was observed, resulting in a significantly higher apparent molar O_2:CH_4 consumption ratio in the dark bottles (Table VIII-2). The higher *in situ* O_2 production in the illuminated bottles was also observed at the end of the testing period, as a significantly higher DO concentration was present in the liquid phase of the illuminated bottles compared to the dark bottles (Table VIII-2).

VIII.4 Discussion

VIII.4.1 Algae assist the methane oxidation

Without the photosynthetic activity of the algae, the whole pond would be anaerobic, except for a surface layer of a few millimetres, where wind and diffusion effects provide some aeration. Photosynthetic O_2 production is a necessity for methanotrophic activity as aerobic MOB can only use molecular O_2 as final electron acceptor (Hanson and Hanson, 1996). It has been reported that photosynthetic organisms like the aquatic grass *Calamogrostis canadensis* or submerged *Spaghnum* mosses can provide O_2 to MOB in anaerobic or microaerophilic conditions, thereby enhancing CH_4 oxidizing activity (Kip et al., 2010). This ability was also observed for algae in laboratory tests, where algae enhanced methanotrophic activity by the *in situ* production of O_2 (van der Ha et al., 2011). This interaction was in this study confirmed in a straightforward set-up, mimicking a shallow stagnant anaerobic water column under an atmosphere of CH_4 and air. In the darkened bottles, the CH_4 oxidizing activity was limited by the slow gas transfer of O_2 to the water column (Melse and Van der Werf, 2005), while in the illuminated bottles algal photosynthetic activity increased the concentration of the limiting dissolved O_2, resulting in a 57% higher CH_4 removal rate. The difference in the apparent O_2:CH_4 ratio can be explained by *de novo* O_2 production by the active algae, which led to a lower consumption of O_2 originating from the gas phase. It can also be concluded that the CH_4 oxidizing activity in these systems is limited by O_2 availability, rather than the amount of available CH_4. This beneficial effect of the algae on MOB is probably even more pronounced in the

pond, as the water column there is much deeper, leading to an even more difficult gas transfer. Moreover, a stronger influence of the algae can be expected in the pond, as light intensities during the day are more than an order of magnitude higher than in the batch test, resulting in a zone of more than 50 cm with algae.

VIII.4.2 Facultative ponds harbor a robust and diverse methanotrophic community

The microbial community from the pond showed a high CH_4 oxidizing activity when incubated with CH_4, demonstrating the presence of CH_4 oxidizing organisms. The maximal observed CH_4 oxidation rate was quickly achieved and those rates were similar to those observed in comparable studies, where enrichments of various inocula were used (Hoefman et al., 2012a, van der Ha et al., 2010). As the activity of the enrichments with filtered pond water and typical dAMS growth medium was similar, no severe nutrient limitations seemed to occur in the pond. Indeed, sufficient NH_4^+-N and PO_4^{3-}-P was present in the pond to support bacterial activity, while the observed pH and temperature ranges are suited for most in literature described MOB. These conditions explain why at least 10^4 cultivable MOB mL^{-1} were observed in pond samples from the upper 50 cm of the water column. This number is undoubtedly an underestimation of the total amount of active MOB as a relatively short incubation period was applied and not all MOB are cultivable in one mineral growth medium (Hoefman et al., 2012a). Still, these numbers show that, in contrast to results by van der Steen et al. (2003), a large pool of MOB is present in the facultative pond. The isolation campaign showed that MOB indeed were responsible for CH_4 oxidation, as species were isolated and identified from all three major types of proteobacterial MOB, i.e. type Ia, Ib and II. This reveals a diverse methanotrophic seed bank and implies that there was no strong selection towards one specialized species. Moreover, a *Methylococcaceae* sp. was isolated that is only very distantly related to all known MOB type strains. This finding could be the result of the specific conditions of the pond and deserves further investigation.

During the incubation period of pond samples with CH_4, short lag-phases were observed compared to similar batch tests with different types of CH_4 oxidizing inocula, showing that many MOB were active or able to resuscitate quickly from dormancy (Hoefman et al., 2012a, Hoefman et al., 2012b, van der Ha et al., 2011). Indeed, fast phenotypic adaptation and formation of resting structures are common characteristics of MOB and would create a major advantage for the MOB in facultative ponds, where drastic changes in growth conditions take place (Hanson and Hanson, 1996, Whittenbury et al., 1970a). Due to the non-sheltered nature of the facultative pond and the algal activity, extreme changes of DO, pH and temperature occurred over time and created temporarily harsh growth conditions. Moreover, the combination of high pH with strong light intensities and O_2 oversaturation could result in an increased production of harmful radicals, which are known to negatively influence MOB and induce dormancy (Murase and Sugimoto, 2005). Also, the presence of toxic H_2S, originating from anaerobic processes in the sludge blanket, was observed regularly. Probably the MOB form resting structures and/or have mechanisms to protect themselves to a certain extent against such negative influences (Hanson and Hanson, 1996, Roslev and King, 1994). These characteristics could be of importance for biotechnological applications where robustness and a fast response towards negative stimuli are required.

VIII.4.3 Importance within the framework of global warming

It can be concluded that algal O_2 production is the driving force for the CH_4 oxidizing activity in facultative ponds. As such ponds are important emission sources of CH_4, understanding and enhancing these interactions could help to decrease the C footprint of these wastewater treatment units. Indeed, although at least part of the CH_4 produced by methanogens in the anaerobic sludge blanket is consumed by the MOB before it reaches the atmosphere, elementary extrapolation of measured pond emissions still shows a total loss of about 3000 m^3 CH_4 y^{-1}, equalling a yearly contribution of 55 ton CO_2-equivalents y^{-1} or an emission of 0.98 kg CH_4 y^{-1} per inhabitant for this relatively small pond. The role of this algal driven CH_4 oxidation seems underestimated and therefore deserves further attention.

Acknowledgements

DVDHA, AKC and NB designed the experiments. DVDHA performed the experiments, analyzed the data and wrote the manuscript. SH performed the miniaturized extinction culturing, including the rep-PCR and identification of MOB isolates. MLI helped with the experiments. SH, PDV, IML, AKC and NB commented on the manuscript. The authors like to thank the EBRU staff firmly for their kind assistance. Also our gratitude to the Research Foundation Flanders (FWO), who made this joint research possible via financial support. This research was also funded by a PhD grant to David van der Ha from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen, SB-83259) and research grants from the Geconcerteerde Onderzoeksactie (GOA) of Ghent University (BOF09/GOA/005).

CHAPTER IX

CONVERSION OF BIOGAS TO BIOPRODUCTS BY ALGAE AND MOB

Chlorophyll extractions, EBRU, Grahamstown

Sometimes our best decisions are the ones that don't make sense at all.

Ted Mosby

Chapter IX. Conversion of biogas to bioproducts by algae and MOB

Abstract

Biogas produced by anaerobic digestion is typically converted into electricity and low value heat. In this study, biogas is microbially transformed into a different set of valuable bioproducts. As proof of principle, the production of feed additives, i.e. lipids and poly-hydroxy-butyrate, out of biogas was evaluated. In a first stage, the CO_2 in a synthetic biogas was photosynthetically fixed by an algal *Scenedesmus* sp. culture at an average rate of 192±9 mg CO_2 L^{-1} liquid d^{-1}, resulting in concomitant O_2 production. After N depletion, more than 30% of the formed lipids (220±7 mg lipids g^{-1} total organic carbon) were unsaturated. In a second stage, the theoretical resulting gas mixture of 60% CH_4 and 40% O_2 was treated by a methane oxidizing *Methylocystis parvus* culture, with oxidation rates up to 452±7 mg^{-1} CH_4-C L^{-1} liquid d^{-1}. By repeated N limitation, concentrations of 295±50 mg intracellular poly-hydroxy-butyrate g^{-1} cell dry weight were achieved. Finally, a one-stage approach with controlled coculturing of both microbial groups resulted in harvestable bioflocs. This is the first time that a total microbial conversion of both greenhouse gases into biomass was achieved without external O_2 provision and could result into a novel sink for CH_4 originating from all types of wastestreams.

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IX.1 Introduction

The major goals of wastewater treatment are the protection of downstream users and the environment (Wilsenach et al., 2003). However, due to the scarceness of freshwater, nutrients and energy, sustainable recovery of the latter has become an additional priority (Driver et al., 1999, McDonough and Braungart, 2002). While conventional aerobic treatment converts waste into low caloric heat and CO_2, anaerobic digestion technology allows partial recovery of energy into biogas, which contains about 60% CH_4 next to 40% CO_2 and small amounts of H_2S, H_2 and NH_3 (Demirel et al., 2010). Up to 70% of the organic matter from sewage can be converted to biogas, hence about 0.35 m^3 CH_4 can be produced out of a kg of sewage chemical oxygen demand (Verstraete et al., 2009). In best case, this biogas is used for green electricity production with conversion yields of merely 40% while the rest becomes heat, making economical sustainable recovery tough in smaller installations (Poschl et al., 2010). Due to these losses, green electricity production through anaerobic digestion is subsidised with amounts of 83-280 euro MWh_{el}^{-1} in the EU to assure competitiveness with non-renewable energy sources (Sakulin and Borroni, 2010). Therefore, alternative sinks for biogas could be of interest on the condition that enough added value is created to motivate the conversion, as biogas has a value of about 0.2 euro m^{-3} (for the EU) (Verstraete et al., 2009).

Scientists and industry have put much interest in microalgae as they can be cultivated in areas that are not of interest for food crop production, while at the same time, production rates of 70 ton cell dry weight ha^{-1} y^{-1} were calculated, comparable with production rates of agricultural crops (Melis, 2009, Zamalloa et al., 2011). Microalgae have been used to transform CO_2 into a whole range of interesting metabolites, like carotenoids and vitamins (Sasso et al., 2012) while their relatively high lipid content make them also interesting for biofuel production (Mata et al., 2010). Aerobic methane oxidizing bacteria (MOB) on the other hand are one of the few bacteria able to oxidize the stable CH_4-molecule, the other major component of biogas. They are known to produce poly-hydroxy-butyrate (PHB) - a biodegradable polyester - as a type of intracellular energy and carbon storage (Sasso et al.,

2012). When grown under nutrient limitation, MOB can incorporate PHB up to 60% of their total cell dry weight (Wendlandt et al., 2010, Zhang et al., 2011). The theoretical yield coefficient lies between 0.51 and 0.73 mg PHB mg^{-1} CH_4 oxidized, but depends strongly on the species and the type of nutrient limitation (Wendlandt et al., 2010). This PHB can be used as a biodegradable alternative for polypropylene plastics and is also applied in aquaculture as an energy source and probiotic compound for fish and *Artemia* nauplii (Pieja et al., 2011).

In this study, synthetic biogas was converted into valuable algal and bacterial biomass by N limited cultivation. In a first of two stages, an algal *Scenedesmus sp*. culture fixed the CO_2 present in the biogas, creating a gas mixture of CH_4 and O_2. In a second stage, this gas mixture was transformed into PHB by a methanotrophic culture. An alternative one-stage coculture approach under controlled conditions allowed *in situ* transformation of both greenhouse gases into microbial biomass without the need of external O_2 addition, thus creating an alternative sink for biogas. An increase in pH hereby resulted in the formation of bioflocs, facilitating the harvesting of the flocs.

IX.2 Experimental section

IX.2.1 Growth characteristics of the *Scenedesmus* sp. culture on different CO_2 concentrations

A non-axenic *Scenedesmus* sp. culture originating from a photobioreactor (Zamalloa et al., 2012a) was pre-cultivated on buffered diluted Nitrate Mineral Salts medium (dNMS) with a pH of 6.8. This growth medium consisted out of 0.2 g KNO_3; 0.2 g $MgSO_4.7H_2O$; 3.59 g $Na_2HPO_4.2H_2O$; 1.36 g KH_2PO_4; 30 mg $CaCl_2.6H_2O$; 5 mg FeNaEDTA; 2.5 mg $CuSO_4.5H_2O$ (10 µM) and 1 mL trace element solution (van der Ha et al., 2010, Whittenbury et al., 1970b) L^{-1}. For optimal algal growth, 0.01 mg cyanocobalamin, 0.01 mg thiamin and 0.002 mg biotin L^{-1} were supplemented. Fifteen similar gastight bottles of 1150 mL were filled with 180 mL of this sterile medium and inoculated with 20 mL of the *Scenedesmus* sp. culture (final concentration of 316 mg cell dry weight (CDW) L^{-1}). Light microscopy confirmed that

no other algal species were present. All bottles were flushed with He and filled with 0%, 10%, 20%, 40% and 60% CO_2 at atmospheric pressure (in triplicate). After 1h of equilibration, 1M NaOH was used to assure that the pH was between 6.75 and 7.10 after which gas pressure and composition were analyzed. The bottles were placed on a shaker (120 rpm) at 20±1°C and illuminated with fluorescent lamps (75 µmol PAR m^{-2} s^{-1}). The gas composition and optical density (OD) at 610 nm were analyzed daily over a period of 72h. On a daily base, the amount of consumed CO_2 was calculated and a similar amount of CO_2 added.

IX.2.2 Lipid production by an algal *Scenedesmus* sp. culture cultivated on synthetic biogas

Six new bottles were filled and handled in a similar way. The only difference between the three test and control bottles was the nitrogen content of the applied medium: 23.6±0.4 mg NO_3^--N L^{-1} versus 136.0±1.0 mg NO_3^--N L^{-1} in the controls. To the bottles, 320 mL medium was added together with 80 mL *Scenedesmus* sp. pre-cultured on 40% CO_2, resulting in 103**±**2 mg total organic C L^{-1}. The gas phase was filled with synthetic biogas, a mixture of 60% CH_4 and 40% CO_2. After equilibration, the gas phase consisted out of 63±3% CH_4 and 28±3% CO_2 next to 5±1% N_2 and 1.5±0.3% O_2. The bottles were placed on a shaker (100 rpm) at 22±2°C under 90 µmol PAR m^{-2} s^{-1}. Daily, 100 mL of liquid was sampled for further analysis: pH, anion concentration, OD, CDW, total inorganic and organic C content (TIC and TOC) and lipid content, next to gas phase analyses. After 120h, 80 mL of the algal cultures from triplicate bottles was mixed with 320 mL of fresh medium and synthetic biogas was added. The total test period consisted out of three cycles.

IX.2.3 Production of poly-hydroxy-butyrate by *Methylocystis parvus*

Three similar test bottles were filled with 200 mL dNMS-medium with 32.0±0.3 mg NO_3-N L^{-1} while three control bottles contained 143.6±3.1 mg NO_3^--N L^{-1}. Inoculation took place with an axenic *Methylocystis parvus* culture (NCIMB 11129^T), resulting into 46.9±0.5 mg CDW L^{-1}. The gas phase of the closed bottles was flushed resulting in a final composition of

58.4±3.7% CH_4, 41.2±2.4% O_2, 4.2±1.3% N_2 and 0.2±0.2% CO_2. The bottles were placed in the dark on a shaker (100 rpm) at 22±2°C. On a one to two day basis, 25 mL of liquid was sampled for further analysis of the pH, OD, CDW, anion concentration and poly-hydroxy-butyrate (PHB) content, together with analyses of the gas phase composition. After 48, 72 and 96h, 100 mL O_2 was added to the gas phase to ensure that the O_2 concentration did not become limiting. At the end of each of three cycles (120h), the liquid phase of triplicate bottles was merged and diluted 15-25 times to obtain an optical density at the start of the new cycle in a range between 0.100 and 0.125. Thereafter, a new cycle started with a fresh gas mixture.

Before each cycle, purity of the culture was checked by plating on tryptic soy agar (Hoefman et al., 2012a). After the third cycle, both a test bottle and a control bottle were again refreshed, however with an addition of 2% C_2H_2, while two other bottles were autoclaved. In all these bottles, no substantial CH_4 oxidation was observed during a period of 120h.

IX.2.4 One-stage biofloc production under controlled conditions

A 3L continuous stirred tank reactor (CSTR, Biostat B reactor, Sartorius, Germany) was used at 20±1°C with pH regulation between 6.95 and 7.05. The reactor was originally filled with 1150 mL dNMS medium (45±3 mg NO_3^--N L^{-1}) and inoculated with a *Scenedesmus* sp. culture, pre-inoculated on synthetic biogas (final concentration of 598 mg CDW L^{-1}; 9.7 mg chlorophyll L^{-1}) and a *Methylocystis parvus* culture pre-inoculated on a gas mixture of 60% CH_4 and 40% O_2 (final concentration of 468 mg CDW L^{-1}). The gas phase of the reactor was flushed with synthetic biogas. During the first 24h, a gastight bag (2.0L) with the same gas mixture was attached to the reactor to assure atmospheric pressure in the reactor. Thereafter, the gas bag was replaced by a gastight syringe (60 mL), filled with He. The reactor was continuously stirred (120 rpm) and a light intensity of 60 µmol PAR m^{-2} s^{-1} was measured inside the reactor. At the start of the test, 150 mL of liquid was sampled for later analyses, leading to a final volume of 1L liquid. Daily, 120-300 mL liquid was sampled and replaced by fresh dNMS medium without N source. This allowed to follow up the OD,

TIC-, TOC- CDW- and chlorophyll content. After 96h, the gas phase was flushed with synthetic biogas after which 40 mL of C_2H_2 was added.

After the original test period without C_2H_2 addition, 200 mL of the liquid phase was taken out of the reactor and diluted with 200 mL fresh dNMS medium (32.4±0.8 mg NO_3^--N L^{-1}). In two of four reactors, the pH was increased from 7.1 to 8.8 with 1M NaOH in order to evaluate the influence of a pH increase on floc formation. All four reactors were incubated on a shaker (100 rpm, 72 µmol PAR m^{-2} s^{-1}) at 20±1°C with synthetic biogas.

IX.2.5 Physico-chemical analyses

The gas phase compositions were analyzed with a Compact gas chromatograph, combined with gas pressure measurements.(van der Ha et al., 2011) The methane oxidation rate (MOR) was calculated and expressed as mg CH_4 L^{-1} liquid d^{-1}. All gas concentrations are expressed as volume percent (% v/v) of the total gas phase. CDW was assessed according to Greenberg et al. (1992). The TIC and TOC contents were determined with a TOC-5000 total organic carbon analyzer (Shimadzu). A SP10B pH electrode, connected to a Consort C532 multimeter analyzer (Turnhout, Belgium) was used to measure pH differences. Filtered samples (0.45 µm filter, Millipore, Brussels, Belgium) were analyzed for NO_3^- and NO_2^- by means of a 761 Compact Ion Chromatograph, equipped with a conductivity detector (Metrohm, Zofingen, Switzerland). There was no sample where the nitrite concentration exceeded 1.4 mg NO_2^--N L^{-1}. Assessment of optical density (610 nm) was performed with a WPA Lightwave II spectrophotometer (610 nm). The chlorophyll content was assessed according to Wilthshire et al. (2000), whereby two extraction cycles (37 kHz; 80 W) took place with sonication at 0°C for 60 minutes.

IX.2.6 Fatty acid and poly-hydroxy-butyrate analyses

For fatty acid analyses, fresh samples of 50 mL were centrifuged (15', 5000 g) and the water phase discarded. The test tubes were then flushed with N_2 and stored at -20°C until extraction according to Lepage and Roy (1984). Dried fatty acids methyl esters (FAME)

were dissolved in 0.5 mL octane and transferred to 2 mL glass vials, flushed with N_2 and stored at -30°C until injection in a Chrompack CP9001 GC, equipped with a CP9010 liquid autosampler, a temperature programmed on-column injector and an FID detector with H_2 as carrier gas.

For PHB analyses, frozen samples of 4 mL were thawed and centrifuged (20', 6000 g). The pellets were then treated according to De Schryver et al. (2009) Crotonic acid, formed after acid hydrolyses of the PHB was analyzed with high-performance liquid chromatography (HPLC), equipped with a Phenomenex Rezex ROA organic acid (H 8%) column. Injection took place at room temperature with a flow of 0.6 mL min^{-1} with 2.5 mM H_2SO_4 in milli-Q water. For each set of analyses, a standard curve was made by means of a crotonic acid solution. The PHB content is expressed per g cell dry weight (CDW), analyzed in parallel.

IX.2.7 Statistical analysis

Results were statistically analyzed by performing a Welch modified two-sample t-test with equal variance and at a significance level of 95% (*n* results, $p<0.05$). Analyses were performed with the Statistical Package for the Social Sciences (SPSS, Duncan, version 19.0).

IX.3 Results

IX.3.1 Activity of a *Scenedesmus* sp. monoculture under increased CO_2 concentrations

A *Scenedesmus* sp. culture (318 mg CDW L^{-1}) was cultivated in the absence of other microalgae after addition of 0%, 10%, 20%, 40% and 60% CO_2 respectively, in the gas phase. Over a period of 72h, no significant growth or photosynthetic O_2 production was observed in the control bottles without CO_2 amendment ($n=3$, $p<0.05$). The growth and activity was comparable in the treatments with 10%, 20% and 40% CO_2 and an average O_2 production of 345±10 mg O_2 L^{-1}_{liquid} d^{-1} (40% CO_2) was found over the three-day period.

The treatment with 60% CO_2 however showed a significantly lower growth rate and photosynthetic oxygen production of 244±89 mg O_2 L^{-1}_{liquid} d^{-1} (p<0.05).

IX.3.2 Growth parameters of a *Scenedesmus* sp. monoculture under N limited growth conditions

In a first of two stages, the growth of a *Scenedesmus* sp. culture was further evaluated under an atmosphere of 60% CH_4 and 40% CO_2, defined as synthetic biogas. The three test and the three control bottles differed only by the applied NO_3^--N dose, i.e. 23.6±0.4 mg NO_3^--N L^{-1} in the test bottles and 136±1 mg NO_3^--N L^{-1} in the controls. During all three subsequent cultivation cycles, the NO_3^--N concentration in the control bottles never decreased below 69 mg NO_3^--N L^{-1}. In the test bottles, the NO_3^--N concentration decreased below 2 mg NO_3^--N after 48-72h in the second and third cycle.

The average CO_2 fixation rate of 192±9 mg CO_2 L^{-1} liquid d^{-1} in the test bottles were not significantly lower than in the control bottles (Table IX-1). When the dissolved inorganic nitrogen was depleted, CO_2 fixation rates in the test bottles decreased slightly, but differed never significantly (p<0.05). The molar ratio of produced O_2 over fixed CO_2 was comparable for both test series and 98±4% of the fixed CO_2-C was found back in the biomass. Due to the N limited growth from 48-72h on, a significantly lower ratio of 0.05±0.01 mg consumed NO_3^--N mg^{-1} produced TOC was observed in the test bottles, compared to 0.11±0.01 mg NO_3^--N mg^{-1} TOC in the control bottles (Table IX-1). In a similar set-up, growth of algae was tested with biogas from a lab scale digester fed with molasses. The same trends were observed with comparable average CO_2 fixation rates of 154±36 mg CO_2 L^{-1} liquid d^{-1}.

Table IX-1. Overview of different activity parameters of a *Scenedesmus* sp. monoculture in the test bottles with N limiting growth and the control bottles without N limiting growth for three subsequent cycles: average CO_2 fixation rate (mg CO_2 L^{-1} liquid d^{-1}), average and maximal O_2 production rate (mg O_2 L^{-1} liquid d^{-1}), average ratio of produced O_2 over consumed CO_2 (mol O_2 mol^{-1} CO_2), average growth yield (mg $TOC_{produced}$ mg^{-1} $CO_{2\text{-consumed}}$), average TOC production (mg TOC L^{-1} liquid d^{-1}) and average ratio of consumed NO_3^--N over produced TOC (mg NO_3^--N mg^{-1} TOC). Standard deviations are given for triplicates. Significant differences between test and control bottles are marked with a * (p<0.05).

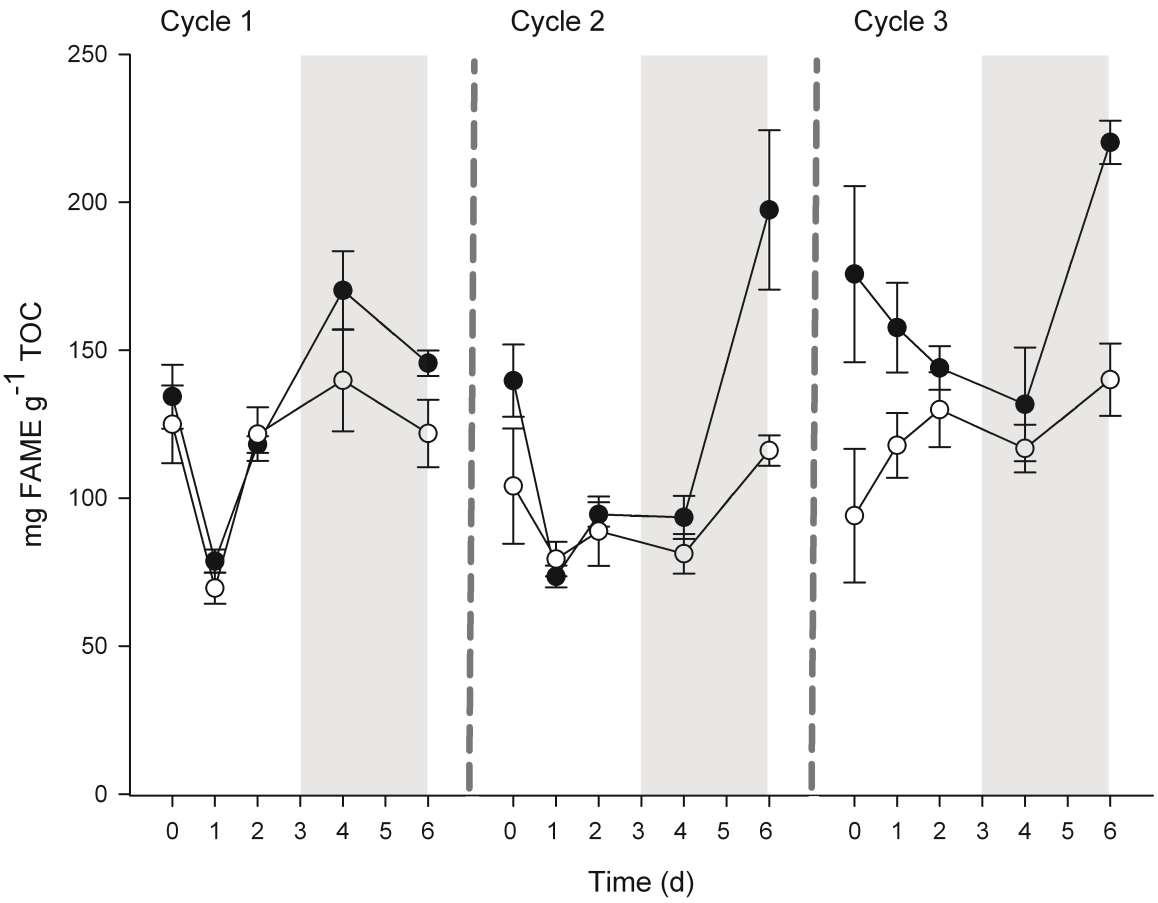
	Test bottles			Control bottles		
	Cycle 1	**Cycle 2**	**Cycle 3**	**Cycle 1**	**Cycle 2**	**Cycle 3**
Average CO_2 fixation rate	163±38	166±9	192±9	163±31	160±11	219±10
Average O_2 production rate	114±30	112±45	121±37	122±26	125±42	139±47
Maximal O_2 production rate	148±39	140±31	140±30	160±25	145±15	148±66
Average molar O_2/CO_2 ratio	0.96±0.17	0.93±0.06	0.87±0.08	1.02±0.12	1.08±0.12	0.87±0.09
Average growth yield	1.12±0.19	1.13±0.10	1.02±0.09	1.11±0.13	1.16±0.09	0.93±0.06
Average TOC production	50±4	51±8	54±9	49±5	50±7	55±7
Average NO_3^-/TOC ratio	0.05±0.01*	0.05±0.01*	0.05±0.01*	0.14±0.01*	0.13±0.02*	0.11±0.01*

IX.3.3 Lipid profile of a *Scenedesmus* sp. monoculture under N limited growth conditions

During the cycles described above, the lipid content and constitution of the *Scenedesmus* sp. cultures were analyzed (Figure IX-1). The N limited growth on synthetic biogas resulted in an increase of the lipid content, measured as fatty acid methyl esters (FAME). While a minimum of 79±4 mg FAME g^{-1} TOC was found at the beginning of the first cycle, 220±7 mg FAME g^{-1} TOC was detected in the test bottles at the end of the third cycle. This was significantly more than the 128±12 mg FAME g^{-1} TOC found in the control bottles ($p<0.05$). During each cycle, the same response was found for the test bottles: when sufficient N was provided, the concentration of lipids decreased. Under N limited growth conditions however, a steep incline of the lipid concentration occurred (Figure IX-1). This increase was only faintly present in the control bottles. Of the detected fatty acids, the following were present in the highest concentrations at the end of the third cycle: oleic acid (C18:1(n-9), 38%), palmitic acid (C16:0, 23%) and alpha linolenic acid (C18:3 (n-3), 18%). The most important influence of the N limited growth was the increase in the concentration of oleic acid from 10.0±1.4% (day 3, cycle 3) to 38.3±4.6% at the end of cycle 3. The total amount of the omega-3 and omega-6 lipids was 21.4 and 10.3% respectively (end of cycle 3) and was not significantly different from the control bottles ($p<0.05$).

IX.3.4 Growth parameters of a *Methylocustis parvus* monoculture under N limited growth conditions

In the second stage, the possibility of PHB production by MOB was tested. An axenic monoculture of *Methylocystis parvus* was cultivated on a gas mixture of 60% CH_4 and 40% CO_2, the theoretical gas composition after total photosynthetic conversion of CO_2 by algae growing on the former synthetic biogas mixture. The control and test series differed again only for the amount of added NO_3^--N: 147.8±2.7 mg NO_3^--N L^{-1} in the control bottles ($n=3$) and 33.4±0.5 mg NO_3^--N L^{-1} in the test bottles ($n=3$) at the beginning of the first of three cycles.



Except for the first 24h of the second and third cycle, no significant differences in the methane oxidation rate (MOR) were observed between the test and control bottles (Table IX-2). Also the CO_2 production and O_2 consumption rates showed only significant differences during the first 24h, as a slightly longer lag phase could be observed for the test bottles. The overall MOR for both test series did not change significantly over the three cycles. The maximum daily MOR always occurred between 24h and 48h after the

start of a cycle, with a maximum of 452±7 and 437±18 mg CH_4-C L^{-1} liquid d^{-1} for the test and control bottles respectively (cycle 3). Although not significant (p<0.05), a slightly higher MOR was found in the control bottles. The average ratio of mol $O_{2\text{-consumed}}$ over mol $CH_{4\text{-consumed}}$ was higher for the test bottles, where an average of 1.59±0.03 was found in the third cycle, compared to 1.46±0.02 for the control bottles (Table IX-2). The average growth yield was always higher in the control bottles, although the growth yield in the test reactors increased each cycle, with a maximum of 0.46±0.04 mg $TOC_{produced}$ mg^{-1} $CH_{4\text{-consumed}}$ in the third cycle, compared to 0.55±0.08 mg $TOC_{produced}$ mg^{-1} $CH_{4\text{-consumed}}$ for the control bottles (Table IX-2). Due to the N limitation, a significantly lower ratio of NO_3^--$N_{consumed}$ over produced TOC was found for the test bottles. In the third cycle, only 0.07±0.01 mg NO_3^--N was consumed per mg TOC increase, where this was 0.21±0.03 mg NO_3^--N for the control bottles without N limitation (Table IX-2).

IX.3.5 PHB production by a *Methylocystis parvus* monoculture under N limited growth conditions

During each cycle of five days, the same trends of bacterial PHB concentrations were observed: during the first 24h of the cycle, when no N limitation occurred, PHB concentrations were low. After 24-48h, N limited growth occurred, followed by a significant (p<0.05) increase of the PHB concentration (Figure IX-2). The PHB concentration decreased thereafter, with a percentage of PHB after 120h that was still significantly higher than after 24h (p<0.05). Due to the alteration of optimal and N limited growth, considerably higher PHB concentrations were found at the start of the second and third cycle, as each cycle started with a PHB enriched inoculum from the former cycle. However, the PHB content always decreased during the first 24h, and was at that moment always below 20 mg PHB g^{-1} CDW.

Table IX-2. Overview of different activity parameters for an axenic *Methylocystis parvus* culture in the test bottles with N limiting growth and the control bottles without N limiting growth for three subsequent cycles: total amount of oxidized CH_4–C (mg CH_4-C L^{-1} liquid), average and maximal CH_4 oxidation rate (MOR; mg CH_4-C L^{-1} liquid d^{-1}), average ratio of consumed O_2 and CH_4 (mol O_2 mol^{-1} CH_4), average growth yield (mg $TOC_{produced}$ mg^{-1} $CH_{4\text{-}consumed}$), average TOC production (mg TOC L^{-1} liquid d^{-1}) and average ratio of consumed NO_3^--N over produced TOC (mg NO_3^--N mg^{-1} TOC). Standard deviations are given for triplicates. Significant differences between test and control bottles are marked with a star (*) (p<0.05).

	Test bottles			Control bottles		
	Cycle 1	**Cycle 2**	**Cycle 3**	**Cycle 1**	**Cycle 2**	**Cycle 3**
Total oxidized CH_4	1032±32	1019±18	1008±11	1081±11	1112±17	1052±25
Average MOR	212±6	209±4	210±2	222±2	228±4	220±5
Maximal daily MOR	316±17	295±11	452±7	299±21	298±50	437±18
Average mol O_2/CH_4 ratio	1.52±0.05	1.55±0.03*	1.51±0.14	1.46±0.01	1.41±0.01*	1.46±0.03
Average yield	0.37±0.03*	0.41±0.04	0.46±0.04	0.50±0.01*	0.46±0.03	0.55±0.08
Average TOC production	79±6*	86±8	97±8	112±2*	105±7	121±17
Average NO_3^--N/TOC ratio	0.09±0.0*	0.08±0.01*	0.07±0.01*	0.18±0.01*	0.21±0.01*	0.21±0.03*

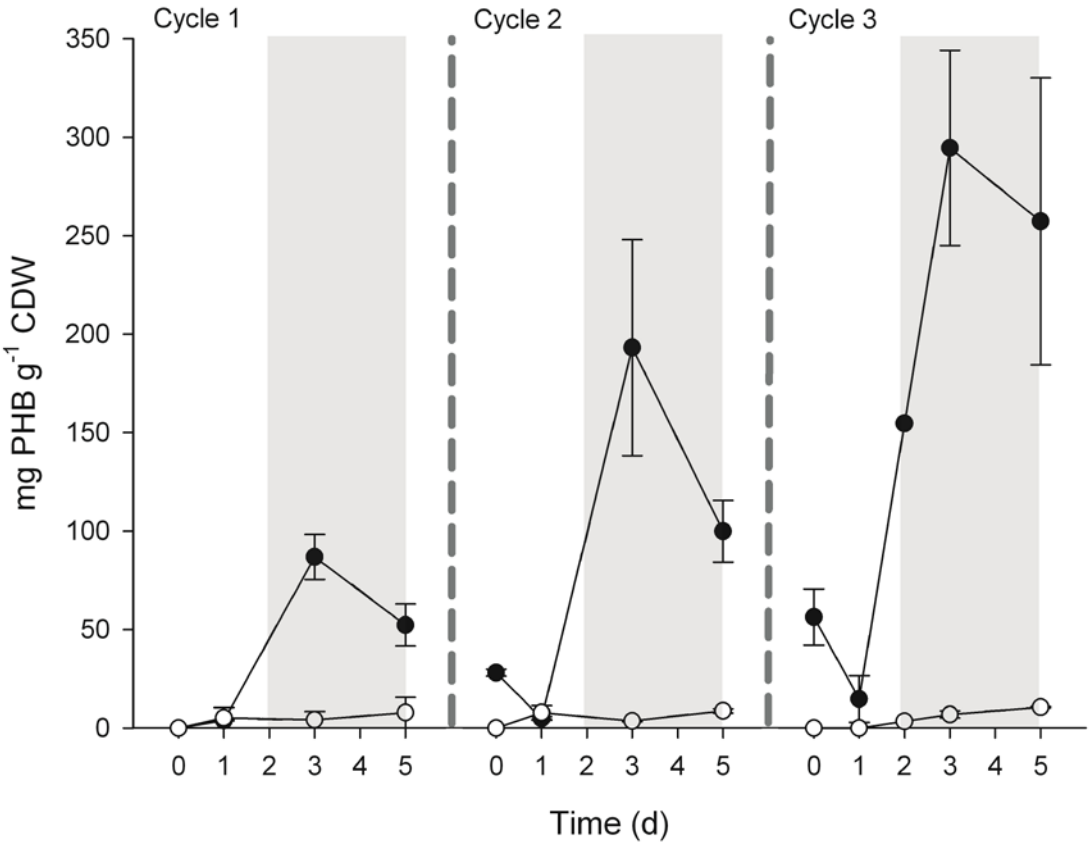


Figure IX-2. The average ratio (*n*=3) of measured PHB per unit of CDW (mg PHB g^{-1} CDW) for the test bottles (filled circles) and the control bottles (open circles) of a *Methylocystis parvus* monoculture over three consecutive cycles of five days. The grey zones indicate where N limited growth occurred.

When comparing the three subsequent cycles, the maximum achieved PHB concentrations in the test bottles increased significantly in each subsequent cycle (p<0.05). The highest concentration in the test bottles was found after 72h during the third cycle: 295±50 mg PHB g^{-1} CDW, where the concentration in the control bottles was always below 1 mg PHB g^{-1} CDW. During the N limited growth (48-120h) period of the third cycle, 243±76 mg PHB was produced per g consumed CH_4-C in the test bottles.

IX.3.6 Biofloc production by a coculture of *Methylocystis parvus* and *Scenedesmus* sp. under controlled N limited growth conditions

Alternatively, a one-stage approach was tested whereby a non-axenic coculture of *Methylocystis parvus* and *Scenedesmus* sp. was cultivated in a continuous stirred tank reactor (CSTR) under an atmosphere of synthetic biogas. From the moment that light energy was provided, the dissolved oxygen (DO) concentration increased from less than 0.2 to 3.2 mg O_2 L^{-1} in 8 min. Thereafter, the DO concentration dropped immediately back to less than 0.2 mg O_2 L^{-1} and never increased above that value for the whole testing period of 96h. The O_2 concentration in the gas phase showed the same trend, as 1 to 3% O_2 was present in all gas samples.

Over the whole testing period (96h), an average MOR of 70 mg CH_4-C L^{-1} d^{-1} was found, resulting in a total oxidation of 281 mg CH_4-C. During those 96h, the apparent amount of CO_2 – the sum of CO_2 in the gas phase and TIC – decreased with 204 mg CO_2-C. As a total production of 474 mg TOC was measured, 98% of the total sum of apparent metabolized CH_4-C and CO_2-C was found back as TOC. It was calculated that 59% of the TOC production originated from CH_4, by direct methanotrophic assimilation or by secondary algal fixation of CO_2 originating from the bacterial dissimilatory processes. In total, 40±4 mg chlorophyll L^{-1} liquid was produced, and the production rates correlated well with the evolution of CDW in the reactor. The NO_3^--N concentration decreased during the first 24h from 45.4±0.6 to 15.15±0.2 mg NO_3^--N L^{-1} and became depleted from the second day on as measured values from then on were below the detection limit of 0.5 mg L^{-1}. At the end of the test period, a similar lipid profile was observed as in the former batch tests.

After the test period of 96h, 2% C_2H_2, a known inhibitor of MOB (Urmann et al., 2008), was added to the gas phase resulting in a decrease of the MOR from 128 to less than 2 mg CH_4 L^{-1}_{liquid} d^{-1}. The DO concentration, which before never reached 0.2 mg O_2 L^{-1} or 2% of the maximal O_2 solubility of 9.1 mg O_2 L^{-1} (21°C), increased immediately after addition of C_2H_2 to 3.3 mg O_2 L^{-1} after 10', 7.3 mg O_2 L^{-1} after 30', 11.2 mg O_2 L^{-1} after 1h and 23.2 mg O_2 L^{-1} after 21h. Gas phase analyses showed that the algal O_2 production during the 21h period

was 211 mg O_2 L^{-1}_{liquid} d^{-1} (770 mg microbial CDW L^{-1}). At the same time 274 mg CO_2 L^{-1}_{liquid} d^{-1} was removed in the reactor, resulting in a ratio of 0.94 mol $O_{2\text{-produced}}$ per mol $CO_{2\text{-fixed}}$.

After the test period without C_2H_2, the coculture was transferred to four Schott bottles. In the two bottles without pH adjustment, 16% of the biomass was present in microbial flocs (mesh of 105 µm, *n*=2) after 48h. Due to the algal activity, the pH increased from 7.1 to 8.3 over the test period. In the other two bottles, which started with a pH of 8.8, floc formation was observed with on average 48% of the biomass present in bioflocs. The higher pH at the start of the test period also led to a 38% lower CH_4 removal rate compared to the non-adjusted bottles. The pH increased in these bottles to 9.8.

IX.4 Discussion

IX.4.1 Algae and MOB can metabolize high CO_2 and CH_4 concentrations

To enable microbial conversions of biogas into bioproducts, algae and MOB are needed which can rapidly metabolize high concentrations of CO_2 and CH_4, respectively. The algal *Scenedesmus* sp. culture did not show a significant loss in fixation or growth rate with CO_2 concentrations up to 40%. Moreover, there was no sign that the build-up of O_2 influenced the fixation activity. These results are in agreement with earlier published data, where a similar 35% decrease in algal activity occurred only at concentrations above 50% CO_2 (Ho et al., 2010) Moreover, preliminary tests with biogas from a lab scale digester indicated that the CO_2 fixation was not hampered by other present gas components. Finding other algae adequate to grow on biogas should not pose problems, as other commercially applied algae species are known to grow on concentrations above 40% CO_2 (Salih, 2011). Less is known about the behaviour of MOB on high concentrations of CH_4 and O_2 particularly. While it has been shown that MOB can metabolize concentrations above 50% CH_4, few other publications mention O_2 concentrations above the atmospheric concentration of 20.8% (Pieja et al., 2011, Scheutz et al., 2009b). No negative effect of the high CH_4 and O_2 concentrations was observed, as the measured average MOR of 210±2 mg CH_4-C L^{-1} liquid d^{-1} was in agreement with former tests with atmospheric O_2 concentrations under comparable conditions (van der Ha et al., 2010). Although other

MOB strains need to be tested, it can be expected that many other methanotrophic species and strains are able to metabolize high concentrations of CH_4 and O_2. Therefore, the intended gas mixtures, i.e. biogas, should not hinder the presented concept.

IX.4.2 The production of bioproducts can be easily induced by nitrogen depletion

In order to create large amounts of biomass with maximal concentrations of valuable bioproducts, a cost-effective approach is needed whereby initially optimal growth conditions are created. Thereafter, the growth conditions should induce high production rates of the biocompounds. In both the two-stage and the coculture approach, N limited growth was chosen as inducing factor because under such conditions algae increased their lipid production while MOB produced high quality PHB if sufficient CH_4 is available (Wendlandt et al., 2010, Zhang et al., 2008). Without any manipulation during the test cycles, growth and biocompound production were successfully combined without a significant loss of activity. Indeed, both the algal *Scenedesmus* sp. culture as the methanotrophic *Methylocystis parvus* culture became N depleted after 24-48h, without expressing lower overall activity or growth rates. N limited cultivation of algae on synthetic biogas led to a significantly higher lipid content compared to controls without N limited growth. Moreover, up to 30% of the lipids had an unsaturated omega-3 or omega-6 nature and are of high value in animal feed and food, as they induce beneficial health effects (Sasso et al., 2012). The same trend was observed in the second stage, where N limited cultivation of a methanotrophic culture on a mixture of 60% CH_4 and 40% O_2 led to an increase of the PHB content up to 30% of the cell dry weight, while this was less than 2% in the control bottles without N limited growth. When optimally controlled, PHB concentrations of MOB like *Methylocystis parvus* can reach values up to 60% of the bacterial CDW, leaving space for further improvements (Wendlandt et al., 2010, Zhang et al., 2008). Also important is the effect of the feast-and-famine regime, whereby alternating phases of optimal growth conditions and N limited growth respectively selected for those algae and bacteria able to switch their metabolism the fastest, leading

to cultures with a higher biocompound production rate. This effect was the strongest for the MOB, as two extra cycles led to a three times higher PHB concentration. However, the obtained results are just an indication of possible yields, as it looks plausible that a higher amount of cycles would further increase the concentrations of lipids and PHB respectively. Also a better fine-tuning of the cycles would increase the yields, for example by harvesting the biomass when the biocompound concentration is maximal.

IX.4.3 A symbiotic cooperation of algae and MOB led to biofloc formation

Whereas a two-stage approach allows to optimize the growth conditions and biocompound production of the algae and MOB separately, a one-stage process is ideal for production of bioflocs while lowering the investment costs. These bioflocs consisted out of randomly organized microalgae and bacteria, as described in our previous work (van der Ha et al., 2011). The results with a coculture in a CSTR showed that both the photosynthetic and methanotrophic processes can take place simultaneously, whereby a symbiotic relationship exists between the algae and MOB. Over the testing period, both the amount of CO_2 - gaseous and dissolved - as CH_4 decreased steadily. Overall, 98% of consumed CH_4-C and CO_2-C was found back as biomass. Algae were actively growing as chlorophyll was produced over the test period, while light microscopy and flow cytometry confirmed an increase in algal numbers of at least two orders (data not shown). Interestingly, light energy immediately triggered the photosynthetic O_2 production followed by an immediate start-up of the aerobic bacterial metabolism due to the sudden availability of dissolved O_2. The produced O_2 was efficiently taken up, as dissolved O_2 values always stayed below the detection limit. Simultaneously, a steady decrease in CH_4 concentration was observed. This CH_4 removal had an obvious methanotrophic nature as the CH_4 removal was stopped when a methanotrophic inhibitor was added. Instantaneously, concentrations of dissolved O_2 increased up to 250% of saturation because MOB were no longer a sink for the produced O_2. At the same time, an increase in the apparent CO_2 removal rate was observed, as MOB no longer produced additional CO_2. The average CH_4 oxidation rate of 93 mg CH_4-C L^{-1} d^{-1} during the normal test period in the CSTR was lower than the 158±2 mg CH_4-C L^{-1} d^{-1} in the batch tests, as the methanotrophic

activity was limited by the low availability of dissolved O_2. To increase the MOR, an increase in photosynthetic activity is needed through better design, a higher ratio of algae over MOB or an increase of the low light intensities.

No important floc formation occurred under controlled conditions. However, an increase of the pH to 8.8 at the start easily induced floc formation, probably due to exopolysaccharides (EPS) formation by the MOB under these non-optimal conditions (Malashenko et al., 2001). This relatively minor increase in pH at the end of the production process could lower the cost of harvesting the biomass considerably.

Acknowledgments

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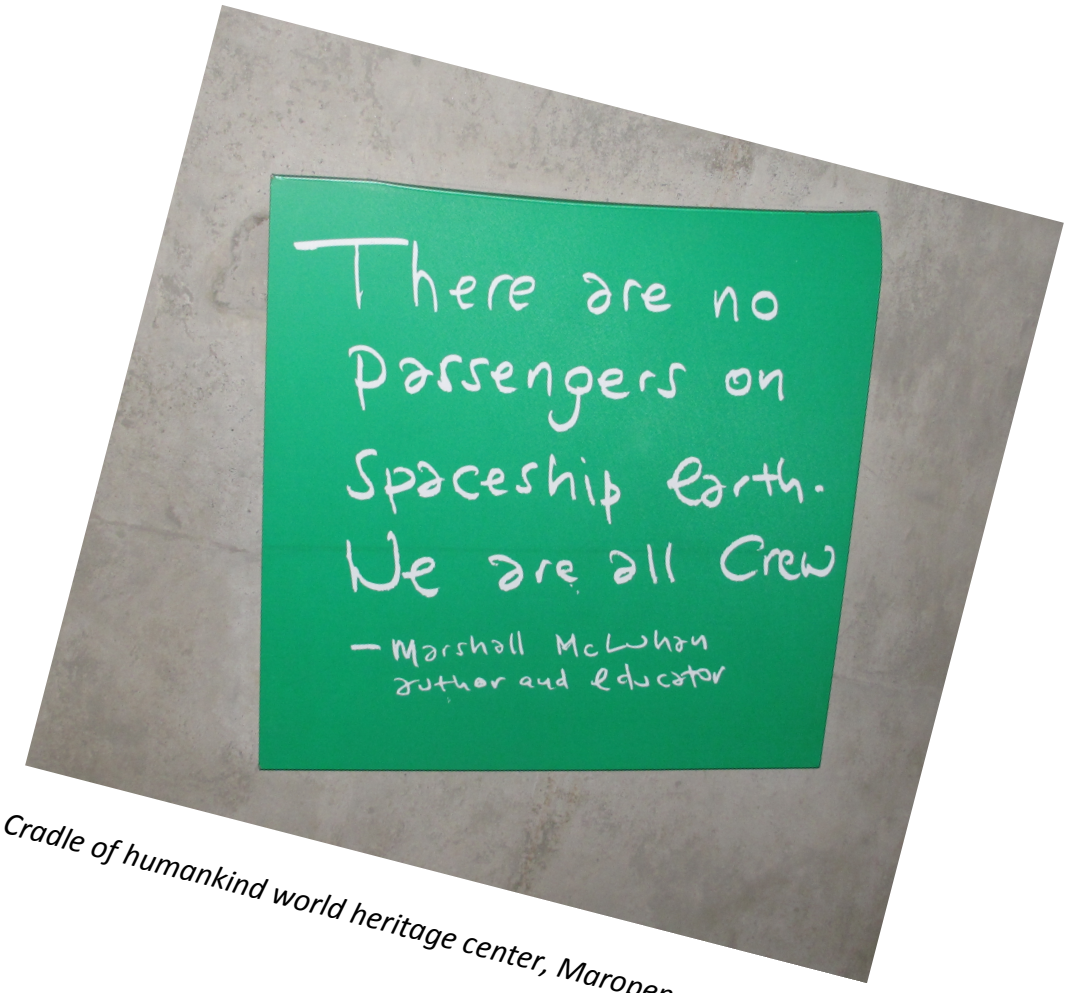
Chapter X

General Discussion And Perspectives



Cradle of humankind world heritage center, Maropeng, South Africa

Laat mij maar dromen…

(till death do us part)

Chapter X. General discussion and perspectives

X.1 General research outcomes

X.1.1 Positioning of this research

When the United Nations Secretary General Ban Ki-moon took the stand at the last UN climate Conference (COP17) in Doha, he gave following warning about the global warming phenomena: "*Let us be under no illusion. This is a crisis. A threat to us all. A threat to our economies, our security and the well-being of our children and those who will come after."* (Abano, 2012). The fact that high profile spokesmen dare to be so blunt on the international scene shows that many policy makers are now aware of the possible long-term threats that climate change can cause.

Traditionally, all spotlights in the climate change debate are on CO_2. Indeed, a majority of the global warming effect is assigned to this GHG. However, CO_2 is to a large extent linked to our behaviour; to considerably decrease our CO_2 emissions, we mostly need to lower our luxury consumption (Kannberg et al., 2009). Anthropogenic CH_4 emissions are however more on the production side, i.e. gas, oil and coal installations, ruminants and agriculture, rice paddies and landfills. These sources often have a potential for engineerable solutions, especially as such sources are often less diffuse. With its 15-20% contribution to the global warming effect (Forster et al., 2007, WMO, 2012), CH_4 mitigation is an important piece in the whole GHG puzzle. The specific global goal is clear: balance the whole CH_4 budget by achieving a decrease of 6% in global CH_4 emissions (Jardine et al., 2004).

Over the last decades, many research groups have focused on the microbiotechnological potential of CH_4 mitigation by means of methanotrophs. While much knowledge has been gathered about the physiology and ecology of methanotrophs, translation to biotechnological applications has been lagging behind. A few of the main reasons are (i)

the low solubility of CH_4 and O_2 in water, (ii) scanty experience with methanotrophic communities in engineered environments and (iii) the fact that there are less economical stimuli for CH_4 mitigation than for CO_2 mitigation. The goal of this dissertation was therefore to increase the knowledge on methanotrophic microbiomes and to explore novel biotechnological applications based on interactions of MOB with other microorganisms.

X.1.2 Main research outcomes

The key concept in this dissertation is the methanotrophic microbiome, i.e. the whole microbial community directly or indirectly depending on CH_4 as carbon and energy source. Hereby two different relations can be distinguished: in the first chapters, the focus lay on the effect of external growth factors on the methanotrophic community, while further chapters zoom in on the interactions within the methanotrophic microbiome and the applied potential that follows out of it.

In order to shed some light in these *black box* systems, it is important that the methanotrophic community can be characterized and the dominant MOB identified and/or isolated. To achieve these goals, it is necessary to combine culture independent and dependent techniques. Conventional isolation techniques are however laborious and time-consuming. Therefore, a novel and miniaturized isolation technique was developed in **Chapter II,** allowing to isolate and screen MOB with desired characteristics for their biotechnological potential. This technique was used to isolate fast-growing MOB from various environments. The applicability of this novel isolation technique was confirmed in **Chapter VIII**, where a diverse set of MOB was isolated from a facultative pond, thereby proving that such environments harbor an interesting methanotrophic potential.

The physiology, behaviour and ecology of MOB have been investigated numerously in lab environments. However, when the goal is to treat CH_4 emissions in or from natural or anthropogenic environments, it is important to know how steering factors influence such methanotrophic communities. In **Chapter III**, the influence of three important steering

factors on the activity and community structure was evaluated, i.e. the N source, salt concentration and Cu^{2+} levels, respectively. It was observed that Cu^{2+}, as stimulator of pMMO expression, increased the methanotrophic activity with a factor 1.5-1.7. Moreover, Cu^{2+} additions increased the resistance against salt additions significantly, indicating that pMMO is more salt resistant than sMMO. Therefore, screening and follow up of Cu^{2+} concentrations is important in environments with increased salt concentrations.

The influence of NH_4^+ - another steering factor - on MOB is still under debate, as both stimulating as inhibiting effects have been observed previously (De Visscher and Van Cleemput, 2003, Stein and Klotz, 2011). In **Chapter IV**, the behavior of methanotrophic communities was evaluated at increased NH_4^+-N concentrations. A methanotrophic community was found able to oxidize CH_4 at a concentration of 2000 mg NH_4^+-N L^{-1}, without loss of methane oxidizing activity. This is by far the highest concentration of NH_4^+-N ever observed whereby MOB are not inhibited by NH_4^+. This sustained methane oxidizing activity was however only observed when the NH_4^+-N concentration was gradually increased. The typical inhibiting effect of NH_4^+ on MOB observed in lab environments as well as in agricultural applications could be counteracted by accomplishing a gradual increase instead of the currently applied shock loads. The increased NH_4^+-N concentrations also resulted into drastic shifts in the methanotrophic community. Although type I MOB were hardly present in the starting inoculum, a type Ia - *Methylosarcina*-like - MOB became highly dominant after NH_4^+-N amendments above 1000 mg NH_4^+-N L^{-1}. This MOB could be useful to mitigate CH_4 in environments where increased NH_4^+ concentrations occur.

When dealing with methanotrophic communities, it is important to pay attention to the present non-methanotrophic members. In all the mentioned methanotrophic communities, heterotrophs formed an important fraction, although no organic carbon sources were amended and the retention times were kept relatively short. This shows that there exist meaningful interactions between the MOB and the associated heterotrophs. In **Chapter V**, the interaction between MOB and associated heterotrophs was investigated at

different copper concentrations. Although continuous shifts were observed in the heterotrophic community of the different subcultures, over time the heterotrophic communities evolved to a similar composition. This indicates that MOB select for certain heterotrophs, possible fulfilling vital processes. These interactions within the methanotrophic microbiome were the subject of **Chapter VI**, where it was shown that C^{13} labelled CH_4-C was metabolized by MOB and quickly transferred to heterotrophs, which build it in into their cell structures. In less than 50 h, half of the bands observed in 16S rRNA gene based DGGE profiles of methanotrophic communities already had C^{13} incorporated. Moreover, it was proven that the CH_4 derived carbon fluxes allowed growth of a methylotrophic yeast with biotechnological potential. Tests with axenic cultures of an MOB strain together with a *Pichia pastoris* strain indicated that only active MOB could sustain growth of the associated heterotroph, indicating an active transfer of CH_4 derived metabolites from the MOB to the methylotroph.

A specific interaction between MOB and another type of microorganisms was the subject of **Chapters VII-IX**, i.e. the symbiotic relationship between MOB and the autotrophic microalgae. In **Chapter VII**, proof of principle was given of this *methalgae* concept. It was observed that MOB and algae can be cocultured, with CH_4 as sole carbon source. Algae were able to grow by metabolizing the methanotrophic end product CO_2 and subsequently, algae supplied O_2 *in situ* to MOB by photosynthetic processes. By means of this cooperation, both GHG are tackled simultaneously, while at the same time MOB are no longer restricted to aerobic environments, enabling more biotechnological applications. In **Chapter VIII**, proof was obtained that this interaction occurs in semi-natural conditions, as the *methalgae* interaction was observed in a facultative pond. It was shown that photosynthetic activity allowed a higher methane oxidizing activity in stagnant waters. Due to the algal O_2 production, such ponds harbor an important methanotrophic community, reducing the carbon footprint of these wastewater treatment plants. Moreover, the methanotrophic community was diverse, as both type Ia, Ib and II MOB were isolated, most probably including members of a new genus. The methanotrophic community in such ponds have a biotechnological potential, as the harsh conditions

wherein they are active could lead to adaptations that might be beneficial when used in applications under tough and/or variable conditions. Lastly, the *methalgae* concept was applied for biotechnological purposes in **Chapter IX**. Instead of the typical conversion of biogas to electricity and low value heat, a conversion of CH_4 into valuable bioproducts was accomplished. In a two-stage approach, synthetic biogas was converted into valuable algal and methanotrophic biomass, respectively, by straightforward N limited growth under a feast-and-famine regime. By means of a one-stage approach with a coculture of algae and MOB, it was shown for the first time that a total microbial conversion of both CH_4 and CO_2 is possible under anaerobic conditions, without external O_2 provision. Moreover, an increase of the pH induced floc formation, facilitating the harvesting of the bioflocs.

A schematic overview of the different research topics is presented in Figure X-1.

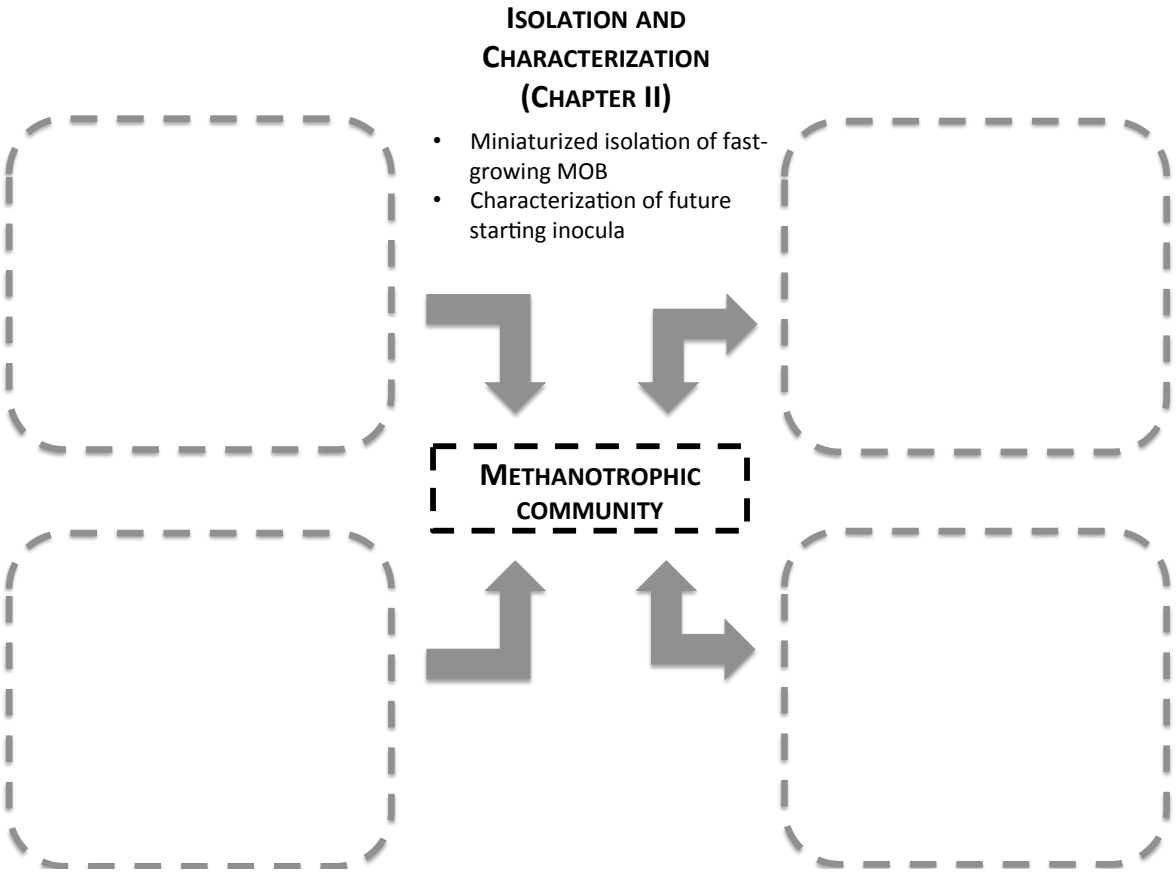


Figure X-1. Schematic research overview indicating the most important conclusions for each chapter.

X.2 Future perspectives

X.2.1 Increasing the available methanotrophic pool

For a long time, the following question has been subject of debate: when a microbial community is performing the required tasks, is there still a need to identify each essential organism of the community? Some people of the engineering-orientated school would state that as long as the job is done, thereby attaining functional stability, there is no need to invest lots of efforts and money on further characterization. Besides, what is the value of a complete screening of a community, if the latter is possibly constraint to only this specific ecosystem? Indeed, bio-augmentation efforts, i.e. transfers of communities from one environment to another, have been less straightforward as hoped for. Microbial communities do not easily occupy niches in a new ecosystem where a native microbial community is already present (Guimaraes et al., 2010). Nonetheless, matching the specific needs for a certain biotechnological application with the characteristics of available microorganisms increases the chance of a successful application considerably. Yet, before the behavior and biotechnological potential of MOB can be tested, they first need to be available. However, like many fastidious organisms, MOB are difficult to preserve (Hoefman et al., 2012a). As a result, many discovered and described strains are currently no longer available in culture collections (Bowman et al., 1993b). Consequently, only a small fraction of the methanotrophic diversity is described and thus, it is no surprise that in the last two years alone, at least four new MOB genera have been described in literature (Geymonat et al., 2011, Hirayama et al., 2012, Luke and Frenzel, 2011, Vorobev et al., 2011). Even often examined environments like wastewater treatment plants, biofilters and wetlands, allow isolation of novel MOB species, as was shown in **Chapter II**. These results also demonstrate that enrichment based techniques can still result in the isolation of novel methanotrophs with potentially interesting characteristics.

Due to troublesome preservation, much of the knowledge gained about the physiology of MOB is based on the limited number of type strains that are available in culture collections. A good example with MOB can be found in **Chapters II and V**, where

Methylomonas spp. were identified, able to express sMMO in addition to pMMO. These results contrast with the for a long time generally accepted idea that sMMO expression is very rare in type I MOB; a statement based on tests with the type strains, which are not able to perform this enzyme switch. Thanks to recent novel culture dependent and independent techniques and the increased interest for MOB, more established MOB facts had to be revised. The old separation between type I and type II MOB concerning their ability to fixate N_2 for instance has also been thorn down (Auman et al., 2001). In general, recent discoveries of new species and new abilities have shown that the flexibility and variability in the methanotrophic gene pool have been underestimated. Moreover, it is clear that more unknown MOB strains are out in the wild, some of them with interesting characteristics. Therefore, uncultured methanotrophic populations can be seen as a latent source of potential industrial catalysts (McDonald et al., 2006).

The in **Chapter II** depicted miniaturized isolation strategy, combined with novel preservation techniques for autotrophs like MOB (Heylen et al., 2012a, Heylen et al., 2012b, Hoefman et al., 2012a) should help to increase the pool of available MOB, allowing to screen more organisms for attractive characteristics. In **Chapter II**, the proof of principle was shown for fast-growing MOB. However, the applied strategy has more potential, as it allows high-throughput isolation of MOB with a whole range of desired characteristics. For example, when the objective is to obtain highly NH_4^+-N resilient MOB or to evaluate the influence of a certain environmental factor like salt concentration on the community, a straightforward adaptation of the growth medium in the 96 well plates allows to simultaneously isolate and screen the methanotrophic members, all in a miniaturized way.

If the presented isolation technique would be used to evaluate the methanotrophic community of a certain environment, it is important to know that the enrichment step will skew the investigated community structure to a possibly large extent (Vorobev and Dedysh, 2008). Still, the relatively small isolation campaign concerning the MOB in a facultative pond, as described in **Chapter VII,** resulted in positive isolation of type Ia, Ib

and II MOB, indicating that the proposed approach is able to retain part of the present diversity. Although it is not clear if these isolated MOB were active and/or highly abundant in the original ecosystem or rather present in the seed bank, those concerns are only secondary from an applied point of view. Indeed, the proposed approach does not aim to understand the community structure of the environment under investigation, but results in the isolation of MOB with a wanted profile. In this particular case, robust and fast growing MOB could be isolated, able to withstand drastic changes in growth conditions like pH, temperature, O_2, CO_2 and H_2S concentrations. The latter could certainly be beneficial for biotechnological applications, for example as adequate members in *methalgae* consortia. Therefore, these isolates - including strains most probably belonging to a new methanotrophic genus - are currently described and characterized by Sven Hoefman et al. in the Laboratory of Microbiology (LM-UGent).

In addition to the culture dependent characterization, culture independent techniques are powerful tools to gain understanding of the methanotrophic microbiome (McDonald et al., 2008). In **Chapter V**, community structures and shifts were evaluated by analyzing the 16S rRNA gene PCR based DGGE patterns using interpretative parameters, i.e. community organization, dynamics and richness (Read et al., 2011). Although next generation sequencing techniques are currently becoming the state-of-the-art technique, due to their ability for a faster and deeper community characterization, these DGGE-based analyses allow a relatively fast screening of the community and its reaction to changes in the environment. However, to gain knowledge on who is actually present and active in those communities, additional techniques are required. Clone libraries as in **Chapter V** can be useful to get an idea of the most dominant species, while microarray analyses as used in **Chapter IV** allow - at least for MOB – to gain taxonomic information at species level. Future studies will undoubtedly incorporate results from the omics technology platform, based on high throughput screening, as this advanced technology allows an even higher resolution characterization of mixed communities. The focal point of attention will thereby not lie on the information gathering, but rather on adequately dealing with the large datasets that are generated (Hudson, 2008).

When looking not only to 'who is doing what', but also to 'who is doing it with whom', other molecular techniques have proven their value. In **Chapter VI**, the strength of isotope labelling is shown for elucidating interactions between different organisms. Even with basic stable isotope probing (SIP), it was possible to visualize the very quick turnover of labelled methanotrophic metabolites from the MOB to the associated heterotrophic community. In further research, isotope based techniques could also prove their value in unravelling the interactions taking place in *methalgae* consortia. While the exchange of CH_4**,** CO_2 and O_2 between MOB and microalgae is clear, it is likely that also other interactions take place within the *methalgae* microbiome, for example the exchange of vitamins or metabolites.

In **Chapter VI**, other high-throughput technologies like flow cytometry helped to screen potentially interesting interactions with only a limited amount of sample volume and time. On top of these established molecular techniques, novel and promising techniques are currently coming available: Illumina MiSeq, protein-SIP, Raman microscopy, NanoSIMS and novel *fluorescent in situ hybridization* methods are only a few techniques that could allow a better, deeper and faster characterization of the methanotrophic microbiome, thereby expanding our knowledge on MOB significantly.

X.2.2 Expanding the field for applications

The ecology of MOB has been examined repeatedly, revealing the flexible nature of this bacterial group (Ward et al., 2004). Yet, throughout this dissertation it has become clear that both from an ecological as well as a biotechnological point of view, more interesting facts are awaiting discovery. In **Chapters II and V**, confirmation was given that the former believe that sMMO expression is rare for type I MOB indeed needs to be revised, as *Methylomonas* spp., type Ia MOB, were observed that are able to express both sMMO and pMMO. These MOB could have a biotechnological potential, since sMMO expressing MOB are popular candidates as catalysts for biotransformation and bioremediation processes. Moreover, being able to shift between both enzymes could be advantageous. This fact was observed in **Chapter V**, where the ability to express both types of MMO resulted into

a higher resistance against Cu^{2+} fluctuations. Another example of the beneficial MMO switch is the application of MOB for TCE degradation. One could take advantage of the higher growth yield of pMMO during the growth phase and couple this to a second phase where the higher rates of chlorinated compound degradation of sMMO could be exploited (Dalton, 2005, DiSpirito et al., 1992, Yu et al., 2009).

Also the never before reported observation in **Chapter III** that expression of pMMO makes MOB more resilient to salt additions, can be useful for environmental applications. This finding could be of interest when non-extremophilic MOB need to be used in wastewaters and brines with an increased salt content. By dosing a small amount of Cu^{2+}, it could be possible to considerably increase the salt resistance of the methanotrophic community. Either way, an analysis of the Cu^{2+} concentration should become standard when applying MOB, as the influence of MMO expression cannot be underestimated. This was highlighted in **Chapter III**, where an increase of the Cu^{2+} addition led to a significantly higher CH_4 oxidation rate; an effect that has also been observed by other research groups (Begonja and Hrsak, 2001). This correlation between Cu^{2+} concentrations and activity is however not a certitude, as was pointed out in **Chapter V**, where no significant increase of the methanotrophic activity was observed when pMMO instead of sMMO was expressed by the MOB. The presence of a dominant MOB able to switch between sMMO and pMMO in all different treatments could have played a role in this particular case. However, the underlying reason is unclear and tough to unravel without a better characterization and identification of the active MOB members.

Next to more insights in the role of MMO expression, attention in this dissertation went to the influence of NH_4^+/NH_3 on the methanotrophic activity and community structure. This choice was made because CH_4 mitigation often occurs in environments where reduced N compounds are present. From a biotechnological point of view, the most important finding was presented in **Chapter IV**, where a methanotrophic community was observed, resilient to high concentrations of 2000 mg NH_4^+-N L^{-1}. This shows that the application field of MOB can be extended to areas with high NH_4^+-concentrations. In further research,

it would also be interesting to evaluate the resilience towards NH_3. Indeed, biotechnological applications like mitigation of CH_4 emissions from animal husbandry often take place in environments with increased NH_3 concentrations (Melse and Van der Werf, 2005, Petersen et al., 2009). Nonetheless, highly NH_4^+-N resistant methanotrophic communities look promising for CH_4 mitigation in anaerobic environments where NH_4^+ could be an inhibiting factor. In a further step, it would be noteworthy to evaluate the possibilities of coculturing MOB and AOB, which would result in a simultaneous treatment of CH_4 and NH_4^+**/**NH_3. Although preliminary tests have shown that the latter is achievable, this coculturing strategy needs further exploration.

The obtained results of **Chapter IV** also call attention to another important element: a gradual adaptation towards an influencing growth parameter could result into an overall higher resilience. Currently, shock loads of NH_4^+-N and other nutrients are still the common practice. However, in lab tests, these prompt doses skew the observed dose-response relationship, while in the agricultural business, fertilizer shock loads prevent the present community to adapt to the new conditions. When fertilizing soils, it would be useful to apply smaller and steadily increasing doses instead of giving all at once, in order to allow a community shift towards more adapted MOB.

X.2.3 Exploring the potential for eco-engineering

Most enrichments and tests in this dissertation where performed in mineral salts medium, without any addition of organic carbon sources. Combined with a relatively short sludge retention time, this created conditions whereby heterotrophs could only establish themselves in the community by employing methanotrophic metabolites to fulfil their carbon and energy needs. As observed in **Chapters II-VI**, MOB form the core of whole microbial food chains, driven by CH_4 derived carbon. Although such interactions have been observed before (Dumont et al., 2011), the underlying reasons and the impact of these interactions are mostly still to be discovered.

From an eco-engineering point of view, these interactions within the methanotrophic microbiome could lead to bacterial life in pristine environments lacking organic carbon source, on the condition that the other nutritional needs like N and P availability are fulfilled. As such, methanotrophic communities could help to reconvert the vast areas where for example former mining activities have resulted in desolate environments without fertile topsoils. As a result, these environments lack the conditions needed for life of higher organisms like plants and invertebrates. However, regulations in countries like South Africa are becoming stricter and companies are demanded to reclaim these areas with weathered or spilled coal. In such environments, MOB could be used as primary colonizers, creating a flux of energy and carbon to the soil. Indeed, due to the nature of such coal beds, CH_4 continuously seeps out (Adams et al., 2001), thereby providing the MOB and indirectly the associated heterotrophs of a relatively continuous flow of carbon and energy. Moreover, as many MOB have a N_2 fixation ability, even a lack of available NH_4^+/NO_3^--N could be circumvented. MOB also have specific mechanisms to attain trace elements like Cu and Fe (Kenney and Rosenzweig, 2012), while sufficient P and S should be found in the coal layer itself. Therefore, methanotrophic communities could be able to start colonization processes that may form the base for further reconversion steps by grasses, fungi, etc. Until now, no attention has gone to this type of eco-engineering, although this could change due to the attention for biochar, a type of charcoal, created by pyrolysis of biomass. Currently, much scientific efforts are made to investigate if the conversion of organic carbon to inert charcoal could be a useful approach for carbon sequestration while at the same time the soil fertility would be increased (Xu et al., 2012). Results from other research groups indicate that at least in the beginning, biochar results into a C, N and probably also a P flux to soils wherein it has been applied (Nelissen et al., 2012). Moreover, biochar amendments seem to result – although temporarily – in an increased microbial activity. When this biochar would be inoculated with MOB before being applied to nutrient poor soils, it could be possible to sustain an active methanotrophic community, whereby the slow release of C, N and P allow the methanotrophic community to establish and adapt to the conditions. After the start up phase, the MOB could take over the C and N sequestration. Additionally, such an

enhanced bio-augmentation strategy could also help to reclaim the oil sands after fracking, while at the same time the CH_4 emissions of such soils could be lowered.

It is also worth mentioning another pristine environment that could be harboring CH_4-based communities, i.e. the sky. It has become clear that diverse bacterial communities are present in the vast atmosphere (Delort et al., 2010). MOB seem to be members of cloud communities, possibly forming an unexpected sink for atmospheric CH_4 (Temkiv et al., 2012). Methanotrophic activity could be possible due to the fact that next to water, CH_4 and CH_3OH, also N and P compounds are available in clouds. Moreover, MOB are able to go into resting stages, thereby able to rely on intracellular energy and carbon storage compounds like PHB (Roslev and King, 1994). Like that, they have a higher chance to sustain the harsh atmospheric conditions. The importance of this air-born community is far from assessed but although the concentration of MOB in clouds will be relatively low, this unacknowledged CH_4 sink could be meaningful due to the enormous volume of the atmosphere.

X.2.4 Creating a future for metabolic networking

Based on the findings in this dissertation, it is clear that under the tested conditions, the MOB selected for specific heterotrophs. This was most clear in **Chapter V**, where the associated heterotrophic community evolved over 10 sequential cycles to the same composition, although the Cu^{2+} concentration was different for all three treatments. There are strong indications that the selection of the associated bacteria does not occur randomly and therefore this interaction is more than just saprofaghy. The hypothesis of an active interaction is confirmed in **Chapter VI**, where only an active MOB culture was able to support the activity and growth of a methylotrophic *Pischia pastoris* strain, while no growth of the yeast was observed when the MOB was not oxidizing CH_4. The metabolites that allow this flux of carbon and energy from MOB to associated microorganisms are still unidentified. Also the reason why MOB seem to release considerable amounts of precious carbon and energy sources is not elucidated, although the heterotrophic removal of toxic intermediates like formaldehyde could for example be beneficial for the MOB (Hrsak and

Begonja, 1998). Not surprisingly, the identified associated heterotrophic communities in **Chapters II and V** consisted mostly of bacteria metabolizing small organic molecules and/or intermediates of the methane oxidizing pathways. The latter fuels the hypothesis that the methanotrophic-heterotrophic interactions consist mostly out of the 'leaking' of smaller molecules from the MOB to the associated community.

However, the carrying capacity based on crossfeeding with MOB is remarkable. The clone library of **Chapter V** even indicates that there are more heterotrophs than MOB present in the methanotrophic community. Although in depth techniques are currently used to confirm these findings, support is given by the observations of other research groups that MOB are able to supply large amounts of carbon and energy to higher organisms (Petersen and Dubilier, 2009, Sanseverino et al., 2012). Yet, the biotechnological potential of these interactions have not been explored yet. The tests in **Chapter VI** create expectations, as proof of principle was given that active MOB are able to support the growth of heterotrophic organisms, *in casu* a methylotrophic biotechnological model-organism. If these results can be confirmed with other MOB and heterotrophic strains possessing a biotechnological potential, a novel sink could be created for numerous CH_4 containing waste streams, including biogas originating from anaerobic digestion processes of biorefinery downstreams, wastes and sediments (Figure X-2). The implementation of such biorefineries comes closer every day, but results in new waste streams to deal with (Vandamme and Verstraete, in press). One of the open questions is what to do with the large amounts of organic waste that results from the different biorefinery processes. A combination of anaerobic digestion and the here presented biogas-to-biomass transformation could validate these waste streams and result into a secondary stream of valuable biotechnological metabolites. Next to methanotrophic biomass, which could be used as a source for single cell proteins, PHB or ectoine, metabolic networking would allow to create additional value by transforming the methanotrophic metabolites to biotechnological high-value products by engineered organisms like *Pichia pastoris* or *Escherichia coli*.

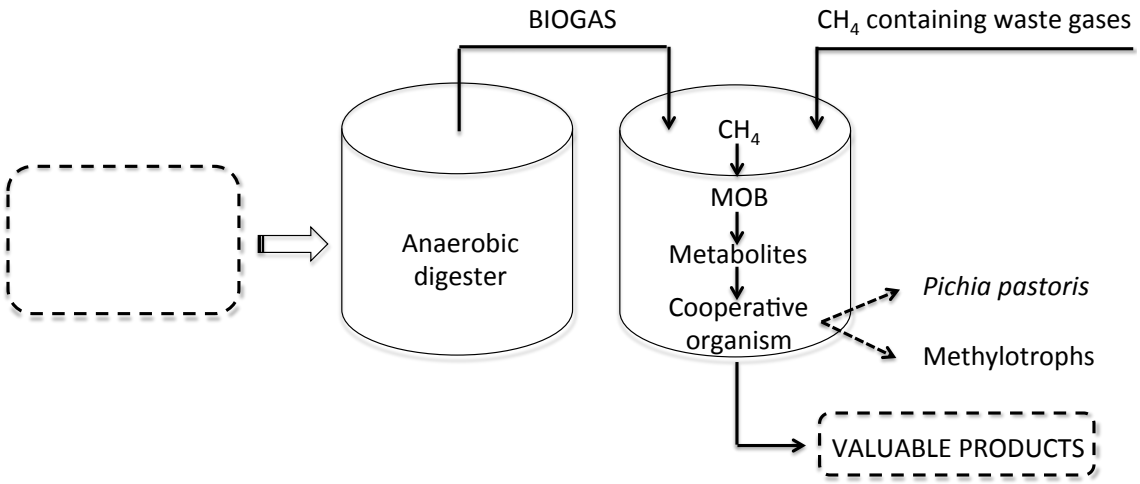


Figure X-2. Conceptual scheme of a potential application of metabolic networking within methanotrophic consortia. The latter would allow to convert CH_4 containing gaseous streams, no matter the origin; both CH_4 containing waste streams and biogas originating from the recycling of biorefinery downstreams, wastes and sediments could be transformed into valuable products.

Still, this metabolic engineering concept with MOB is only in its first stage of development. Further tests are currently taking place in order to evaluate the identity, amount, rate and potential of the transfer of metabolites from MOB to associated microorganisms. Only then, the true potential of this interaction can be judged on its merits.

X.2.5 Revealing the ecological relevance of the *methalgae* interaction

Except for *Candidatus Methylomirabilis oxyfera*, all methane monooxygenase based methanotrophs need free O_2 as final electron acceptor (Ettwig et al., 2012, Hanson and Hanson, 1996). Therefore, applications with MOB are normally restricted to aerobic environments. Yet, CH_4 is microbially produced and therefore mostly present in anoxic environments. As a result, MOB are in natural stagnant water bodies typically dominantly present in the thin transition layer between the anaerobic and aerobic zones (Blumenberg et al., 2007, Schmale et al., 2012). However, in fully anoxic environments, only the small

upper water layer in contact with the atmosphere is aerated. In **Chapter VIII**, where a facultative pond was used as model for such environments, it was indeed observed that on dark days, the whole pond was anoxic with exception of the upper millimetres. Consequently, not all CH_4 can be oxidized before it escapes to the atmosphere. However, algae have the potential to aerate a large zone of the water column. This effect is demonstrated in Figure X-3, whereby diffusion columns are used with a counter diffusion grade of synthetic biogas from the bottom and ambient air from the top, inoculated with an algal *Chlorella* sp. culture. It is clear that algae drastically increase the depth of the zone wherein aerobic bacteria like MOB can be active, thereby increasing the methane oxidizing potential in the water column.

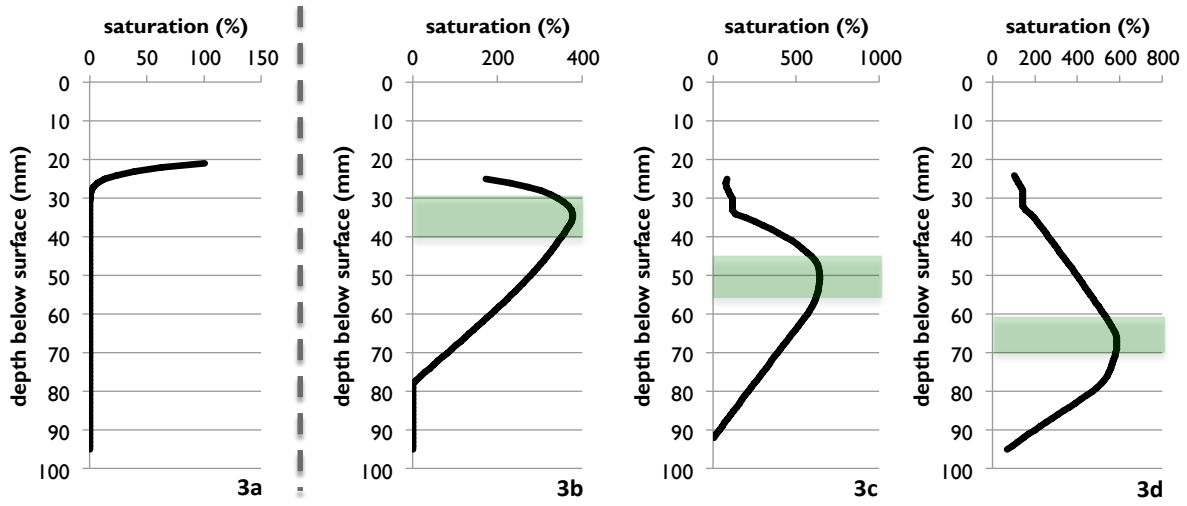


Figure X-3. Depth profiles of diffusion columns with soft agar, inoculated with an algal *Chlorella* sp. culture. Passive diffusion of synthetic biogas occurred from the bottom, while the top of the columns where open to the atmosphere. The most left profile (3a) was obtained from a column that was completely shielded from the light. It can be observed that only the upper zone of 8 mm was aerated. The other columns were shielded from the light, expect for a zone of 10 mm, where algal growth could occur (marked in green). This illuminated zone was present at a depth of 30-40 mm (3b), 45-55 mm (3c) and 60-70 mm (3d), respectively.

This algae-MOB interaction is probably not limited to facultative ponds, as the conditions needed for the *methalgae* interaction to occur are present in many water bodies. Various lakes, rivers, swamps and estuaries, but also engineered water bodies like wastewater treatment ponds, constructed wetlands and organic waste pits have a bottom layer where organic material is degraded under anaerobic conditions, with on top a surface layer wherein photosynthetic micro- or macroalgae are active. If actions can be taken to increase the amount and diversity of algae in such aquatic systems, it would not only lead to increased CO_2 sequestration, but could also indirectly increase the CH_4 oxidation rates.

At the same time, it is important to realize that the *methalgae* interaction is actually a triad, as also heterotrophic interactions occur with both methanotrophs and microalgae. The most important interactions are depicted in Figure X-4, indicating that there are a lot of processes taking place in the *methalgae microbiome*. It also shows the potential of such microbial communities for wastewater treatment purposes, as next to GHG mitigation, the whole community is also removing BOD, N and P.

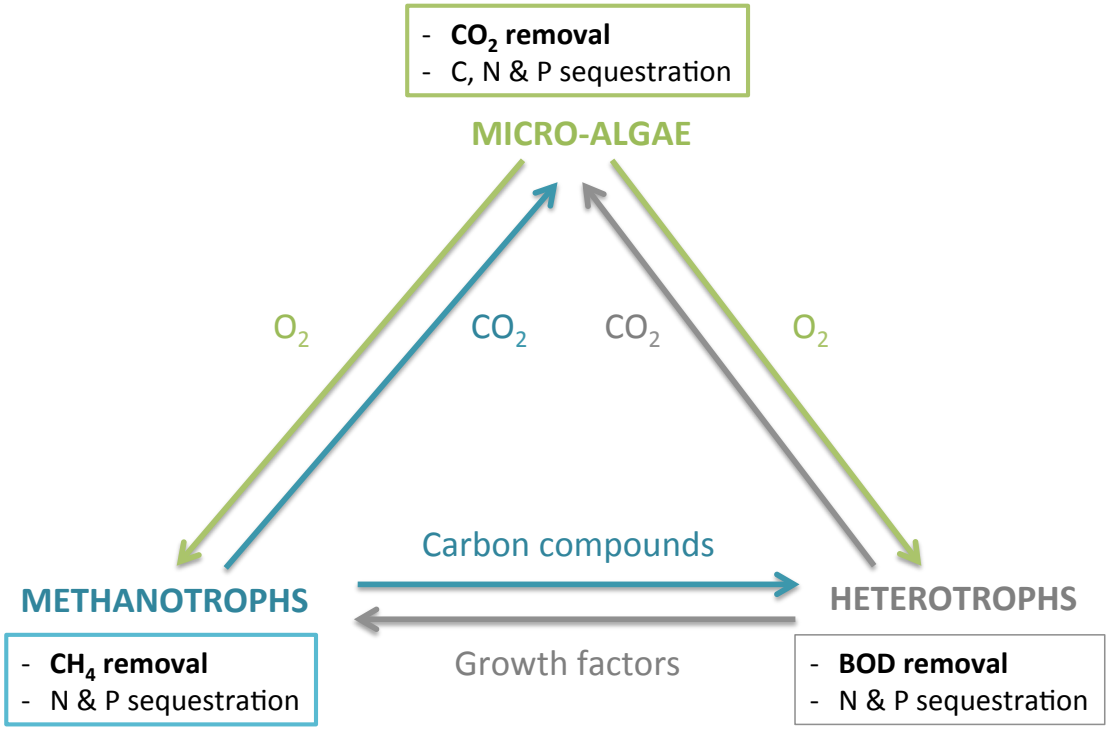


Figure X-4. Schematic overview of the most important interactions between methanotrophic bacteria, microalgae and heterotrophic bacteria, respectively.

X.2.6 *Methalgae* for CH_4 mitigation in anaerobic effluents

Although anaerobic digestion is often perceived as a more sustainable version of the traditional activated sludge processes, there are concerns about the carbon footprint of such installations (Cakir and Stenstrom, 2005, Hartley and Lant, 2006). Due to oversaturation phenomena and the fact that part of the CH_4 and CO_2 dissolves in the continuously outflowing effluent, these installations can become huge GHG emitters; a matter that only recently got attention. Physical treatments based on gasification and extraction are currently rolled out, but will still need to prove their economical and ecological feasibility (Bandara et al., 2011, Matsuura et al., 2010).

As wastewater treatment is one of the most successful microbiotechnological stories, it is no surprise that research groups have looked into the potential of microorganisms to treat dissolved CH_4 in anaerobic effluents. From a microbiotechnological point of view, *Candidatus Methylomirabilis oxyfera* has the most potential, as this species is able to produce its own oxygen in anaerobic conditions. Therefore, it does not need to be supplied of free O_2 like all other known MMO based methanotrophs (Ettwig et al., 2012). However, more knowledge on this peculiar methanotroph is needed before such applications could become possible. Anaerobic archaeal methanotrophs are also prone candidates, but due to their long doubling times and challenging cultivation needs, progress in the applied field is slow. As no anaerobic organisms can be applied (yet), aerobic MOB came into the picture (Hatamoto et al., 2011, Hatamoto et al., 2010). This research group used a down-flow hanging reactor with sponges whereon MOB could attach, resulting in the removal of a large fraction of the dissolved CH_4. However, there was a need for air sparging in order to supply the MOB of necessary O_2, which comes with a cost and can result into stripping of CH_4 and CO_2 to the gas phase. Therefore, an alternative strategy is proposed in this dissertation, whereby O_2 is microbially produced *in situ* in the anaerobic effluent by micro-algal photosynthesis. To our knowledge, the *methalgae* concept as described in **Chapter VII, VIII and IX** is the first microbial technique that allows complete mitigation of both GHG under anaerobic conditions without the need of air/O_2 sparging.

The strength of the *methalgae* approach lies in the fact that CO_2 mitigation and CH_4 oxidation occur simultaneously. It should be stressed that every mitigation strategy by MOB results into production of CO_2, which is also a GHG. Due to the fact that CH_4 is a 25 times stronger GHG than CO_2, policy makers easily minimize the consequences of this transformation of CH_4 into a weaker GHG, CO_2. However, it is often forgotten that the GWP is a weight-based factor; the oxidation of 1 mol CH_4 results in 1 mol CO_2, but the latter is 2.8 times heavier. Therefore, only a factor 9 instead of 25 should be used when evaluating the GHG mitigation potential of MOB, and more general, every CH_4 mitigation strategy that results into CO_2 production. The *methalgae* approach is one of few CH_4 mitigation strategies allowing a true carbon neutral CH_4 removal, as overall, no CO_2 is produced, but instead additional CO_2 is consumed by the algae. Moreover, algae and bacteria are a powerful combination for treatment of anaerobic wastewaters. Indeed, both do not only mitigate the GHG but are at the same time removing N, P and possible hazardous contaminants (Molinuevo-Salces et al., 2010, Munoz and Guieysse, 2006). Therefore, GHG mitigation by *methalgae* can be successfully combined with a tertiary treatment of wastewaters. In **Chapter VIII**, this 'double action' has been demonstrated in an existing WWTP; while the goal of facultative ponds is primarily wastewater treatment, it is clear that the *methalgae* interaction is also responsible for a decrease of the carbon footprint of such installations.

In this dissertation, proof of principle has been given for the application of *methalgae* for treatment of dissolved CH_4 in anaerobic environments. Yet, more efforts are needed to valuate the feasibility for implementation of this approach in full-scale wastewater treatment plants. The most important challenges are the following:

- Wastewaters, especially with an industrial origin, are harsh environments for MOB, algae and microorganisms in general, due to the presence of inhibiting factors. Still, many successes have been booked with algal-bacterial consortia for the treatment of industrial anaerobic effluents (Molinuevo-Salces et al., 2010, Munoz and Guieysse, 2006, Van den Hende et al., 2011). Moreover, tests in this dissertation with *methalgae*

cultures on anaerobic effluents from a domestic WWTP (**Chapter VIII),** a potato factory (**Chapter VII)** and a paper factory (data not shown) did not indicate unconquerable issues.

- As with all algae based reactors, provision of light energy and heat is the most critical factor. Although the cocultures in **Chapters VII-IX** were active under low light intensities and a lot of progress in efficient light provision has been made over the last years (Chen et al., 2011), artificial light energy stays costly. Therefore, optimal use should be made of available 'free' natural light and heat. For example, as effluents from anaerobic digesters are typically warmer than 20°C, no additional energy should be needed for heating.
- Digestate is often turbid. Although the algae that were cultivated in the digestate of a potato factory had no problem capturing light with a low intensity (**Chapter VII)**, mixing will be needed to assure optimal algal activity and low hydraulic retention times.
- The reactor design should optimize the amount of light that can penetrate in the effluent. Therefore, a high surface to volume ratio is needed, which at the same time allows a high enough residence time of the flocs in the illuminated zone. Moreover, the reactor should be covered, to minimize the loss of (oversaturated) CH_4 and CO_2 to the atmosphere. Fortunately, both conditions can easily and at moderate costs be accomplished in installations like the one in Agristo NV, a Belgian potato factory, which has the digestate running out of the digester tank by means of an easily modifiable gutter system (Figure X-5). Concerning the dark night periods, buffer tanks could be useful in order to allow all digestate being treated during the illuminated period.

Figure X-5. A partial view on the wastewater treatment plant of the potato factory Agristo, Harelbeke, Belgium. The digestate originating from the anaerobic digestion process is pumped into a gutter system for further processing. The basin has a depth of less than a meter and the effluent is constantly in motion.

- Reactor design should also allow sufficient retention time for the microorganisms. Although doubling times of different MOB and microalgae vary to a large extent, a doubling time of one day is for both organisms a good guideline. Carrier material could be used to decouple the SRT from the HRT, avoiding washout of the *methalgae* biomass and allowing a decrease of the HRT. Tests in the lab indicated that MOB can easily attach on many conventional carrier materials (data not shown), while the sponge-like type used by Hatamoto (2010) could also be a worthy alternative. However, next to the cost, attention should be given to the shading effect that these carrier materials could generate.
- Lastly, a useful sink will be needed for the produced biomass. For most industrial wastewaters, it will not be possible to convert the biomass to valuable biocompounds due to regulatory rules, as the risk for toxins and hazardous products is high. If allowed by the local legislation, the biomass could however be useful as organic fertilizer. Also

re-digestion could be a solution, although the digestibility of algae is not superb and yields therefore relatively low (Munoz and Guieysse, 2006, Zamalloa et al., 2012a, Zamalloa et al., 2012b). Interestingly, bioflocs originating from wastewaters in food processing factories would be allowed as feed additive. As was mentioned in **Chapter IX**, such *methalgae* flocs could have a nutritional and prebiotic added value. Therefore, such companies would be the logic partners to start up a pilot installation, in order to evaluate the feasibility of the *methalgae* concept.

It is also important to highlight the not yet mentioned benefits of a *methalgae* approach for wastewater treatment:

- The main reason that methanotrophic biofilters have rarely made it to the full-scale level is due to the poor solubility of CH_4 and O_2. In studies with a CSTR and MOB for example, mass transfer coefficients of 19.1 h^{-1} for O_2 and 16.3 h^{-1} for CH_4 were calculated (Yu et al., 2006). Due to the slow mass transfer of these gases, enormous reactor volumes are needed to threat CH_4 containing gas flows. Even for a 1,000 m^3 manure storage tank with high concentrations of CH_4, a biofilter of 20-80 m^3 is required to remove 50% of the CH_4 emitted from the tank (Melse and Van der Werf, 2005). However, when (over-)saturated waters are treated, CH_4 is already dissolved maximally in the water phase, decreasing the effect of slow mass transfer to a large extend. As the intrinsic oxidation rate of MOB is much higher than the observed mass transfer limited rate, a decrease of the HRT can be expected, the latter being a prerequisite for successful implementation.
- When working with CH_4, there is always an explosion hazard. Especially when degassing processes are involved, the risk of calamities cannot be eliminated. However, when treating dissolved CH_4, this risk can be neglected. Therefore, a *methalgae* approach would be one of the safest solutions.
- No air sparging is needed, as the algae produce the oxygen *in situ*. Moreover, no heavy pumps are needed, as some current installations could quite easily be altered into a *methalgae* reactor. Both factors allow to decrease the operational cost.

Conclusively, it will take more extensive tests before *methalgae* will be used for the treatment of dissolved CH_4 and CO_2 in anaerobic effluents. However, there seems to be no reason why the *methalgae* concept should be ruled out beforehand. Nonetheless, it is clear that this concept has the highest change to succeed in low-cost WWTP with year round high amounts of solar energy.

X.2.7 *Methalgae* as driver for valuable biocompound production

In **Chapter IX**, another application for *methalgae* was explored in order to create an alternative sink for biogas. *Methalgae* consortia were used to convert biogas into valuable biomass. This production of valuable biocompounds out of biogas could be an element in the biorefinery concept, whereby the conversion of plant material to biomolecules results in a waste stream containing about 50% of the incoming C (Vandamme and Verstraete, in press). Anaerobic digestion coupled to the presented approach could result in a secondary stream of interesting biocompounds, increasing the overall conversion efficiency of the biorefinery in a sustainable way. However, as biogas has already a value (about 0.2 euro m^{-3} in the EU), conversions are only economically feasible if the end product is worth more than the biogas, augmented with the cost of the conversion. Two approaches were proposed as schematically represented in Figure X-6: a two-stage process which allows maximal optimization of the separate processes, leading to the creation of two separate high value end products and a one-stage approach which exhibits a lower operational cost, and therefore could be interesting for the production of feed components.

While many of the mentioned remarks about *methalgae* implementation are also valid here, some extra arguments should be pointed out:

- Also for this application, optimal use should be made of natural daylight, due to the high cost of artificial light. However, the proposed microbial conversions are perfectly complementary with green electricity production out of the produced biogas. Therefore, an elegant solution is here present to handle the dark hours. At the same time, the produced free low caloric heat could be useful to maintain optimal growth temperatures for the microorganisms.

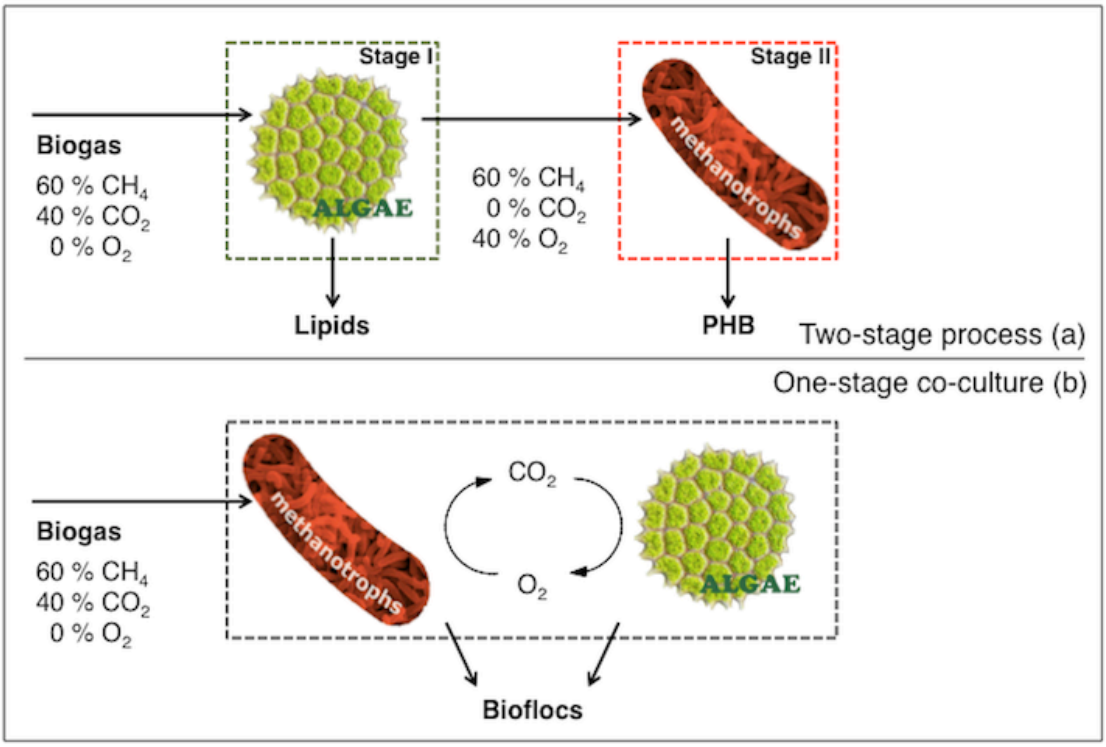


Figure X-6. Overview of the two possible pathways to apply the *methalgae* concept for conversion of biogas to valuable biomass. The two-stage process (a) allows to fine-tune the growth conditions for the algae and MOB separately. In the one stage approach (b), the MOB and microalgae are cultivated together. While the first approach allows the creation of two separate high-value end products, the second approach is more fit for low-cost production of nutritional feed flocs.

- Secondly, as with all methanotrophic bioreactors, reactor design should focus on an optimal gas transfer of the relatively low-soluble CH_4 and O_2, as this is still the Achilles' heel of such biofilters. The membrane biofilm reactor technology might be promising, as it allows to tackle many challenges: the specific design allows to fine-tune the gas flows, optimize the gas transfer and lower the safety hazard as was well described for methanotrophic applications by Hamer (2010) and Modin et al. (2010).
- Floc formation could also affect the mass transfer rate negatively, but that has not hindered the application of bacterial-algal biofloc technology for wastewater and

flue gas treatment (Munoz and Guieysse, 2006, Van den Hende et al., 2010). Moreover, in the current study, algae and bacteria were well dispersed until stress conditions were induced, minimizing a possible decrease in mass transfer due to floc formation.

- Lastly, it should be stressed that this approach allows to aim for the production of biocompounds with high values. Promising biocompounds made by MOB are epoxypropane and ectoine (Reshetnikov et al., 2011), while algal production of astaxanthin and β-carotene is commercially established (Sasso et al., 2012). The most important strength of the proposed approach is the putative flexibility, as all growth conditions can be controlled and therefore optimized. At the same time, the concept allows to create clean biocompounds out of all kinds of gas streams with CH_4 and CO_2.

A quite extra-ordinary environment where *methalgae* could also be of value is space travelling. To allow a long-term manned space mission, closed loop systems are needed, allowing to minimize losses of energy, water and carbon on board of the spaceship. Therefore space agencies have been developing artificial ecosystems whereby all waste is recycled back to food for the crew, by means of physico-chemical techniques, higher plants, algal and bacterial processes. The MELiSSA (Micro-Ecological Life Support System Alternative) project of the European Space Agency has proposed and tested some possible settings (Tikhomirov et al., 2007, Verstraete et al., 2005). Some proposed closed loop systems include anaerobic digestion processes, thereby creating CH_4 (Poughon et al., 2009). However, the hazards of this explosive gas are unacceptable on a spaceship and therefore CH_4 should be vented to outer space, leading to a loss of carbon. As algae will be key-players of such artificial ecosystems, a *methalgae* implementation could be of help to close the carbon balance. By co-cultivating the algae and MOB on the digestate, CH_4–C could be recuperated and converted into food additives, without a risk for calamities.

X.2.8 Conclusions

This dissertation had as central concept the methanotrophic microbiome. The behavior of the methanotrophic community as a whole was evaluated during changes of environmentally relevant parameters like the Cu^{2+}-, NaCl- or NH_4^+-concentration, respectively. Even more imperative was that the interactions within the community were followed up, which allowed gaining knowledge on a community level. Indeed, since environmental biotechnological applications will be based mainly on whole communities rather than axenic cultures, it is important to couple the physiological, ecological and biotechnological capacities and needs. Obtained outcomes like the increased resilience towards salt or NH_4^+-additions have their merits, but need now translation to the applied field. Some efforts were made to exploit the interactions of methanotrophs with the associated heterotrophs and microalgae, respectively. Novel concepts were presented and tested on a lab scale. By means of methanotrophic communities, it was possible to create novel CH_4 sinks, thereby combining CH_4 mitigation in wastewaters and gases with the production of added value products. This value creation is an important benefit, as the exploitation of methane oxidizing reactors comes with a capital and operational cost.

In conclusion, it has become clear that there is still a lot of knowledge about the methanotrophic microbiome waiting for us to discover. While innovative molecular technologies will definitely result into an expansion of the knowledge on the ecology and physiology of MOB and methanotrophic communities, biotechnological obstacles seem harder to tackle. Especially towards the treatment of poorly soluble gases, new ideas and concepts are required. These truly remarkable methanotrophs deserve that attention.

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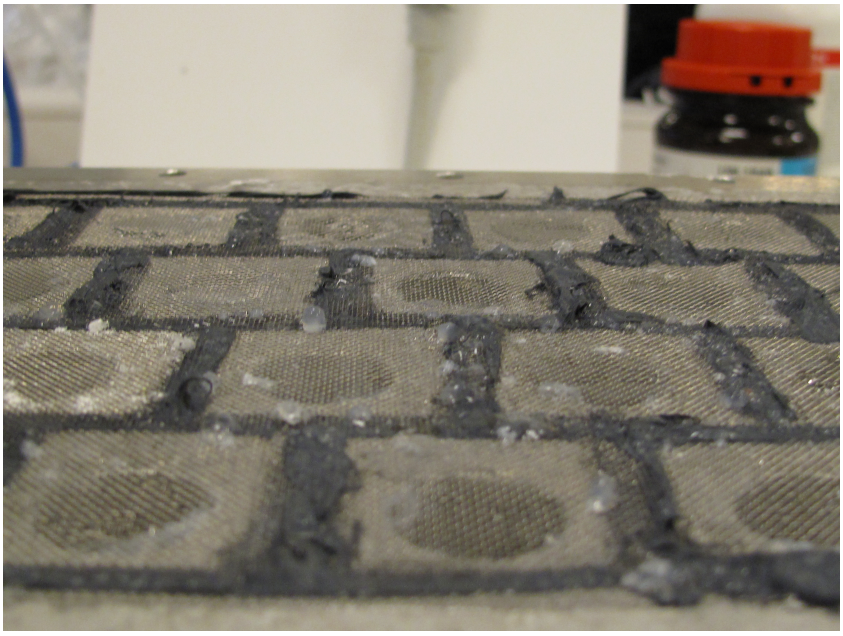

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SUMMARY

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Summary

Since the industrial revolution, the average temperature on Earth has risen considerably, mainly due to the enormous emissions of anthropogenic greenhouse gases. While CO_2 mitigation gets most of the attention, it is important to realize that CH_4, on weight basis a 25 times stronger greenhouse gas than CO_2, is responsible for about 18% of the global warming effect. In order to balance the worldwide CH_4 budget, a 6% decrease of current CH_4 emissions is needed. Physico-chemical technologies exist which enable the mitigation and transformation of CH_4, but these techniques are energy demanding. A microbiological alternative is available, as methanotrophic microorganisms have developed methods to oxidize the stable CH_4 molecule. A subgroup of these methanotrophs consists of aerobic alpha- and gamma-proteobacterial methane oxidizing bacteria (MOB), which are the key players in this dissertation. MOB are able to use CH_4 as their sole carbon and energy source and they form the core of methanotrophic microbiomes, the latter being entire microbial communities directly or indirectly depending on CH_4 for their growth and activity. In this dissertation two factors were examined that are important for utilizing these communities for applications in environmental biotechnology. The first factor concerns external parameters that influence the activity and structure of such communities. The other factor relates to the interactions taking place within the methanotrophic microbiome.

To facilitate this examination, an extinction-based isolation technique was developed in this dissertation to increase the available methanotrophic pool. In a microbiological framework, the chance of a successful application increases considerably when the specific needs to achieve a certain biotechnological purpose can be matched with the characteristics of available microorganisms. However, MOB are notoriously difficult to isolate and preserve and therefore only a small fraction of the methanotrophic diversity has been described so far. The developed miniaturized isolation technique allowed to isolate and screen MOB with desired characteristics for their biotechnological potential. By applying this novel approach, 22 fast-growing MOB were isolated from various

environments, even when traditional plating was unsuccessful. The effectiveness of this isolation technique was also confirmed by isolating a diverse set of MOB from a facultative waste stabilization pond. In that case, strains of all three groups of MOB, i.e. type Ia, Ib and II MOB, were isolated, of which some strains most likely belong to a new genus. In conclusion, miniaturized extinction culturing can be seen as a technique that is efficient in rapidly isolating numerous diverse MOB with desired characteristics, requiring little effort and few materials.

When MOB are applied for environmental biotechnological purposes, it is important to know how external growth parameters influence methanotrophic microbiomes. A potential niche for methanotrophic microbiomes is the mitigation of dissolved CH_4 in effluents from anaerobic digesters. These anaerobic effluents are an underestimated source of greenhouse gases, as they are often saturated with CH_4 and CO_2. Semi-continuously fed stirred reactors were used as model systems to evaluate the influence of key parameters on the activity of methanotrophic communities. By increasing the Cu^{2+} concentration, the expression of particulate methane monooxygenase (pMMO) was enhanced, which led to an increase of the methane oxidation rate by a factor of 1.5. Moreover, communities with pMMO expressing MOB exhibited also a higher resilience against NaCl. Additionally, the influence of increased loads of NH_4^+-N on the activity and composition of a methanotrophic community was assessed. In contrast to a sudden increase, MOB showed remarkable resilience to gradually increasing ammonium loads. For the first time, substantial methane oxidation activity was observed in a methanotrophic community exposed to ammonium concentrations of up to 2500 mg NH_4^+-N L^{-1}. All these obtained results demonstrate that the treatment of CH_4 saturated effluents, even those with increased NH_4^+-N and salt levels, can be mitigated by implementation of methanotrophic communities.

It is also important not to lose sight of the non-methanotrophic members present in the community when dealing with methanotrophic microbiomes. In all the evaluated methanotrophic communities, heterotrophs formed an important fraction, although no

organic carbon sources were added. This shows that there exist meaningful interactions between the MOB and the associated heterotrophs. Furthermore, when a methanotrophic community was exposed for 30 days to three different copper concentrations, i.e. 0.1, 1.0 and 10 µM Cu^{2+} respectively, the associated heterotrophic communities showed continuous shifts, but over time all subcultures evolved to a comparable composition, independent of the copper concentration. This indicates that the MOB selected for certain heterotrophs, possibly fulfilling vital processes like removal of potentially inhibitory intermediary compounds.

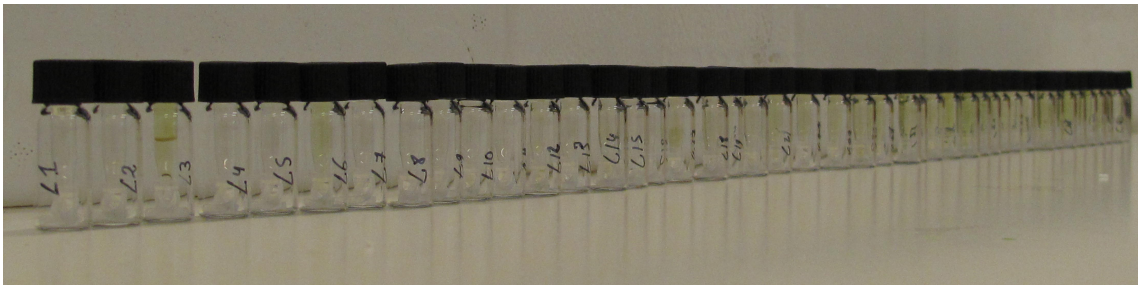
At the same time, carbon fluxes occur between MOB and associated microorganisms. When cultivating axenic MOB strains with CH_4 as sole C source, about 25% of the organic matter was present in the culturing media. Moreover, stable isotope probing showed that after 96h, up to 100% of bands observed in 16S rRNA gene DGGE profiles of three different methanotrophic communities were also present in the ^{13}C gradient fractions. These results provided evidence that the associated community rapidly incorporated ^{13}C-compounds originating from CH_4 oxidation processes by MOB. In an additional test, this metabolic networking allowed a methylotrophic yeast to grow indirectly on CH_4 in the presence of an active MOB culture. These results prove that metabolic networking takes place within the methanotrophic microbiome, creating CH_4 derived food webs with possible industrial potential.

Lastly, the symbiotic interaction between MOB and autotrophic microalgae, described as the *methalgae* concept, was developed. It was observed that MOB and algae can be cocultured with CH_4 as sole carbon source. While algae metabolize the methanotrophic end product, CO_2, they produce *in situ* O_2, which is subsequently used by the MOB to oxidize CH_4. By means of this cooperation, both CO_2 and CH_4 are treated simultaneously, while at the same time applications with MOB are no longer restricted to aerobic environments. Moreover, *methalgae* cultures adapted easily to, and grew readily on, an industrial digestate. Even under anoxic conditions, considerable methanotrophic activity was observed, due to the fact that MOB could rely on *in situ* O_2 production by algal

photosynthetic processes. Proof was obtained that this *methalgae* interaction occurs in a facultative waste stabilization pond, a key unit of many low-cost wastewater treatment plants. A pilot pond was used to evaluate the fate of CH_4 when it reaches the oxygenated pond surface layer, after being produced at the bottom of the pond. On site emission analyses showed that such facultative ponds are substantial CH_4 sources. Yet, part of the produced CH_4 is consumed by MOB before it can reach the atmosphere. At least 10^4 cultivable MOB mL^{-1} were observed in the upper layer of the pond, while enrichments showed a high methane oxidizing activity of 190±5 mg CH_4 L^{-1}_{liquid} d^{-1}. As algae were needed to supply the MOB with O_2, they have an essential supportive role in the CH_4 oxidation and could therefore influence the carbon footprint of such ponds to a substantial extent. This *methalgae* interaction could take place in other water bodies too, and therefore deserves further attention. Finally, a biotechnological application for *methalgae* communities was explored. While biogas produced by anaerobic digestion is typically converted into electricity, a new sink was created by transforming biogas into valuable bioproducts. As proof of principle, the production of feed additives, i.e. lipids and poly-hydroxy-butyrate was evaluated. In a first stage, the CO_2 in a synthetic biogas was photosynthetically fixed by an algal *Scenedesmus* sp. culture at an average rate of 192±9 mg CO_2 L^{-1} liquid d^{-1}, resulting in concomitant O_2 production. After an N depleted growth phase, more than 30% of the 220±7 mg lipids g^{-1} total organic carbon were unsaturated. In a second stage, the theoretical resulting gas mixture of 60% CH_4 and 40% O_2 was treated by a methane oxidizing *Methylocystis parvus* culture, with methane oxidation rates up to 603±9 mg CH_4 L^{-1} liquid d^{-1}. By repeated N limitation, concentrations of 295±50 mg intracellular poly-hydroxy-butyrate g^{-1} cell dry weight were achieved. Finally, a one-stage approach with controlled coculturing of both microbial groups resulted in harvestable bioflocs. This is the first time that a total microbial conversion of both greenhouse gases into biomass was achieved without external O_2 provision. Based on these results, a biotechnological approach is discussed whereby all kinds of biogas can be transformed into valuable bioproducts.

The current study has increased the knowledge on the behavior of methanotrophic microbiomes. While innovative molecular technologies will definitely result into an expansion of the knowledge on the ecology and physiology of MOB and methanotrophic communities in general, biotechnological obstacles seem harder to tackle. An effort was made to approach methanotrophic applications from a non-traditional angle, thereby creating novel CH_4 sinks, relying on the interactions between MOB and associated microorganisms. While these concepts were positively evaluated on a lab scale, future research should focus on a better understanding of the potential of these approaches. This should allow further evaluation of the feasibility to apply methanotrophic microbiomes as drivers for environmental biotechnology.

SAMENVATTING



Extracts in vials for HPLC analysis, LabMET

Samenvatting

Sinds de start van de industriële revolutie is de gemiddelde temperatuur op aarde significant gestegen, voornamelijk door de enorme toename in antropogene uitstoot van broeikasgassen. Terecht wordt veel aandacht besteed aan het verminderen van koolstofdioxide (CO_2) emissies. Hierbij mogen de methaan (CH_4) emissies echter niet uit het oog verloren worden. Dit gas is immers op gewichtsbasis een 25 maal sterker broeikasgas dan CO_2 en verantwoordelijk voor ongeveer 18% van de huidige wereldwijde opwarming. Een verlaging van de huidige CH_4 emissies met minstens 6% is noodzakelijk, teneinde de globale CH_4 balans terug in evenwicht te krijgen. Er bestaan fysico-chemische technologieën om CH_4 te behandelen, maar deze vergen een grote hoeveelheid energie. Een microbieel alternatief is voorhanden in de vorm van methanotrofe micro-organismen, welke in staat zijn de stabiele CH_4-molecule te oxideren. Een methanotrofe subgroep bestaat uit de aerobe alfa- en gamma-proteobacteriële Methaan Oxiderende Bacteriën (MOB), welke het voornaamste onderwerp vormen van deze verhandeling. Deze MOB zijn in staat CH_4 als enige koolstof- en energiebron aan te wenden. Zij vormen de kern van methanotrofe microbiomen, i.e. gehele microbiële gemeenschappen die voor groei en activiteit direct of indirect afhangen van CH_4. In deze verhandeling werden twee belangrijke aspecten onderzocht die belangrijk zijn met het oog op milieubiotechnologische toepassingen. Het eerste aspect betreft de invloed van externe parameters op de activiteit en structuur van methanotrofe gemeenschappen. Het tweede aspect betreft de interacties welke plaats vinden tussen de verschillende leden van zulke methanotrofe microbiomen.

Om deze onderzoeksvragen eenvoudiger te kunnen beantwoorden, werd in deze verhandeling vooreerst een extinctie-gebaseerde isolatietechniek ontwikkeld die toeliet de beschikbare methanotrofe diversiteit te vergroten. Binnen een microbieel kader verhoogt immers de kans op succesvolle applicaties aanzienlijk wanneer de specifieke noden voor een biotechnologische toepassing kunnen gelinkt worden met de karakteristieken van beschikbare micro-organismen. MOB zijn echter berucht voor hun

moeilijke isolatie en bewaring, wat ertoe geleid heeft dat de methanotrofe diversiteit slechts beperkt beschreven is. De ontwikkelde geminiaturiseerde techniek liet toe MOB met gewenste eigenschappen te isoleren en te screenen op biotechnologisch potentieel. Met behulp van deze nieuwe aanpak werden 22 snel groeiende MOB succesvol geïsoleerd uit verschillende omgevingen, hoewel simultane traditionele uitplatingen geen enkele isolaat opleverden. De toepasbaarheid werd eveneens bevestigd door de geslaagde isolatie van een diverse groep MOB uit een facultatieve lagune. Hierbij werden MOB species geïsoleerd behorende tot de drie beschreven types, i.e. type Ia, Ib en II, waarbij enkele stammen hoogstwaarschijnlijk behoren tot een nieuw genus. Hieruit kan besloten worden dat geminiaturiseerde extinctie-gebaseerde cultivatie een efficiënte en effectieve techniek is die een snelle isolatie toelaat van diverse MOB met gewenste karakteristieken.

Met het oog op milieubiotechnologische toepassingen, is het belangrijk de invloed van externe parameters op de methanotrofe microbiomen in kaart te brengen. Een potentiele niche voor methanotrofe microbiomen is de behandeling van opgelost CH_4 in effluenten van anaerobe vergisters. Deze anaerobe effluenten vormen een onderschatte bron van broeikasgassen aangezien ze vaak (over)verzadigd zijn met CH_4 en CO_2. Semi-continu gevoede reactoren werden gebruikt als modelsystemen om zodoende de invloed te evalueren van beïnvloedende groeifactoren op de activiteit en samenstelling van methanotrofe gemeenschappen. Zo leidde een verhoging van de Cu^{2+}-concentratie tot de expressie van particulair methaan monooxygenase (pMMO), hetgeen resulteerde in een verhoging van de CH_4 oxidatiesnelheid met een factor 1,5. Bovendien vertoonden deze gemeenschappen een verhoogde resistentie ten opzichte van zouttoedieningen. Daarnaast werd de invloed van verhoogde NH_4^+-N concentraties op de activiteit en samenstelling van methanotrofe gemeenschappen onderzocht. Methanotrofe gemeenschappen bleken een opmerkelijke resistentie te vertonen ten opzichte van een graduele NH_4^+-N verhoging. Dit was in tegenstelling met een plotse concentratieverhoging, waarbij een significant lagere methanotrofe activiteit werd waargenomen. Voor het eerst werd substantiële methaan oxidatie activiteit vastgesteld bij concentraties tot 2500 mg NH_4^+-N L^{-1}. Het geheel van deze resultaten toont aan dat methanotrofe gemeenschappen

kunnen worden ingezet voor de behandeling van CH_4 verzadigde effluenten, zelfs wanneer verhoogde zout of NH_4^+-N concentraties aanwezig zijn.

Hoewel MOB (vaak) de kern vormen van methanotrofe microbiomen, dient voldoende aandacht te worden besteed aan de aanwezige niet-methanotrofe leden. In alle geobserveerde methanotrofe gemeenschappen vormden heterotrofe bacteriën immers een belangrijke fractie, hoewel geen organische koolstofbronnen werden toegediend. Dit toont aan dat er een betekenisvolle interactie plaatsvindt tussen MOB en de geassocieerde heterotrofen. Bij testen waarbij een methanotrofe gemeenschap voor 30 dagen werd blootgesteld aan drie verschillende koperconcentraties, i.e. 0.1, 1.0 en 10 µM Cu^{2+}, bleek de geassocieerde heterotrofe gemeenschappen continu verschuivingen te vertonen. Met de tijd bleken alle gevormde gemeenschappen echter naar een vergelijkbare samenstelling te evolueren, onafhankelijk van de koperconcentratie. Dit geeft aan dat MOB specifieke heterotrofen uitselecteren. Deze laatsten voeren mogelijk vitale processen uit, zoals de verwijdering van inhiberende intermediaire metabolieten.

Terzelfdertijd vindt er tussen de MOB en de geassocieerde micro-organismen een uitwisseling plaats van koolstof. Wanneer pure MOB culturen werden opgekweekt met CH_4 als enige koolstofbron, bevond 25% van het organisch materiaal zich in het groeimedium. Daarenboven kon met behulp van *stable isotope probing* technieken worden aangetoond dat na 96h tot 100% van de banden welke werden geobserveerd in DGGE profielen (op basis van het 16S rRNA gen) ook aanwezig waren in de ^{13}C gelabelde gradiënt fracties. Deze resultaten tonen aan dat de geassocieerde gemeenschap snel ^{13}C-metabolieten incorporeert, afkomstig van CH_4 oxidatie processen door MOB. Dit metabolisch netwerken liet eveneens toe een methylotrofe gist indirect op CH_4 te cultiveren in aanwezigheid van een actieve MOB cultuur. Hieruit valt te concluderen dat het metabolisch netwerken binnen methanotrofe microbiomen tot CH_4 afhankelijke voedselketens leidt met mogelijk een industrieel potentieel.

In de laatste hoofdstukken werd het *methalgen* concept ontwikkelt, i.e. de symbiotische interactie tussen MOB en autotrofe micro-algen. In eerste instantie werd aangetoond dat

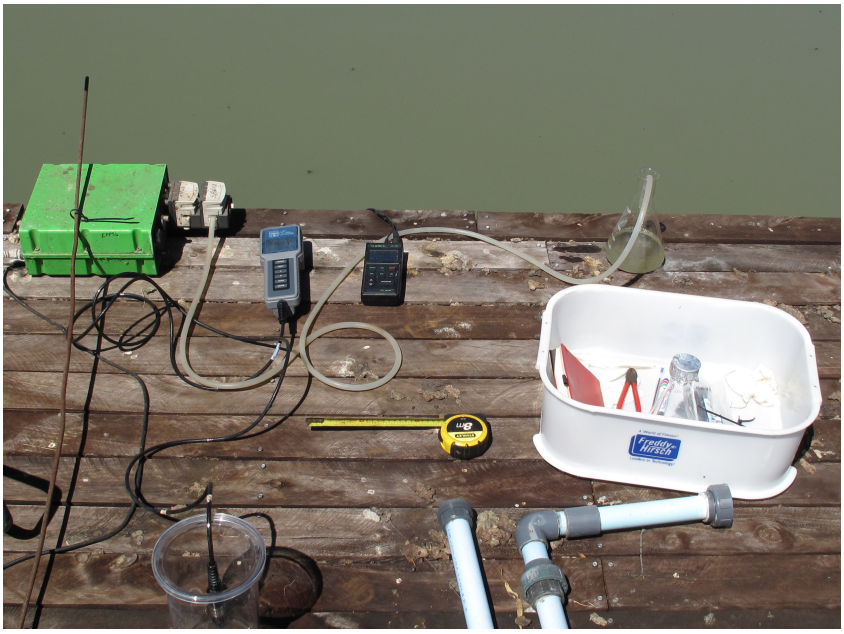
MOB en algen samen gecultiveerd kunnen worden met CH_4 als enige koolstofbron. Algen incorporeren hierbij het methanotrofe eindproduct CO_2, waarbij terzelfdertijd via fotosynthese O_2 wordt geproduceerd. Dit laatste wordt vervolgens door de MOB gebruikt als finale elektron acceptor voor hun methanotrofe activiteit. Deze interactie leidt ertoe dat CO_2 en CH_4 simultaan worden behandeld. Bovendien laat het toe om MOB in te zetten in anoxische omgevingen. Daarenboven bleken *methalgen* gemeenschappen zich te kunnen handhaven in industrieel anaeroob digestaat. Zelf onder anoxische omstandigheden werd een beduidende methanotrofe activiteit vastgesteld doordat de MOB konden rekenen op *in situ* O_2 voorziening door de aanwezige micro-algen. Deze *methalgen* interactie werd eveneens vastgesteld in een facultatieve lagune, een belangrijke eenheid van vele rudimentaire afvalwaterzuiveringsinstallaties. Op basis van een kleine lagune werd geëvalueerd wat het lot is van CH_4, gevormd in de diepere lagen, wanneer het migreert naar de beluchte oppervlaktelaag. Emissiemetingen toonden aan dat zulke lagunes substantiële CH_4 bronnen zijn. Een gedeelte van het geproduceerde CH_4 werd echter door MOB geconsumeerd voordat het werd vrijgesteld in de atmosfeer. Minstens 10^4 cultiveerbare MOB mL^{-1} waren aanwezig in de aerobe oppervlaktelaag (50 cm) van de lagune. Bovendien vertoonden aanrijkingen een hoge methaan oxidatie activiteit die opliep tot 190±5 mg geoxideerd CH_4 L^{-1}_{liquid} d^{-1}. Algen hebben in deze omgevingen een essentiële ondersteunende rol ten opzichte van de CH_4 oxidatie aangezien zij de MOB voorzien van noodzakelijk O_2. Daardoor verkleint de *methalgen* interactie vrij zeker de *carbon footprint* van zulke installaties. Deze wisselwerking tussen MOB en algen verdient daarom verdere aandacht, zeker ook omdat deze interactie eveneens in menig andere aquatische omgevingen kan plaatsvinden.

Tot slot werd een biotechnologische toepassing van *methalgen* gemeenschappen onder de loep genomen. Biogas (vaak een samenstelling van ongeveer 60% CH_4 en 40% CO_2), afkomstig van anaerobe vergisters wordt typisch omgezet naar elektriciteit. Als alternatief werd een nieuwe *sink* onderzocht, waarbij het biogas microbieel wordt getransformeerd naar waardevolle biocomponenten. De productie van voederadditieven, i.e. lipiden en poly-hydroxy-butyraat (PHB), werd conceptueel geëvalueerd. In een eerste compartiment

werd het CO_2 uit een synthetisch biogas fotosynthetisch gefixeerd door een *Scenedesmus* sp. algencultuur aan een gemiddelde snelheid van 190±5 mg CH_4 $L^{-1}_{vloeistof}$ d^{-1}, wat resulteerde in stijgende O_2 concentraties. Na een N gelimiteerde groeifase bleek bovendien meer dan 30% van de 220±7 mg lipiden g^{-1} totale organische koolstof onverzadigd. In een tweede compartiment werd het theoretisch resulterende gasmengsel, bestaande uit 60% CH_4 en 40% O_2**,** behandeld door een methanotrofe *Methylocystis parvus* cultuur, welke resulteerde in CH_4 oxidatiesnelheden met waarden tot 603±9 mg CH_4 $L^{-1}_{vloeistof}$ d^{-1}. Door herhaalde N gelimiteerde groei werden bovendien concentraties van 295±50 mg intracellulaire PHB g^{-1} cel droog gewicht behaald. Parallel werd een aanpak geëvalueerd waarbij de MOB en de algen samen in eenzelfde compartiment werden gecultiveerd, hetgeen resulteerde in oogstbare biovlokken. Voor het eerst werd een totale microbiële omzetting bewerkstelligd van beide broeikasgassen naar biomassa zonder externe zuurstofvoorziening. Op basis van deze resultaten werd een biotechnologisch concept voorgesteld dat toelaat allerhande biogassen te transformeren in waardevolle biocomponenten.

De voorliggende studie heeft geleid tot een kennisuitbreiding wat betreft methanotrofe microbiomen. Innovatieve moleculaire technologieën zullen zonder twijfel leiden tot een verdere expansie van de kennis rond de ecologie en fysiologie van MOB en - meer algemeen - methanotrofe gemeenschappen. Daartegenover staan de noodzakelijke evoluties van technologisch aspecten, welke momenteel een grotere uitdaging vormen. In deze verhandeling werd getracht om methanotrofe toepassingen vanuit een nieuwe invalshoek te benaderen. Hierbij werden nieuwe CH_4 *sinks* voorgesteld, waarbij werd uitgegaan van het potentieel dat de interacties tussen MOB en geassocieerde micro-organismen bezitten. Hoewel deze concepten positief werden geëvalueerd in labo-omstandigheden, dient verder onderzoek zich te richten op een beter begrip van deze processen op grotere schaal, teneinde het ware potentieel van de voorgesteld concepten te bepalen. Dit moet toelaten de toepasbaarheid van methanotrofe microbiomen als drijvende kracht voor milieubiotechnologische toepassingen beter in te schatten.

CURRICULUM VITAE



Work in progress, EBRU, Grahamstown

Curriculum Vitae

Personal information

Name: David J. M. van der Ha
Date of birth: May 16, 1983
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Education

2007-2012 Doctoral School of (Bioscience) Engineering

2004-2007 Master in Bioscience Engineering, option environmental technology, Faculty of Bioscience Engineering, Ghent University
Master thesis: New forms of added value for the microbial fuel cell technology
Promotor: Prof. dr. ir. Willy Verstraete

2001-2004 Bachelor in Bioscience Engineering, Faculty of Bioscience Engineering, Ghent University

1995-2001 High school, option Science and Mathematics, St. Lodewijkscollege, Lokeren, Belgium

Professional activities

2009-2012 **PhD in Applied Biological Sciences** (personal IWT grant SB-83259), Faculty of Bioscience Engineering, Ghent University
Methanotrophic microbiomes as drivers for environmental biotechnology
Promotors: Prof. dr. ir. Nico Boon and Prof. em. dr. ir. Willy Verstraete

2007-2012 **Scientific collaborator at the Laboratory of Microbial Ecology and Technology**
- Tutor of 9 master thesis students, 2 bachelor projects and 1 internship
- Teaching activities:
    - Practical exercises “Molecular-microbial techniques”
    - Calculations “Environmental Technology: biotech processes”
    - Calculations “Microbial re-use technology”
    - Calculations “Introduction to environmental microbiology”
- Reviewer for Industrial & Engineering Chemistry Research

2007-2008 **Scientific collaborator with Dehousse scholarship** on the Industrial Research Funds project (IOF-Vlaanderen) ‘Valorization of the anti-microbial potential of Microsil, nanosilver produced with a probiotic matrix’

Professional training

Workshops and summer schools

2012 **Ghent Bio-based Economy summer school** - 07/08-10/08/2012, Ghent, Belgium – organized by Ghent University and the 'Biotechnology for a sustainable economy' consortium

2011 **Summer course in Environmental Biotechnology** - 10/06-23/06/2011, Lausanne, Switzerland – organized by the US-EU Working Group on Environmental Biotechnology, the University of Lausanne and the BACSIN consortium

2011 **Workshop for Young Water Professionals at the IWA Leading-Edge Conference** - 06/06/2011, Amsterdam, The Netherlands – organized by the International Water Association (IWA)

2011 **Workshop 'Algae Harvesting and Processing for Value Added Applications' -** 26/05-27/05/2011, Brussels, Belgium – organized by the Algae Technology Platform

Specialized courses and skills

2012 **Seminar in transferable skills 'Popular Scientific Writing'** – 18/06-20/06/2012, Ghent, Belgium – organized by the Doctoral School of Bioscience Engineering

2010 **Seminar in transferable skills 'Academic Posters'** – 03/11-11/11/2010, Ghent, Belgium – organized by the Doctoral School of Bioscience Engineering

2010 **Specialization course 'Basic course Gas Chromatography'** – 29/04/2010, Ghent, Belgium – organized by Interscience Netherlands

2010 **Specialization course 'Environmental Impact of Global Change'**, Ghent, Belgium – organized by the Doctoral School of Bioscience Engineering

2010 **Specialization course 'Sociomicrobiology'** – 12/10/2009 - 28/02/2010, Ghent, Belgium – organized by the Doctoral School of Bioscience Engineering

2010 **Seminar in transferable skills 'Teaching skills for assistants'** – 23/02-04/03/2010, Ghent, Belgium – organized by the Doctoral School of Bioscience Engineering

2009 **Seminar in transferable skills 'Research Methodology'**, Ghent, Belgium – organized by the Doctoral School of Bioscience Engineering

2008 **Specialization course 'Molecular-Microbial Techniques'**, Ghent, Belgium – organized by the Doctoral School of Bioscience Engineering

Scientific awards

First poster price at the 1th international symposium on MRM with "Sustainable carbon neutral methane oxidation in anaerobic effluents through a partnership of methanotrophs and microalgae". Ghent, Belgium, June 30th – July 1th 2011.

EOS-award for most creative research presentation at the 16th PhD Symposium on Applied Biological Sciences with "Methalgae: the road to applications". Ghent, Belgium, December 20th 2010.

Services and collaborations

International study leaves

2012 Research on the modeling of interactions between algae and methane oxidizing bacteria, in cooperation with the Department of Environmental Engineering, Technical University of Denmark (DTU), Lyngby, Denmark (08-09/2012)

2012 On-site research on co-cultures of algae and bacteria on wastewater treatment plants, in cooperation with the Institute for Environmental Biotechnology (EBRU), Rhodes University, Grahamstown, South Africa (01-04/2012)

Collaboration with industry

2011 Research on microbial assisted removal of methane in anaerobic wastewater treatment plants, in cooperation with Agristo NV, Harelbeke, Belgium

2008 Joint research on the influence of electrolysis on bacterial contaminants in drinking water, in cooperation with Ecodis NV, Schoten, Belgium

2007 Joint research on the valorization of the anti-microbial potential of Microsil, nanosilver produced with a probiotic matrix resulting in two patents, in cooperation with Avecom NV, Wondelgem, Belgium

Internal services

2010-2012 Representative of the scientific personnel, Faculty Bioscience Engineering, Ghent University in the 'Doctoral Committee' and 'Faculty Board'

2011 Chairman of the 'Board of Scientific Personnel', Faculty Bioscience Engineering, Ghent University

2009-2012 Representative of the scientific personnel in the 'Doctoral School Committee for Life Sciences', Ghent University

Scientific contributions

International peer reviewed articles

CLAUWAERT P., **VAN DER HA D.**, BOON N., VERBEKEN K., VERHAEGE M., RABAEY K., VERSTRAETE W. (2007). Open air biocathode enables effective electricity generation with microbial fuel cells. *Environmental Science and Technology*, 41(21), 7564-7569.

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WANG Y., CLAEYS L., **VAN DER HA D.**, VERSTRAETE W., BOON N. (2010). Effects of chemically and electrochemically dosed chlorine on *Escherichia coli* and *Legionella beliardensis* assessed by flow cytometry. *Applied Microbiology and Biotechnology,* 87(1), 331-341.

GUIMARÃES B. C. M., ARENDS J. B. A., **VAN DER HA D.**, VAN DE WIELE T., BOON N., VERSTRAETE W. (2010). Microbial services and their management: recent progresses in soil remediation technology. *Applied Soil Ecology*, 46(2), 157-167.

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VAN DER HA D., BUNDERVOET B., VERSTRAETE W., BOON N. (2011). A sustainable, carbon neutral methane oxidation by a partnership of methane oxidizing communities and microalgae. *Water Research,* 45, 2845-2854.

SINTUBIN L., AWOKE A.A., WANG Y., **VAN DER HA D.**, VERSTRAETE W. (2011) Enhanced disinfection efficiencies of solar irradiation by biogenic silver. *Annals of Microbiology*, 62 (1), 187-191.

HARTER T., BOSSIER P., VERRETH J., BODÉ S., **VAN DER HA D.**, DEBEER A., BOON N., BOECKX P., VYVERMAN W., NEVEJAN N. (2012). Carbon and nitrogen mass balance during flue gas treatment with *Dunaliella salina* cultures. *Journal of Applied Phycology*, doi:10.1007/x10811-012-9870-9.

HOEFMAN S.*, **VAN DER HA D.***, DE VOS P., BOON N., HEYLEN K. (2012). A two-step liquid isolation procedure allowing high-throughput isolation of novel methane oxidizing bacteria. *Microbial Biotechnology,* 5 (3), 368-378. (* co-first authorship)

VAN DER HA D.*, VANWONTERGHEM I.*, HOEFMAN S., DE VOS P., BOON N. (2012). Selection of associated heterotrophs by methane-oxidizing bacteria at different copper concentrations. *Antonie Van Leeuwenhoek Journal of Microbiology*, doi:10.1007/s10482-012-9835-7. (* co-first authorship)

VAN DER HA D., NACHTERGAELE L., KERCKHOF F.M., RAMEIYANTI D., BOSSIER P., VERSTRAETE W., BOON N. (2012). Conversion of biogas to bioproducts by algae and methane oxidizing bacteria. *Environmental Science and Technology*, 46, 13425-13431.

VAN DER HA D., HOEFMAN S., ISAACS M.L., DE VOS P., COWAN A.K., BOON N. Facultative ponds harbor methane-oxidizing communities supported by algal oxygen production. *Applied Microbiology and Biotechnology* (submitted)

VAN DER HA D., GORTEMAN D., HO A., HOEFMAN S., SCHNEIDER B., DE VOS P., BOON N. Selection and characterization of a methane oxidizing consortium resistant to high ammonium loads. *Environmental Microbiology Reports* (submitted)

VANWONTERGHEM I.*, **VAN DER HA D.***, KERKHOF F.M., HOEFMAN S., DE VOS P. AND BOON N. Enrichment of a methane-driven heterotrophic community in mineral salts media. (in preparation) (* co-first authorship)

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HOEFMAN S., **VAN DER HA D.**, BOON N., VANDAMME P., DE VOS P., HEYLEN K. Miniaturized medium screening enables rapid growth optimization of methane-oxidizing bacteria within the genus *Methylomonas.* (in preparation)

National peer-reviewed articles

CLAUWAERT P., DESLOOVER J., **VAN DER HA D**., VERBEKEN K., BOON N., VERSTRAETE W. (2009). Bio-elektrochemische systemen: microbiële high-tech op zoek naar toepassingen. *Afvalwetenschappen*, 8, 1569-2361.

VAN DER HA D. (2011). Microteam against methane. *EOS Magazine*, Belgium

National and International conferences

VAN DER HA D., NACHTERGAELE L., KERCKHOF F., GANENDRA G., VERSTRAETE W., BOON N. Fourteenth International Symposium on Microbial Ecology (ISME). A cooperation of algae and methane oxidizing bacteria allows the creation of added value products from biogas. Copenhagen, Denmark, 19-24th August 2012. *Contributed presentation.*

VAN DER HA D., GORTEMAN D., HO A., BOON N. Fourteenth International Symposium on Microbial Ecology (ISME). Methane oxidizing bacteria are adaptable to increasing ammonium load. Copenhagen, Denmark, 19-24th August 2012. *Poster presentation.*

VAN DER HA D., LEYRE G., BUNDERVOET B., VERSTRAETE W., BOON N. Eighth Knowledge for Growth life science convention. Sustainable carbon neutral methane oxidation in anaerobic effluents by a partnership of methane oxidizing communites and microalgae. Ghent, Belgium, 24th May 2012. *Poster presentation.*

VAN DER HA D., LEYRE G., BUNDERVOET B., VERSTRAETE W., BOON N. Fourth international conference on Biotechniques. Co-cultures of methane oxidizing bacteria and microalgae allow sustainable carbon neutral methane oxidation in anaerobic effluents. La Coruna, Spain, 12-14th October 2011. *Platform presentation.*

VAN DER HA D., BUNDERVOET B., VERSTRAETE W., BOON N. Sustainable carbon neutral methane oxidation in anaerobic effluents through a partnership of methanotrophs and microalgae. First international symposium on MRM. Ghent, Belgium, June 30th – July 1th 2011. *Poster presentation.*

VANWONTERGHEM I., **VAN DER HA D.**, READ S., VERSTRAETE W., BOON N. The influence of nitrogen on the activity and composition of methane oxidizing communities. First international symposium on MRM. Ghent, Belgium, June 30th – July 1th 2011. *Poster presentation.*

VAN DER HA D., BUNDERVOET B., VERSTRAETE W., BOON N. Sustainable carbon neutral methane oxidation in anaerobic effluents by a partnership of methane oxidizing communities and microalgae. Eight IWA Leading-Edge Technology Conference. Amsterdam, Netherlands, June 7th – June 10th 2011. *Poster presentation.*

VANWONTERGHEM I., READ S., **VAN DER HA D.**, VERSTRAETE W., BOON N. Methanotrophic microbiome. Plantpower symposium, February 10th 2011. *Poster presentation.*

VAN DER HA D., VERSTRAETE W., BOON N. A sustainable, carbon neutral methane oxidation by a partnership of methane oxidizing communities and microalgae. First IWA conference on microbes in wastewater & waste treatment, bioremediation and energy production**.** Goa, India, January 24-27th 2011. *Platform presentation.*

READ S., **VAN DER HA D.**, BOON N. Syntrophic relationships of Methanotrophs and Heterotrophs in the context of methane mitigation. First IWA conference on microbes in wastewater & waste treatment, bioremediation and energy production. Goa, India, January 24-27th 2011. *Platform presentation.*

VAN DER HA D., LEYRE G., BUNDERVOET B., VERSTRAETE W., BOON N. Methalgae: the road to applications. Sixteenth PhD Symposium on Applied Biological Sciences. Ghent, Belgium, December 20th 2010. *Platform presentation*.

VERSTRAETE W., BOON N., **VAN DER HA D.**, VAN DE WIELE T., ARENDS J.B.A., GUIMARÃES B.C.M. Ten questions about contaminated sites, microbial services and their management. International conference on contaminated site management in Europe. Ghent, Belgium, October 18-20th 2010. *Keynote lecture*.

VERSTRAETE W., BOON N., **VAN DER HA D.**, VAN DE WIELE T., ARENDS J.B.A., GUIMARÃES B.C.M. Ten questions about contaminated sites, microbial services and their management. International conference on contaminated site management in Europe. Ghent, Belgium, October 27-29th 2009. *Keynote lecture*.

SINTUBIN L., AWOKE A.A., WANG Y., **VAN DER HA D**., VERSTRAETE W., BOON N. Enhanced disinfection efficiencies by biogenic nanosilver and solar irradiation strategies. Second international colloquium of microbiology. Fes, Morocco, June 2-3th 2010. *Platform presentation.*

SINTUBIN L., DE WINDT W., DICK J., MAST J., **VAN DER HA D.**, THIBO E., VERSTRAETE W., BOON N. Antimicrobial bio-nanosilver produced by *Lactobacillus sp*. International conference on the environmental implications and applications of nanotechnology. Massachusetts, United States, June 9-11th 2009. *Platform presentation*.

CLAUWAERT P., TOLEDO R., **VAN DER HA D.**, CRAB R., HU H., UDERT K.M., RABAEY K., VERSTRAETE W. Combining biocatalyzed electrolysis with anaerobic digestion. IWA Specialist conferences 11th congress on anaerobic digestion. Brisbane, Australia, September 23-27th 2007. *Platform presentation*.

VAN DER HA D., CLAUWAERT P., VERSTRAETE W. Plant derived substrates for a sustainable microbial fuel cell. Third international conference on renewable resources and biorefineries. Ghent, Belgium, June 4-6th 2007. *Poster presentation*.

DANKWOORD

BAIE DANKIE – MERCI – DANKE – OBRIGADA – THANKS – GRACIAS – Ευχαριστώ

GRAZIE – TAK SKAL DU HAVE – ኣመሰገናልኡ – 謝謝 – AKO – ENKOSI – TERIMA KASHI

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Ik had de luxe om vòòr mijn ‘echt’ doctoraat, te mogen opwarmen op allerhande topics. Het grootste deel van die hectische periode ging op aan nanozilver (en zowat de halve tabel van Mendeljev). Het waren niet altijd de beoogde lessen, maar desalniettemin was het een zeer leerrijk jaar in een volledig nieuwe biotoop, letterlijk en figuurlijk. Wim, bedankt voor de opportuniteiten. Jan, bedankt voor de relativerende babbeltjes, de broodnodige humor, handige tips and tricks en het overdragen van de beste stek in het moleculair labo. Liesje, aan jou heb ik de nodige leercurve in semi-moleculair werk te danken. Plaatjes gieten, pipetten gebruiken zonder blaren te krijgen, groeicurves opstellen, noem het maar op, het is later een groot voordeel gebleken.

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Sven, we zijn samen begonnen aan een onbekend avontuur. Het was en is een ongelooflijke steun om iemand te hebben die het bloed, zweet en tranen heeft gedeeld en weet van het bestaan van het vele 'niet-neergeschrevene'. Ik weet niet goed waar juist te beginnen, maar ik wil je alleszins bedanken voor alle hulp bij het uitdenken, uitvoeren, verwerken en neerschrijven van het onderzoek. Evenzeer voor het onvoorstelbare professionalisme waar ik heel veel van opgestoken heb. Niet alleen het gestructureerd werken à la de 'Sventabel', maar ook de doordachte opzetten, ingenieuze testen en perfecte communicatie hebben mij vaak eerbiedig het hoofd doen buigen. En ik beloof, vanaf nu hou ik 'than' and 'then' uit elkaar; dat bespaart je direct de helft verbeterwerk. Meer nog wil ik je bedanken als persoon. Dat eerste toogklapke buiten het labo moeten we dringend regelen, maar alleszins bedankt om dienst te doen als relativerend klankbord en me er telkens aan te herinneren dat het hele doctoraatsgebeuren in the end om die ene vraag draait: "… maar heb je er veel uit geleerd?". Kim, ook jou ben ik een grote dankjewel verschuldigd om verscheidene redenen. In de eerste plaats om me van tijd tot tijd scherp te houden, om me op sleeptouw te nemen in het academisch wereldje, voor het vertrouwen en de wijze woorden wanneer ze nodig waren.

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<< Halfweg en ik heb mijn vooropgesteld pagina-aantal bereikt. >>

Toen ik vier jaar geleden nietsvermoedend de trap naar 't tweede' werd opgestuurd bleek mijn komst een ware volksverhuizing teweeg te brengen. Ineens had ik een allegaartje van redelijk onbekende collega's rondom mij. Six completely different characters put together on an island; it is a guarantee for some memorable moments. It were though but interesting times for all of us, and I for one am very happy I could share them with you.

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D.avid Berlin, February 14th, 2013