Methanotrophic microbiomes from North Sea sediment

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List of abbreviations

A	
AAI	amino acid identity
ANOVA	analysis of variance
AOB	ammonia-oxidizing bacteria
B	
BLAST	basic local alignment search tool
BCA	bicinchoninic acid
BOF	Bijzonder OnderzoeksFonds
BRC	Biological Resource Centers
C	
CTAB	hexadecyltrimethylammonium bromide
CTD	instrument used to determine the conductivity, temperature, and depth of the ocean
CPA	cryoprotectant
Cu_ANOR	nitric oxide reductase accepting electron from cytochrome c
D	
(d)AMS	(diluted) ammonium mineral salts
(d)ANMS	(diluted) ammonium nitrate mineral salts
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
(d)NMS	(diluted) nitrate mineral salts
E	
EPS	extracellular polymeric substance or exopolysaccharide
F	
FADH	formaldehyde dehydrogenase
FCM	flow cytometry
FDH	formate dehydrogenase
FISH-MAR	fluorescence in situ hybridization combined with microautoradiography
FWO	Fonds wetenschappelijk onderzoek

G

GC	gas chromatography
GGDH	genome to genome distance hybridization
GOA	Geconcentreerde onderzoeksactie

H

HGT	horizontal gene transfer

I

IC	ionen chromatograph
ICM	intracytoplasmic membrane
ICP-MS	inductively coupled plasma mass spectrometry
ICP-OES	inductively coupled plasma optical emission spectrometry
IMI	International Microbiome Initiative
IPCC	International Panel for Climate Change
IWT	Agentschap voor Innovatie door Wetenschap en Technologie

K

KEGG	Kyoto Encyclopedia of Genes and Genomes

L

LM-UGent	Laboratory of microbiology – Ghent University

M

MDH	methanol dehydrogenase
ML	maximum likelihood
MO	monoogygenase
MOB	methane-oxidizing bacteria
MOR	methane oxidation rate
MPN	most probable number
MUMM	Management Unit of the North Sea Mathematical Models and the Scheldt estuary
mxaF/MxaF	calcium dependent methanol dehydrogenase gene/ enzyme

N

nanoSIMS	nanoscale secondary ion mass spectrometry
nap/NapA	periplasmic nitrate reductase gene / enzyme
nar/NarG	membrane-bound nitrate reductase gene / enzyme
NOB	nitrite-oxidizing bacteria

nifH/NifH	nitrogenase reductase gene / enzyme
nirK/NirK	periplasmic copper-dependent nitrite reductase gene / enzyme
nirS/NirS	periplasmic cytochrome cd1-dependent nitrite reductase gene / enzyme
O	
OD	optical density
OLAND	Oxygen Limited Autotrophic Nitrification Denitrification
OTU	operational taxonomic unit
P	
ppbv	parts per billion by volume
ppmv	parts per million by volume
Psi-Blast	position-specific iterated BLAST
PSU	Practical Salinity Unit
(p/s)MMO	(particulate/soluble) methane monooxygenase
pmoA	methane monooxygenase subunit A
pXMO	sequence-divergent particulate monooxygenase
PQQ-ADH	pyrroloquinoline quinone dependent alcohol dehydrogenase
Q	
qPCR	quantitative polymerase chain reaction
R	
RAST	Rapid Annotation Subsystem Technology
rep-PCR	repetitive element sequence based polymerase chain reaction
rpm	rotations per minute
Rubisco	ribulose-1,5-bifosfaat carboxylase oxygenase enzyme
RuMP	ribulose monophosphate
RV	research vessel
S	
SPSS	Statistical Package for the Social Sciences
SIP	Stable Isotope Probing
T	
Tg yr^{-1}	teragram ($=10^{12}$gram) per year
TOC	total organic carbon
TOM	total organic matter
TON	total organic nitrogen

TSA	trypticase soy agar
TSB	trypticase soy broth
TT	1% trehalose in 10-fold diluted trypticase soy broth
U	
UMI	Unified Microbiome Initiative
USCα or γ	Upland Soil Cluster Alpha or Gamma
V	
VBNC	viable but non-culturable
VLIZ	Vlaams Instituut voor de Zee
v/v	Volume to volume
W	
WFCC	World Federation of Culture Collections
w/v	Weight to volume
WWTP	waste water treatment plant
X	
xoxF/XoxF	Lanthanide dependent methanol dehydrogenase gene/enzyme

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Chapter 1

General Introduction

Redrafted from:

Vekeman B. & Heylen K., (in press). Preservation of microbial pure cultures and mixed communities, Hydrocarbon and Lipid Microbiology Protocols: isolation and cultivation, in press (McGenity TJ, Timmis KN & Nogales B, eds.). Springer-Verlag, Berlin Heidelberg. DOI: 10.1007/8623_2015_51

1.1. Marine methanotrophic microbiomes

1.1.1. Methane, an important greenhouse gas!

Methane was first discovered by the Italian scientist Alessandro Volta in 1776 in Italy, by poking with a stick in marsh sediment, resulting in the escape of gas bubbles through the water column, that had the characteristic of igniting readily [1]. Nowadays, it is known to be the major component in biogas (50-75%), an economical important combustible gaseous fuel used in households and industries. In addition, it is an important climate-affecting trace gas in the atmosphere [2, 3]. Methane is the most abundant organic greenhouse gas in our atmosphere, and has a strong infrared absorbance, being 25 to 30 times more effective than carbon dioxide on a 100 years scale [4, 5]. Methane therefore plays an important role in the climate warming regulation. Like with other greenhouse gasses, the methane concentration in the atmosphere has increased since the industrial revolution. With concentrations below 700 ppbv before the industrial revolution to concentrations around 1575 ppbv, 1700 ppbv and 1800 ppbv respectively in the 1980's, 1990's and 2011 [6, 7]. In fact the growth rate of atmospheric methane concentrations has outweighed that of carbon dioxide, mainly driven by anthropogenic activities [8]. This increase is driven mostly by increased anthropogenic emissions from fossil fuels, industry, agriculture (including biomass burning) and waste (Table 1.1). According to the International Panel for Climate Change (IPCC) the rise in atmospheric methane concentrations has slowed down significantly the last (2-3) years [9, 10]. More recently it was shown by Dr. Paul Steele and colleagues that this was the result of a decline in the emissions of methane from human activities in the 1990s. Further prolonged drying of wetlands, caused by draining and climate change, resulted in a further reduction of the amount of methane released from these systems, masking the resumed rise in emissions from human activities.

1.1.2. Methane sources in marine systems

The different sources of methane can be natural or anthropogenic in origin. Based on methane amounts, turnover rates and isotopes, together these sources emit a quasi-steady state of approximately 500-600 Tg yr^{-1} of methane to the atmosphere [8, 11] (Table 1.1). Despite the high confidence in the current total methane budget, less certainty exists in the specific contribution of the various sources and predictions concerning future methane concentrations in the atmosphere. Furthermore identification of novel methane emission sources to the

atmosphere are discovered and added to the budget gradually [12, 13]. Around 70-80% of the methane emitted to the atmosphere originates from the activity of methanogenic *Archaea* that anaerobically decompose organic matter in natural ecosystems (e.g. wetlands, ruminants, termites, organic rich oceans sediments) and anthropogenic environments as (e.g. rice paddies, landfills, coal mining) [11, 14–16]. The other 20-30% is the result of thermal decomposition of organic matter (>80°C) within the earth's crust [11, 14, 17]. These locations are called geothermal areas and include seeps, mud volcanoes, hydrothermal vents, etc. The focus of this study will be on marine systems, therefore a more detailed overview of methane sources in this ecosystem is described.

Table 1.1. Global methane budget (sources and sinks) on the earth and atmosphere

Methane sources	**Methane sinks**	**Tg methane yr^{-1}**				
		[18]	[14]	[19]	[20]	[21]
Natural						
Wetlands (incl. lakes)		115	265	100		92-237
Termites		40		20		20
Oceans		20	5	4		10-15
Anaerobic marine sediment				5	75-320	
Geological sources				14		
Wild fires				2		
Agricultural anthropogenic						
Ruminants		80	75	81		80-115
Rice cultivation		110	110	60		25-100
Non-agricultural anthropogenic						
Energy						75-110
Natural gas		45	95	30		
Coal mining		35	35	46		
Other fossil fuels				30		
Landfills		40	40			35-73
Waste water treatment						14-25
Biomass burning		55	40	50		23-55
Waste disposal			25	61		
Other						
Total sources		***540***	***690***	***518***		***500-600***
	Chemical processes					
	Tropospheric hydroxyl radicals (OH-) reaction		530	445		
	Removal to stratosphere		40	40		
	Biological aerobic oxidation					
	Soil uptake		30	30		
	Biological anaerobic oxidation					
	Marine sediments				70-300	
	Total sinks		***600***	***515***		

Earth's interconnected waters form a gradient from freshwater rivers, to estuaries where fresh and salt waters mix, through the relatively shallow coastal ocean on the continental shelves, to

the deep-water open ocean. Oceans cover roughly 71% of the earth's surface and have a surface area of 361 million km². They are critical in controlling the planets temperature. Marine environments, including both oceans and estuaries, currently account for only a minor part of the methane emitted to the atmosphere on a yearly scale. The oceanic methane contribution to the global methane budget has been evaluated extensively and independent research demonstrated that the oceans' emissions vary around 5-20 Tg yr^{-1} (1-3,33% of the global production) [14, 18, 19, 21–24]. Approximately 75% of this is restricted to the shelf and estuarine environment, where tectonic activity facilitates petroleum and natural gas leakage. These areas, although accounting for only 10% of the total area of the world's oceans [22, 25], contribute up to 15% of the oceanic primary production, half of the carbonate burial and most of the burial of organic carbon. A variety of geological and biological methane sources can be identified in these areas, including seafloor cold seeps [26, 27], hydrothermal vents [28], mud volcanoes [29], serpentinization [30–32], coastal organic-rich sediments [33], gas hydrates [34], influent water from rivers [35], and the aerobic ocean surface mixed layer [36]. Estimations of contribution of the different sources to the net methane emission of marine environments have considerable uncertainties due to high spatial heterogeneity and large scale inter annual variability, and are therefore more difficult to predict [37]. Natural seeps contribute around 25 Tg yr^{-1}, mud volcanoes emit around 5 Tg yr^{-1}, micro seeps emit 7 Tg yr^{-1}, other geothermal areas emit around 3 Tg yr^{-1}, oceanic ridge systems emit 0.1 Tg yr^{-1} [13]. Summarized, together the geological sources contribute around 44-48 Tg yr^{-1} to the atmosphere with the potential of increasing an extra 5-10 Tg yr^{-1}, by contributions of gas hydrates [18, 38]. Currently, alternative long distance methods are being applied using satellite sensors detecting mid and thermal infrared [39–41] to improve emission estimates of these sources.

All above-mentioned values are based on methane concentrations actually entering the atmosphere, and thus represent net methane emissions. However methane sinks occur alongside methane sources. A microbial filter in marine sediments has the potential to oxidize more than 80% of the produced methane before its emitted to the atmosphere [19]. The importance of biological methane oxidation is clearly illustrated by the role of the oceans in the global methane budget (Table 1.1). While the oceans have the potential to produce enormous quantities of methane, a series of very effective microbiological methane oxidation processes i.e. anaerobic oxidation in anoxic sediment and anoxic water columns followed by

aerobic oxidation in the top few millimeters of the sediment and aerobic water column, results in the ocean being one of the smallest net global methane sources (Fig. 1.1) [30]. Once methane has reached the atmosphere, 30 Tg methane per year is further removed by the so called high affinity methane oxidizing bacteria, through upland soils. The major sink of atmospheric methane, removing around 445-530 Tg yr^{-1}, is the chemical oxidation in the troposphere by the hydroxyl radical (OH-), the major radical in this part of the atmosphere, leading to the formation of carbon dioxide and water vapor, with potential formation of formaldehyde (CH_2O), carbon monoxide (CO) and ozone (O_3) in the presence of nitrogen oxides (NO_x). From the remaining methane, about 40 Tg per year is transported to the stratosphere where further destruction takes place (Table 1.1) [14, 42].

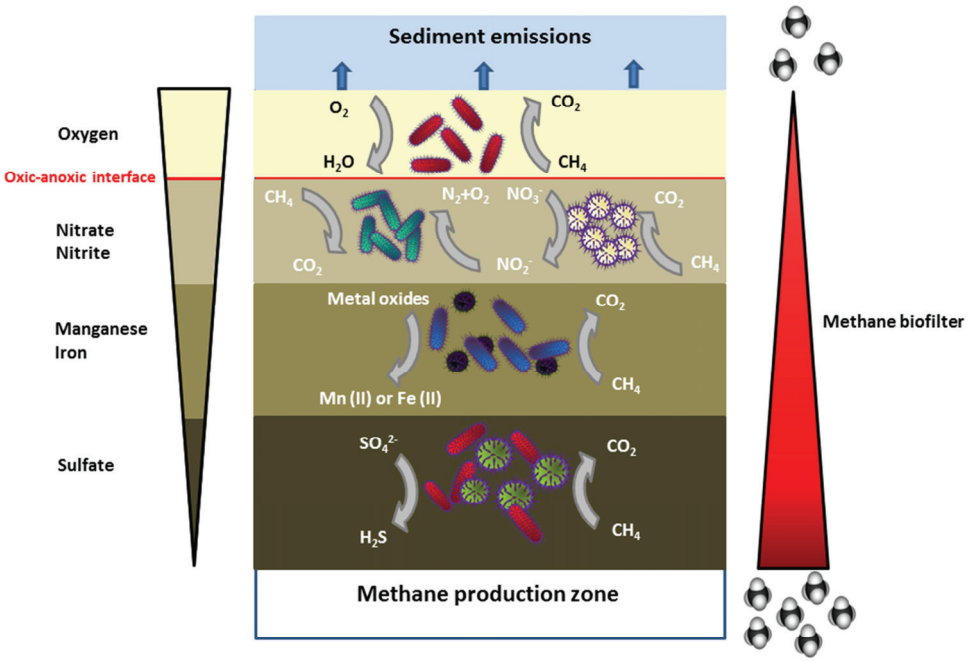

Figure 1.1. Schematic illustration representing the stratified nature of the sediment. In the deeper anaerobic layers, methanogenic archaea produce large amounts of methane. As this methane diffuses through the sediment different methanotrophic members oxidize the methane using different electron acceptors. As such these members form a natural biofilter resulting in the oceans being one of the smallest net global methane sources.

1.1.3. Introduction to the study area

The study area of this thesis is located from the Western Scheldt estuary up to the Southern bight of the North Sea (Fig. 1.2). The North Sea is part of the European shelf and can be classified as a coastal sea. Furthermore it is characterized by the great influence of tidal motions on the ecological conditions. The main water currents transport Atlantic water in a Northeast direction through the English Channel towards the Belgian part of the North Sea, where it meets the Southwest-oriented Western Scheldt estuary outflow in the East [43, 44]. Owing to the tides, pronounced horizontal and vertical exchange and transport effects occur. The salinity distribution is dominated by water masses entering from the North Atlantic and the English Channel [45]. The Western Scheldt estuary is characterized by a macro-tidal current regime, which keeps the water column (average depth 30m) well mixed [46]. The Western Scheldt connects the North Sea with important ports e.g. Terneuzen and Antwerp through busy shipping lanes.

The main source of methane in these areas is sedimentary release following biological methanogenesis [47], supplemented with water discharge from rivers (in the range of 500-5000 nmol L^{-}1 methane from the Western Scheldt estuary [48, 49]), tidal flats and marshes [22, 50, 51]. Bacterial methane oxidation has been identified as an important sink [52] in these areas, together with degassing and dilution with methane poor waters entering from the Atlantic Ocean (i.e. 2.5-3.5 nmol L^{-1}methane [35]) [45].

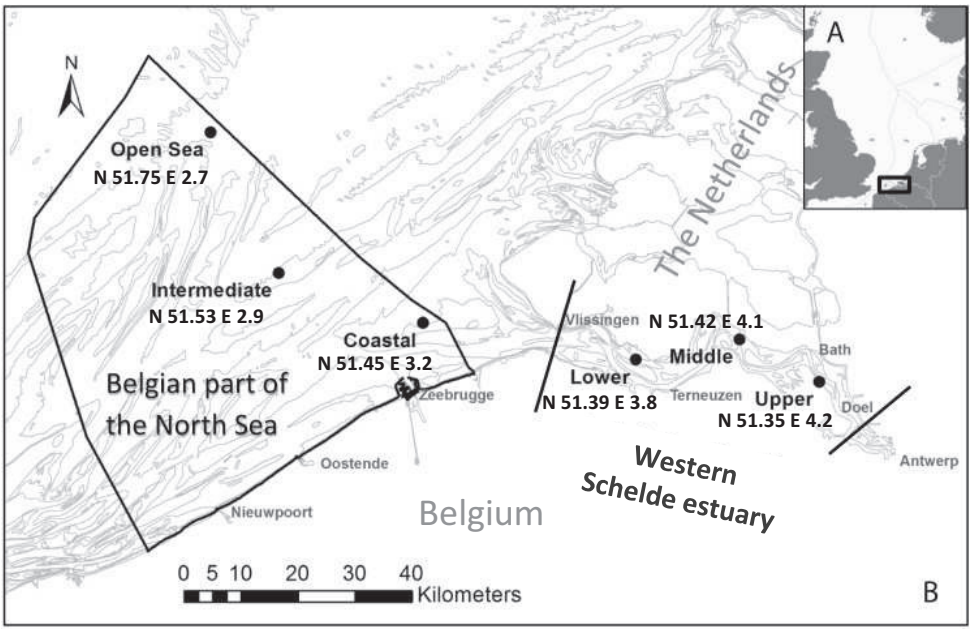

Figure 1.2. Schematic representation of the study area. (A) North Sea with indication of the study area. (B) Position of the six sampling locations in the Belgian part of the North Sea and adjacent Western Scheldt estuary. Modified from [44]

1.1.4. Methanotrophs or Methane-oxidizing bacteria (MOB)

Methanotrophs or methane-oxidizers have the unique ability to use methane as their sole source of carbon and energy [53, 54]. In this way all methanotrophs fully oxidize methane to carbon dioxide [55]. They are remarkably phylogenetically diverse, belonging to *Archaea* and *Bacteria*, and metabolic versatile, capable of aerobic and anaerobic oxidation of methane. Anaerobic oxidation of methane occurs through the process of reverse methanogenesis [56], in which methane oxidation is coupled to reduction of sulphate [57], manganese [58], nitrate [59], nitrite [60] or iron [58] (Fig. 1.1). This is performed by either a bacterium, an archaeon alone or in an association with other microbial partners. The anaerobically produced methane, ranging from 75-320 Tg yr^{-1}, is nearly all oxidized to carbon dioxide under anaerobic conditions. As such anaerobic methane oxidation is the most important marine sink preventing the escape of methane to the atmosphere [20, 61]. The remainder of the methane seeps through to the oxygenated upper layers of the marine sediments where it can be aerobically oxidized by *Proteobacteria* and *Verrucomicrobia* [54, 62–65]. An exception is

Candidatus Methylomirabilis oxyfera, assigned to the NC10 phylum, that performs aerobic methane oxidation in an anaerobic setting, by producing its own oxygen via partial denitrification and a nitric oxide dismutase [66]. While methane is the sole source of carbon and energy for most methanotrophs, members of the *Verrucomicrobia* grow autotrophically by fixing carbon dioxide, while using methane as energy source [67]. Further facultative MOB are able to use alternative carbon sources containing C-C bonds, like ethanol, acetate, propanol, etc as sole source of carbon and energy [68–71]. This thesis will focus specifically on aerobic methane-oxidizing bacteria. Therefore the term methanotroph or methane oxidizing bacteria (MOB) will further refer to the aerobic methane oxidizers, if not stated otherwise.

1.1.5. Diversity and ecology of aerobic methane oxidizers

MOB are Gram-negative bacteria that were first discovered by Kaserer (1905, 1906) and by Söhngen (1906, 1910). However, it took until the 1970s for significant advances to be made in MOB research, when Whittenbury and colleagues isolated around 100 obligate methanotrophs from different environments [72]. The characterization of these isolates resulted in the first classification framework of aerobic methanotrophs. Based on physiology, morphology, ultrastructures and chemotaxonomy, MOB have been classified into two major groups, type I and type II confined within the *Gammaproteobacteria* (family *Methylococcaceae* and *Methylothermaceae*) and the *Alphaproteobacteria* (family *Beijerinckiaceae* and *Methylocystaceae*) respectively [73, 74]. Major differences between both groups included, the arrangement of internal membranes as vesicular discs (type I) or paired membranes aligned to the cell periphery (type II), the carbon fixation mechanisms via the ribulose monophosphate pathway (type I) or the serine cycle (type II), the formation of resting stages and the predominance of specific C16 (type I) or C18 (type II) fatty acids [53, 54]. Since the first isolation report of MOB, the number of isolates has increased importantly with currently twenty genera of *Gammaproteobacteria* and five genera of *Alphaproteobacteria*, in total represented by approximately 60 different species [75]. The diversity of aerobic methanotrophs was further expanded by the discovery of methanotrophic *Verrucomicrobia*, comprising the genera *Methylacidiphilum* [76] and *Methylacidimicrobium* [77], each containing three species. The discovery of the methanotrophic *Verrucomicrobia* [76, 77] and the characterization of several new genera and species within the *Proteobacteria* [69, 73, 78–88] led to a revision of the type I and II division as most of the discriminating

characteristics are no longer exclusively found in one or the other group. As such it has recently been proposed to abandon this system [76]. Nevertheless an adapted, more comprehensive division, is still frequently used, but should only be considered as synonyms of the different phylogenetic groups of currently known methanotrophic diversity (Fig. 1.3). In this context the methanotrophic *Alphaproteobacteria* have been divided in type IIa (*Methylocystaceae*) and type IIb (*Beijerinckiaceae*) methanotrophs [89]. Likewise the methanotrophic *Gammaproteobacteria* are further divided into several subgroups matching different clades. Type Ib consists of the clade containing *Methylococcus*, *Methylocaldum*, *Methylogaea*, *Methylomagnum* and *Methyloparacoccus*; type Ic contain members of the *Methylothermaceae* and type Ia representing the rest of the *Gammaproteobacteria* [75]. An extra group, comprising the clade of *Nitrosococcus* and related uncultured diversity is referred to as type Id. Methanotrophic *Verrucomicrobia* are referred to as type III.

Methane oxidizing bacteria occur ubiquitous in nature. Typically they reside at oxic-/anoxic interfaces where oxygen is available as electron acceptor and methane as carbon and energy source. As such, MOB have been isolated from rivers, lakes, ponds, marine environments, wetlands, rice paddies, ground water, waste water, coal mines, and sludge [65, 90]. Typically most MOB isolated so far are mesophilic (with an optimum temperature for methane oxidation around 25°C) and neutrophilic (with an optimal pH for methane oxidation of 5.0 to 6.5 in pure cultures) [91]. However to highlight the physiological diversity of these bacteria, MOB have also been isolated from thermal terrestrial, alkaline and athalassic, low temperature and acidic ecosystems resulting in the retrieval of thermotolerant/philic strains with growth optima varying from 6°C till 65°C in temperature [76, 88, 92–95], acidotolerant/philic and alkalitolerant/philic strains with pH 0.8 till pH 10.5 growth optima [76, 96]and halotolerant/philic strains with a salinity optima up till 15% NaCl [84, 96, 97]. In all these ecosystems MOB play a key process in the removal of as much as 90% of the produced methane. However several environmental factors seem to control methane oxidation. Manipulative investigations with laboratory cultures or in-situ experiments have shown that methane, oxygen, inorganic nitrogen, pH, copper, phosphate, water availability, temperature and biotic factors seem to play a role [91, 98]. To cope with unfavorable conditions many MOB from different types of resting stages [99]. In contrast to vegetative cells, which die off within days when deprived of methane [100], all forms of resting stages survive for weeks without methane [101], providing a useful survival strategy in environments with variable

methane levels. *Methylosinus* cells elongate and form pear and comma shapes when entering the stationary phase, after which spores are budded off. These exospores are heat-resistant (surviving 15min at 85°C) and desiccation-resistant (surviving at least 18 months in a dried state deprived of methane). Under optimal conditions, spores germinate within days up to two weeks. Besides spores, some MOB also form cysts, of which three types gave been observed: lipid cysts (*Methylocystis* strains), *Azotobacter*-type cysts (*Methylobacter* strains) and immature cysts (*Methylomonas* and *Methylococcus* strains). While the lipid and *Azotobacter*-type cysts provide resistance to desiccation but not to heat, the immature cysts are neither desiccation nor heat resistant and seem to only provide survival in situations in famine [101]. Understanding the ecology of the MOB is important for gaining insight in the effect of different stressors and their effect on net methane production, C fluxes and ultimately management strategies to reduce net emissions of methane to the atmosphere.

Since the isolation of the first MOB in 1906 it took more than 80 years before the first aerobic marine MOB, *Methylomonas pelagica* (later renamed as *Methylomicrobium pelagicum*) was isolated [102]. Since then only a handful of other marine isolates have been reported and characterized, consisting of *Methylobacter marinus* [103], *Methylomicrobium japanense* [27], *Methylomonas methanica* [78], *Methylomarinum vadi* [79], *Methylocaldum marinum* [28], *Methylomarinovum caldicuralii* [10] and *Methyloprofundis sedimenti* [80], all belonging to the type Ia and Ib MOB *Gammaproteobacteria*. This reflects the dominance of type I methanotrophs affiliated to the *Methylococcaceae* as the most important aerobic methanotrophs present in many marine environments [104–109], with occasional reports of type II and type III methanotrophs inhabiting methane rich ocean sediment [104, 105, 108, 110, 111]. However, using the *pmoA* gene, encoding a subunit of the particulate methane monooxygenase present in almost all methanotrophs, as a biomarker has revealed that a huge diversity of methanotrophs is present in nature that is so far not represented by isolates in the laboratory (Fig. 1.3). Also in marine ecosystems, *pmoA* amplicon sequencing have revealed that a more than 80% of the diversity of *pmoA* sequences in these different environments may represent novel species of currently uncultivated methanotrophs [106, 107, 112]. Therefore the current isolates might not represent the dominant and/or active methanotrophic populations in marine environments. *PmoA* sequences retrieved from marine environments seemed to form distinctive operational taxonomic units (OTU) compared to those from other environments that are more widespread [75]. The majority of the marine sequences can be

grouped into five major clusters referred to as deep-sea clusters 1-5 [113]. Deep-sea cluster 1-3 belong to type Ia, deep-sea cluster 4 is distantly related to type Ia and Ib and deep-sea cluster 5 forms a deep branching lineages related to type Ib and Ic methanotrophs (Fig. 1.3). Current insights in the ecology, ecophysiology and habitat preference of these groups is very limited. Representatives of deep-sea cluster 1 and to a lesser extend deep-sea cluster 2 and 4 have been found in association with marine animals such as mussels, tube worms or shrimps, as endosymbionts [114–116] or epibionts [114, 117, 118] and might indicate a habitat preference. Only recently cultured representatives of deep-sea clusters 1 and 2 belonging to the genera *Methyloprofundus* and *Methylomarinum* have been described [119, 120]. However, these genera only cover a small portion of the sequence diversity present in these clusters, assuming that more as yet undiscovered genera are delineated in these clusters. Several of these clusters contain sub-groups with are sometimes given as synonyms for the deep-sea cluster to which they are affiliated to. Cluster 2 contains the subcluster PS-80, cluster 3 contains the sub-clusters OPU3 and EST and cluster 5 contains the cluster OPU1. Besides these large clusters some smaller clusters are sometimes detected in marine systems positioned within the *Gammaproteobacteria*, LS-mat [121], ATII-Icluster3 [106], and those that are distantly related to the well-known *pmoA* sequences, group X [122] and ATII-I cluster 4 [106].

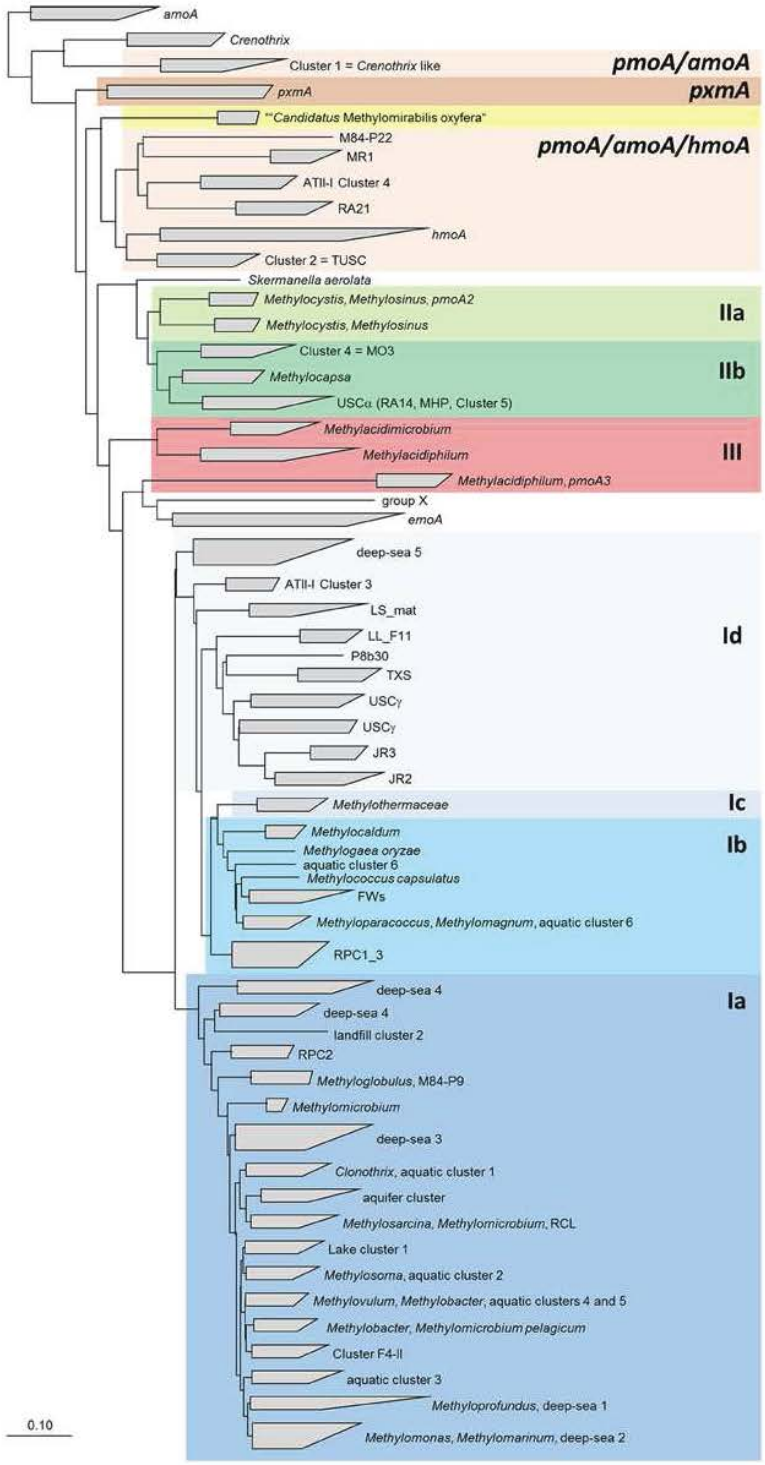
amoA
Crenothrix
Cluster 1 = Crenothrix like
pmoA/amoA
pxmA
pxmA
"Candidatus Methylomirabilis oxyfera"
M84-P22
MR1
ATII-I Cluster 4
RA21
pmoA/amoA/hmoA
hmoA
Cluster 2 = TUSC
Skermanella aerolata
Methylocystis, Methylosinus, pmoA2
Methylocystis, Methylosinus
IIa
Cluster 4 = MO3
Methylocapsa
USCα (RA14, MHP, Cluster 5)
IIb
Methylacidimicrobium
Methylacidiphilum
Methylacidiphilum, pmoA3
III
group X
emoA
deep-sea 5
ATII-I Cluster 3
LS_mat
LL_F11
P8b30
TXS
USCγ
USCγ
JR3
JR2
Id
Methylothermaceae
Ic
Methylocaldum
Methylogaea oryzae
aquatic cluster 6
Methylococcus capsulatus
FWs
Methyloparacoccus, Methylomagnum, aquatic cluster 6
RPC1_3
Ib
deep-sea 4
deep-sea 4
landfill cluster 2
RPC2
Methyloglobulus, M84-P9
Methylomicrobium
deep-sea 3
Clonothrix, aquatic cluster 1
aquifer cluster
Methylosarcina, Methylomicrobium, RCL
Lake cluster 1
Methylosoma, aquatic cluster 2
Methylovulum, Methylobacter, aquatic clusters 4 and 5
Methylobacter, Methylomicrobium pelagicum
Cluster F4-II
aquatic cluster 3
Methyloprofundus, deep-sea 1
Methylomonas, Methylomarinum, deep-sea 2
Ia
0.10

Figure 1.3. A condensed neighbor joining tree showing the gene phylogeny of uncultured copper containing membrane bound monooxygenases in relation to those from type strains of methanotrophic species (taken from [75]**).** The tree includes *pmoA* sequences from all OTUs that were assigned to uncultivated clusters. It was calculated based on 480 nucleotides with Juke Cantor correction. The scale bars display 0.10 changes per nucleotide position. Different colors indicate the different divisions correlating to the different phylogenetic groups of currently known methanotrophic diversity.

1.1.6. Metabolism of aerobic methane oxidizers

The unique feature of methanotrophs enabling them to use methane is the presence of the key enzymes methane monooxygenase (MMOs). These enzymes break the O-O bond in the dioxygen molecule by utilizing two reducing equivalents [53]. One of the oxygen atoms is converted to water and the other one is incorporated into methane to from methanol. Two MMOs can be distinguished, the particulate, membrane-bound type (pMMO) and the cytoplasmic, soluble type (sMMO) (Fig. 1.4). All methanotrophs described so far express the pMMO, with exception of the genera *Methylocella* and *Methyloferula* [79, 81], which contain solely the soluble variant. The sMMO is further only found in a small subset of MOB associated with the genera *Methylocystis*, *Methylosinus* and some members of the genera *Methylomonas*, *Methylomicrobium*, *Methylococcus* and *Methylocaldum* [123–128]. The expression of these enzymes is dependent on the copper availability to the cell. Copper is translocated from the environment to the cells by the production of methanobactin. Both MMOs are mutually exclusively expressed in the cell dependent on the copper concentration in its environment. The pMMO predominates at a high copper/biomass ratio (>0.85-1µmol (g dw)$^{-1}$), whereas sMMO is expressed at low copper/biomass ratios [129, 130]. The switch between both MMOs is called the copper switch [131]. The sMMO is encoded by the operon *mmoXYZBC*, and consists of three components, a hydroxylase, a regulatory protein B and a reductase component C. The pMMO is composed of three subunits encoded by the gene operon *pmoCAB*. Unlike sMMO, pMMO has a relative narrow substrate specificity and can oxidize alkanes and alkenes of up to five carbons in length. However pMMO-grown MOB have a higher substrate to biomass conversion and a higher affinity to methane [54]. Recently through culture independent studies *pmoA* paralogs and related monooxygenases (MOs) have been detected. In most *Methylocystis* and *Methylosinus* spp. a second *pmoA2* gene was discovered supporting growth at lower methane-mixing ratios [132, 133]. Further a homologue for the pMMO has been discovered in members of the genera *Methylomonas*, *Methylobacter* and *Methylomicrobium*, called the pXMO coded by the *pxmABC* operon [134].

However, the function of the gene product and regulation of gene expression remain currently unknown. In *Methyloglobulus morosus* an additional *pxmA*-like gene is present. Further, other MOs of the super family of copper containing membrane bound MOs have been identified, which seem involved in the oxidation of short chain hydrocarbons, but not methane [135–138]. These include the *hmoCAB* operon, referring to the hydrocarbon monooxygenase that has a broader substrate range for ethane, propane, butane and ethylene [136], *emoA* gene oxidizing ethane, and possibly belonging to *Methylococcaceae* members unable to oxidize methane [135], and finally another type of MO found in the genus *Haliea* and in *Skermanella aerolata*, different from *hmoA* and *emoA*, but more related to the *pmoA* of type II MOBs. This MO oxidizes ethylene, ethane, propane and propylene [138].

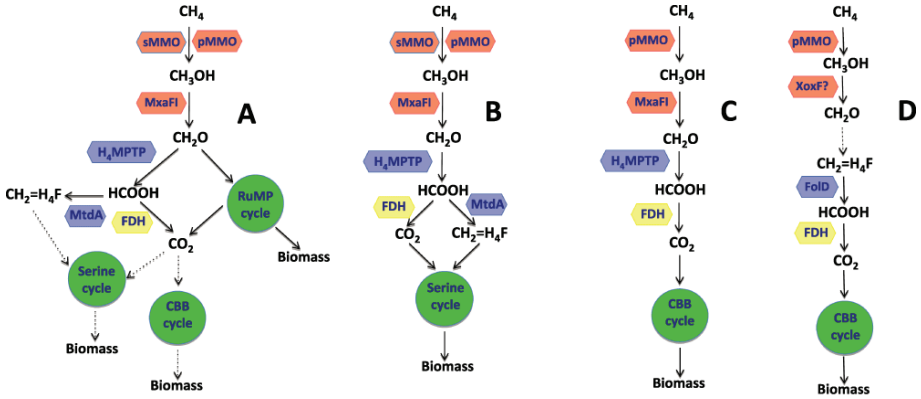

Figure 1.4. Examples of the classic and the novel combinations of methanotrophy metabolic modules. (A) Type I methanotrophs (exemplified by *M. capsulatus*); (B) Type II methanotrophs (exemplified by *M. trichosporium*); (C) Phylum NC10 (exemplified by "*Candidatus* Methylomirabilis oxyfera"); (D) Type III, methanotrophic *Verrucomicrobia.* Primary oxidation (demethylation, dehalogenation) modules are shown in red, formaldehyde (methyl-H_4F) handling modules are shown in blue, formate dehydrogenase module is shown in yellow and assimilation modules are shown in green. Modified from [139].

The PQQ-dependent methanol dehydrogenase (MDH) is responsible for the second step in the aerobic oxidation of methane, and catalyzes the conversion of methanol to formaldehyde (Fig. 1.4). Till recently it was believed that all methanotrophs relied on a calcium-containing MDH, which is coded by the gene operon *mxaFJGIRSACKLD.* Indications of the presence of other alcohol dehydrogenases were found, however their function has been enigmatic for quite

some time [140, 141]. Recently, it is was discovered that the methanotrophic *Verrucomicrobia* lack the calcium-containing MDH and rely solely on a lanthanide-containing MDH coded by the *xox* gene. The Xox-MDHs are phylogenetically divers and at least five clades can be distinguished [55, 142]. Because of the phylogenetically widespread occurrence of the *xox* genes it is thought to be of ecological importance. This is especially true in coastal marine and aquatic environments where a high diversity of these *xox* genes have been found [143] and where rare earth elements are available from sediment and coastal runoff [144–146].

Formaldehyde (HCHO) is the central metabolite in the anabolic and catabolic pathway of methanotrophs (Fig. 1.4). In the catabolic pathway, HCHO is further converted to formate, by the action of formaldehyde dehydrogenase (FADH) and finally to CO_2 by the formate dehydrogenase (FDH). The assimilation of HCHO takes place via two different pathways in type I and type II MOB. Type I methanotrophs use the ribulose monophosphate (RuMP) pathway for assimilation of formaldehyde, whereas type II MOB use the serine pathway [54] (Fig. 1.4). Type III assimilate carbon by means of the Calvin cycle and are independent of formaldehyde (Fig. 1.4). A variant of the serine cycle may however also be present [147]. In the RuMP pathway formaldehyde is combined with ribulose-5-phosphate to produce hexulose-6-phosphate, which are further converted to glyceraldehyde-3-phosphate (C3 molecule) for assimilation via action of hexulosephosphate synthase and hexulosephosphate isomerase. In the serine pathway, formaldehyde is combined with glycine to form serine. Generally the serine pathway requires 2 moles of formaldehyde and 1 mole of CO_2 to form 1 mole of 2-phosphoglycerate (C3 molecule). The key enzyme involved in this pathway is the hydroxypyruvate reductase [53, 55]. The formation of one C3 molecule by the RuMP pathway requires less energy than the formation by the serine pathway [148]. Therefore carbon assimilation is more efficient in type I MOB than in type II.

1.2. PRESERVATION OF MICROBIOMES

1.2.1. Preservation of microbial resources – why should we care?

Archiving of biological material is a very important aspect of microbiology and microbial ecology as microbial pure cultures, communities or original environmental samples are often irreplaceable [149–152]. Stable and long-term storage of microbial resources (i) allows validation of previously obtained results, (ii) avoids accidental contamination or losses during research, (iii) catalogs genetically identical biodiversity for future research, and (iv) ensures long-term accessibility of well-documented cultures well after their discovery for biotechnological or commercial use. Despite the general consensus on its importance and in contrast to data archiving, robust storage of biological material is often neglected and still not widely incorporated in good research practices [152].

Long-term storage is most often achieved by cryopreservation [153]. Unfortunately, when standard freezing of a glycerol stock (commonly at 10-15 % (v/v)) at -20°C or -80°C is unsuccessful, one generally resorts to storage at 4-15°C in appropriate conditions (i.e. medium and headspace) and periodical subcultivation. But storage in a cold-room is not a 'stable' long-term preservation method and does not guarantee identical biological material after several years. In addition, subcultivation is a time-consuming process and can easily result in contamination or genetic drift. In addition to the above-mentioned advantages to systematically preserve biological material, another major incentive is the increasingly strict rules of journal and funding agencies to deposit described pure cultures in a public culture collection, available for the scientific community upon request [154, 155]. This type of open source might become mandatory with science performed by public funds. However, researchers are often unaware that they are obliged to provide the suitable preservation conditions of their pure cultures upon deposit in public culture collections [155] and deposits might be refused when this information is not available.

1.2.2. The basics of preservation

The two major long-term preservation methods for bacteria are cryopreservation and lyophilization (i.e. freeze-drying). The choice between both methods depends on in-house facilities but, more importantly, also on the characteristics of the target organism(s), the reason for preservation and preferred storage period. The term cryopreservation is used for all

storage at freezing temperatures. Residual cell activity decreases with decreasing temperature. Rule of thumb is the lower the storage temperature and frequency of temperature variation, the higher the stability and chance of successful preservation of a viable culture. Above -135°C (i.e. glass transition temperature of pure water), traces of unfrozen water are still possible, rendering preservation at these temperatures unstable [156]. This means that cryopreservation in electrical freezers (at -20°C and -80°C), even when successful in the beginning, will not guarantee that the culture will remain viable indefinitely [157]. Still, it can be sufficient to keep a culture for only a couple of years in the lab before deposit in a public culture collection. In that case, electrical freezers are the easiest and cheapest means of preservation. If researchers want to keep cultures for over 5 years, then storage at ultra-low temperatures in liquid nitrogen containers (at -196°C) is advisable [157], with reports of successful storage up to 35 years (which is the longest that tests have run).

Lyophilization is a controllable process of removing water from frozen cell suspensions by vacuum desiccation. It is a very complex procedure [158] where seemingly trivial changes in an established protocol, such as the use of another container, can render the process unsuccessful [159]. Because of the potentially damaging drying process, freeze-drying is generally considered as less successful than cryopreservation for the storage of a broad diversity of bacteria. However, if a culture can be successfully freeze-dried, it can be kept "indefinitely" (>35 years; note that currently available studies only span several decades) when stored in a dark, preferably acclimatized chamber. The sealed containers also allow an easy and low-cost dissemination of the cultures. As a result of these advantages and the requirement of specific equipment and trained staff, its use is mainly limited to public culture collections.

Many parameters influence the success rate of a specific preservation method. The most important are growth phase of microbial cells, amount of biomass, cooling rate, choice of medium and protective agent, warming rate and resuscitation conditions [160]. Unfortunately, preservation of biological material is mostly an empirical research field, with mainly reports on one particular strain or species, and reasons for general observations are not always clear. In addition, it is widely recognized that different bacterial species and even strains within one species can exhibit great variability in success rate of different preservation conditions [161].

1.2.3. Cryopreservation of MOB and other key players in biogeochemical cycles

Freezing of biological material is normally lethal and protective agents are added to cell suspensions before storage to ensure a higher cell survival rate than in its absence (Fuller 2004). Cryoprotective agents (CPA) reduce the solution effects that play during freezing by inducing osmosis (i.e. movement of water) and diffusion (i.e. movement of solutes). Therefore, any CPA must (i) be highly soluble in water and remain so at low temperatures and (ii) have a low toxicity so that it can be used in the high concentrations that are required to produce these effects [162]. A wide range of CPAs are known and were already successfully applied for cryopreservation of microorganisms. Of these, cryopreservation with dimethylsulfoxide (DMSO) is comparatively more successful than the commonly used glycerol [163]. In addition, cryoprotective function of complex substrates (e.g. yeast extract, blood serum and peptones), sugars or alcohols (e.g. sucrose and mannitol respectively) has previously been reported but is not so well-known [163, 164]. Because most preservation research to date has been performed on heterotrophs grown on complex media, addition of these compounds in freezing media are often forgotten as important cryoprotective measure for autotrophic microorganisms or microorganisms grown in defined oligotrophic media.

Work on MOB indeed confirmed the use of DMSO and complex preservation media for successful cryopreservation [165]. Ten MOB type strains were preserved under fifteen cryopreservation or lyophilization conditions. After three, six and twelve months of preservation, the viability (via live-dead flow cytometry) and cultivability (via most-probable number analysis and plating) of the cells were assessed. All strains could be cryopreserved without a significant induction of the viable-but-non-culturable state (VBNC) using 1% trehalose in 10-fold diluted trypticase soy broth (TT) as preservation medium and 5% DMSO as cryoprotectant. Several other cryopreservation and lyophilization conditions, all of which involved the use of TT medium also allowed successful preservation but showed a considerable loss in cultivability due to VBNC. So, MOB could easily be cryopreserved at -80°C and in liquid nitrogen without great loss of cells. The success of cryopreservation with TT as preservation medium and 5% DMSO as cryoprotectant was further confirmed with aerobic ammonia-oxidizing bacteria [166], highly enriched anammox cultures, both flocs and single cells [167], and nitrite-dependent anaerobic methane oxidizers (Heylen & Ettwig, unpublished).

1.2.4. Preservation of non-axenic cultures, enrichments and environmental samples

Environmental or applied research on bacterial co-cultures, enrichment cultures, consortia or even environmental samples approximates the real biological *in situ* conditions more than work with axenic cultures, thus is generally deemed more relevant. However, experimental work cannot be reproduced or verified because cultures or samples are not stored in a stable manner and batch or continuous cultivation in any kind of reactor will result in a different community composition. In addition, the risk of losing biological material due to calamities such as power failures or contaminations are higher than with pure cultures, with no back up material available in private or public repositories. Research on storage of these kinds of samples is in its infancy. Furthermore, although public culture collections currently do not have appropriate quality control tools at their disposal to handle these types of samples, public deposits of non-pure cultures will become increasingly relevant in the near future.

Still, the limited reports available demonstrate that their stable cryopreservation is possible using similar simple protocols as for pure cultures [160, 168–171]. Embedding of cells in a matrix such as exopolysaccharides in aggregates or biofilm-covered particles in soils or sediments provide extra protection to the cells during preservation and is a major advantage compared to planktonic pure cultures. Maintained functionality and community structure after cryopreservation has already been successfully demonstrated for denitrifying biomass of a fluidized, methanol-fed denitrification reactor [169]. Recently, and using above-described preservation approaches developed at LM-UGent, a methanotrophic co-culture, an oxygen limited autotrophic nitrification/denitrification biofilm and fecal samples from a human donor were successfully preserved [171]. After three months of cryopreservation at -80°C, metabolic activity, in terms of the specific activity recovery of MOB, aerobic ammonium oxidizing bacteria and anammox in the OLAND mixed culture, resumed faster when 5% DMSO was added as CPA with or without TT preservation medium. However, the activity of the fecal community was not influenced by the CPA addition, although the preservation of the community structure (as determined by 16S rRNA gene sequencing) was improved by addition of CPA. These successful examples of preservation of non-axenic cultures, enrichments and environmental samples should encourage further preservation attempts of mixed communities and environmental samples to provide backup biological material for future research.

1.3. CONCEPTUAL FRAMEWORK OF THE THESIS

1.3.1. Context and aims

Cultivation and subsequently isolation of marine methane-oxidizing bacteria is time-consuming and laborious, underestimated by many microbiologists resulting in a discouraging lack of attention and dedication from the scientific community. Marine bacteria are notoriously difficult to cultivate. Therefore most studies on marine MOB are molecular surveys, resulting in the current limited availability of *ex situ* cultures. Nevertheless, they are indispensable to link physiology to genomic features and expand our knowledge about the specific habitat preferences of marine MOB clades. Both low-tech approaches as direct plating and serial subcultivation [62, 63] described over a 100 years ago as well as more sophisticated reactor technology for continuous culturing [66] have yielded completely novel methanotrophic clades over the last decade. However, their potential of capturing novel diversity requiring dedication over many years is in sharp contrast to the number of isolates. Therefore, ***The approach of this thesis aimed at designing a large-scale enrichment and isolation strategy aiming to retrieve a maximal MOB diversity from marine sediments.*** This was done by combining initial enrichment with serial subcultivations and miniaturized extinction culturing, followed by isolation on plate and on floating filter. As the marine sediments are known to be very heterogeneous both in space and time, a range of realistic microniches were mimicked to maximize the retrieval of methanotrophic diversity by (i) varying concentration of inorganic nitrogen, (ii) different methane, oxygen and carbon dioxide concentrations and (iii) addition of sand as adhesion material, reflecting the high microniche heterogeneity in marine sediment. The followed strategy also incorporated the state of the art on bacterial isolation, such as the use of aged seawater from the sampling sites as medium base, Gellan gum as solidifying agent instead of agar, long incubation periods and realistic incubation temperatures.

Cultivation, isolation and characterization of novel microbes is meaningless without guaranteeing their stable and long-term availability. Only then will phenotypic and genotypic features remain unaltered over time and is the biological material available for future research. Storage of non-axenic cultures, enrichment cultures and whole environmental samples is still underexplored, but is as tremendously important for (i) microbial ecology studies allowing world-wide comparative research, (ii) allowing revisiting of samples with other or novel technologies or another research focus and (iii) supporting a sustainable bio-based economy

where environmental microorganisms are essential resources. The availability of the physical sample allows future retrieval or isolation, study and use of the associated microbiota future discoveries in biotechnology. Continuing on previous research on cryopreservation performed at LM-UGent, the effect of various organic CPA was studied as well as the concentration of DMSO on the cryopreservation success of nitrite-oxidizing bacteria, key players of the global nitrogen cycle. Included in the strain panel were several marine strains. The optimized cryopreservation protocol was validated and later used throughout this thesis to store all obtained marine enrichment and axenic MOB cultures. In addition, the effect of cryostorage of marine sediment on aerobic methane oxidizing activity was investigated.

1.3.2. Thesis outline

Chapter II reports on the effect of gas mixture composition and the effect of sand as a carrier material for culturing marine MOB *ex situ*. The addition of a carrier material seemed indispensable and resulted in a significant increase in methane oxidation activity. Comparable results were obtained for low oxygen concentrations and high carbon dioxide concentrations.

Chapter III reports on an extreme example of niche differentiation among marine methylotrophs affiliated to the genus *Methyloceanibacter*, discovered via shotgun sequencing of highly enriched methane-oxidizing cultures and the subsequent isolation of the methylotrophs from these enrichments. Four novel species were obtained of which one is capable of methane oxidation, using solely the soluble methane monooxygenase (sMMO), making this the first type II methanotroph isolated from a marine system, the third methanotrophic taxon only possessing sMMO and the first methanotroph within a non-methanotrophic genus. Furthermore all species displayed a wide ecotypic variation related to growth kinetics on methanol, and preferences for nitrogen, pH, temperature and salt.

In **Chapter IV**, the genomic landscape of two novel gammaproteobacterial cultures, belonging to the deep-sea clade 2, is described. Draft genomes were obtained by shot gun sequencing of two enrichment cultures and subsequent binning by comparing GC content to sequencing depth allowed the extraction of both genomes. The draft genome contained the genes typically found in type I methanotrophs (e.g. *pmoCAB*, tetrahydromethanopterin (H4MPT)- and tetrahydrofolate (H4F)-dependent C1-transfer pathways, RuMP). The most distinctive features compared to other available gammaproteobacterial genomes is the absence of a calcium dependant methanol dehydrogenase.

Chapter V reports on a large scale MOB cultivation survey from marine sediment collected at six stations located along a transect from inside the Western Scheldt estuary up to the Southern bight of the North Sea which coincided with a decrease in nitrogen and increase in salinity. A miniaturized enrichment approach, including realistic nitrogen and oxygen conditions, resulted in a high number of active marine methanotrophic cultures. Subsequent functional marker gene sequencing resulted in the identification of half of the cultures, several of which were affiliated with previously uncultured deep-sea clusters. A clear decreasing trend of cultivability and detectability was observed along the transect from estuary to open sea. Furthermore widely applied *pmoA* primers failed to amplify biomarkers in a large number of active methanotrophic cultures, suggesting enormous underestimation of methanotrophs *in situ* in PCR-based molecular surveys. Unfortunately, despite the numerous attempts we were not able to grow these methanotrophs in pure culture in the laboratory.

In **Chapter VI**, we demonstrated that long-term cryopreservation of fastidious nitrite-oxidizing bacteria assigned to the genera *Nitrobacter*, *Nitrospina*, *Nitrococcus*, *Nitrotoga* and *Nitrospira* was possible using a simple and rapid protocol. Their survival was tested with different cryoprotecting agents, DMSO and Hatefi, and in various carbon-rich preservation media, ten-fold diluted TSB, and ten- fold diluted TSB supplemented with 1% trehalose, and 1% sucrose. Optimal preservation conditions were strain-dependent and marine strains appeared to be more sensitive to freezing than non-marine strains. Nevertheless, a general cryopreservation protocol using 10% dimethyl sulfoxide with or without ten-fold diluted trypticase soy broth as a preservation medium allowed successful preservation of all tested strains.

In **Chapter VII** marine sediment samples were successfully cryopreserved by the same protocol allowing successful storage of different key players in the carbon and nitrogen cycle. Specific activities of two functional groups (methane oxidation by MOB and anaerobic nitrous oxide production) were evaluated. A clear protective matrix effect could be observed from the sample. As the recovery of general viability of all members of a mixed culture is directly linked to the recovery of specific activity, it was assumed, though not verified, that the community remained unaltered after preservation.

Finally, **Chapter VIII** is dedicated to general conclusions and lessons learned from this work and highlights ideas for future perspectives.

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Part I:

Methanotrophs from North Sea sediment

Chapter 2

Factors controlling methane oxidation in marine ecosystems: the effect of adhesion material and gas composition

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Author's contributions:

Bram Vekeman (B.V.), Charles Dumolin (C.D.), Paul De Vos (P.D.V.) and Kim Heylen (K.H.). designed the research, B.V. and C.D. performed research, B.V., C.D. and K.H. analyzed data, B.V., C.D. and K.H. wrote the paper.

2.1. INTRODUCTION

Cultivation of microbial representatives of specific functional guilds from environmental samples essentially depend on the suitability of the applied growth conditions. For methanotrophic bacteria, methane is given as a sole carbon and energy source, most commonly in an initial enrichment, in which over time methane oxidizers numerically dominate heterotrophic bacteria. In the 1970's Whittenbury and colleagues developed two carbon free isolation media, NMS (Nitrate Mineral Salt medium) and AMS (Ammonium Mineral Salt medium) and described their use for isolation of methanotrophs [1]. Until today they are still frequently used [2, 3]. Nevertheless, over time it became evident that specific growth medium compounds influence activity and growth of methane oxidizers, and might have a differential effect on enriched methane-oxidizing communities. The most well-known is copper. Both key enzymes for methane oxidation, the soluble and particulate methane monooxygenase (MMO), are mutually exclusively expressed in the cell dependent on the copper concentration in its environment and the switch between both MMOs is called the copper switch [4]. The pMMO predominates at a high copper/biomass ratio (>0.85-1 μmol (g dw)$^{-1}$), whereas sMMO is expressed at low copper/biomass ratios [5]. The PQQ-dependent calcium-containing methanol dehydrogenase (MDH) is responsible for the second step in the aerobic oxidation of methane, and catalyzes the conversion of methanol to formaldehyde, making calcium an essential medium compound [6, 7]. However, recently it was discovered that the methanotrophic verrucomicrobium *Methylacidiphilum fumariolicum* SolV relies solely on a lanthanide-containing MDH [8]. This explains why its growth *ex situ* was stimulated by the addition of soil extract [9], later found to contain rare earth metals [8]. Other compounds reported to stimulate methane activity or differentially influence MOB growth are multi-carbon compounds [10, 11], nitrogen [12], iron [1, 12, 13], phosphates [1, 10, 14], chlorides [7, 12, 15], magnesium [16], pH [12], buffering capacity of the medium [12], trace elements [1, 12] and minerals [17]. These insights gave rise to modified isolation media, e.g. 5-fold diluted NMS or AMS (dNMS & dAMS) [18, 19], ANMS [20], vitamin and copper rich NMS [21], or media more reflecting the natural *in situ* nutrient conditions of the environment [22, 23].

Specifically for enriching MOB from marine sediments, two aspects have remained underexplored, namely the headspace composition and the role of adhesive material. The

majority of microorganisms in marine sediments are known to be attached to particles, which results in micro-scale heterogeneity [24] that is linked to opposing gradients of methane and oxygen. Both gases overlap at very low concentrations in the top few millimeters below the sediment surface where methanotrophic growth is limited by the diffusive transport of both substrates [25]. For example, in paddy soil, over 30 operational taxonomical units (OTUs) corresponding to the MOB species level [26] were found that shared the same microenvironment but were physically separated according to substrate availability (high methane/low oxygen in deeper layers and low methane/high oxygen in shallower layers) [27]. Lower oxygen concentrations can select for a different MOB diversity [28, 29] by avoiding self-intoxication [30, 31] or selectively stimulate low oxygen adapted species [29]. The use of low to atmospheric methane concentrations have led to the discovery of high-affinity MOB, found especially in upland soils [32, 33]. In addition, higher than atmospheric concentrations of carbon dioxide are essential for autotrophic verrucomicrobial methanotrophs but also stimulate type II MOB using the serine pathway for carbon assimilation, which requires one carbon dioxide for each formaldehyde [34]. Opposing gradients of methane and oxygen have already been exploited in a gradient cultivation system [25]. And the use of adhesion to inert solid surfaces in liquid enrichment media also has shown promise for the cultivation of as-yet uncultured bacteria from various environmental samples. Here, we investigate the effect of headspace composition and solid surface material on methane-oxidizing activity in enrichments of marine sediments.

2.2. MATERIAL AND METHODS

Sediment and seawater sampling

Sediment samples from six marine stations, located in the Western Scheldt (Stations LS03, LS02, and LS01), the Belgian coastal zone (Station W04) and the open sea in the Southern Bight of the North Sea (Station W07tris and W09) were sampled mid-September 2012. All sampled stations are part of a routinely analyzed set of stations by the MUMM (Management Unit of the North Sea Mathematical Models and the Scheldt estuary) and reported by the Belgian Marine Datacenter. A large set of different physico-chemical parameters, measured over the last 25 years, from different marine matrices are freely accessible via www.mumm.ac.be/datacentre/. Using this dataset, stations for sampling were selected based on their location along an increasing salinity gradient and a decreasing inorganic nitrogen gradient from near to further off shore (both parameters were systematically monitored in the water column). Sampling was carried out with the RV Simon Stevin (http://www.vliz.be). Sediment samples were collected by means of a Reineck box-core at all stations, not disturbing the vertical sediment profiles. Sub-samples were taken from each sediment core with a single Plexiglas tube (internal diameter Ø 6.2cm; H: 25cm) and stored at 4°C till further processing in the lab. Surface seawater at 3m depth was collected per station with Niskin Bottles and stored in polycarbonate containers at 4°C.

The effect of attachment

Ten gram of marine sediment, collected at 0-2 cm depth, from station W04 was used as inoculum for enrichments under a headspace of 20:80 v/v methane/air. An initial enrichment was performed in a 60 mL serum bottles sealed with grey butyl rubber stoppers, using media mimicking the *in situ* low nutrient conditions. Low nutrient medium was prepared with natural *in situ* collected seawater supplemented with 25 µM KNO_3, 0.5 µM $NaNO_2$, 10 µM NH_4Cl, 4 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$ 30 µM FeNaEDTA and 5 mM HEPES (pH7.8). The enrichment was incubated at 20°C shaken at 100 rpm. A dense culture was observed after two months and several headspace and nutrient replenishment cycles. The culture was homogenized mechanically by use of 3 mm glass beats during vortexing. The obtained cell suspension (100 µL) was used to inoculate serum vials containing 20 mL low nutrient medium with and without adhesion material (n=2), as well as high nutrient medium (i.e. dNMS medium prepared with natural *in situ* seawater [19]) with and without adhesion material (n=2). Different adhesion materials were tested: (i) no sediment, (ii) 10 g of natural

sterilized *in situ* sediment, (iii) 10 g of acid-washed silicium dioxide 400-500 µm particles (Sigma Aldrich, CAS 60676-86-0), (iv) acid-washed silicium dioxide 0.5-10 µm particles (Sigma Aldrich, CAS 14808-60-7) and (v) acid-washed silicium dioxide 10-20 nm particles (Sigma Aldrich, CAS 7631-86-9). Silicium dioxide particles were added in three different concentrations: 0.2 g (1%), 0.02 g (0.1%), 0.002 g (0.01%). Cultures were incubated under a 25% methane headspace at 20°C and shaken at 100 rpm. *In situ* collected sediment was sterilized by rinsing thoroughly with distilled water, followed by an autoclaving cycle (20 min, 121°C, 2 atm), and finally dried at 96°C for 6h. Sterility of the sand was tested by plating on marine agar and inoculating liquid nutrient rich media. The particle size ranged from 130 to 330 µm with a d_{50} of 213µm.

The effect of headspace composition

Two gram of sediment from 0-2 cm depth from each station was suspended in 20 mL media mimicking the *in situ* nutrient concentrations. All media were prepared with aged seawater and consisted of 400 µM KNO_3, 5 µM $NaNO_2$, 100 µM NH_4Cl, 50 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 100 µM FeNaEDTA for LS03; 200 µM KNO_3, 3 µM $NaNO_2$, 80 µM NH_4Cl, 30 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 70 µM FeNaEDTA for LS02; 50 µM KNO_3, 1.5 µM $NaNO_2$, 50 µM NH_4Cl, 10 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 50 µM FeNaEDTA for LS01; 25 µM KNO_3, 0.5 µM $NaNO_2$, 10 µM NH_4Cl, 4 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 30 µM FeNaEDTA for the station W04; 15 µM KNO_3, 0.5 µM $NaNO_2$, 5 µM NH_4Cl, 2.5 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 20 µM FeNaEDTA for W07tris; and 5 µM KNO_3, 0.3 µM $NaNO_2$, 3 µM NH_4Cl, 1.5 µM KH_2PO_4, 1µM $CuSO_4.5H_2O$, 10µM FeNaEDTA for W09. Per station, triplicate enrichments were set up under different methane (200 ppmv & 10,000 ppmv), oxygen (2% & 20%) and carbon dioxide (400 ppmv & 50,000 ppmv) concentrations. Enrichments were incubated at 20°C and shaken at 100 rpm. Headspace was refreshed every two weeks. After two months, enrichments were homogenized by vortexing with 20-30 sterile 2 mm glass beats. The obtained cell suspension (10 ml) was used to inoculate 120 mL serum vials containing 3 g silicium dioxide (400-500 µm) and 10 mL double strength medium prepared with natural in-situ seawater. Final media composition was 250 µM KNO_3, 250 µM NH_4Cl, 1 mM KH_2PO_4, 1 µM $CuSO_4.5H_2O$ 40 µM FeNaEDTA, 5 mM HEPES (pH7.8) and trace and salt elements according to Whittenbury [1] for all stations. Serum bottles were sealed with grey butyl rubber stoppers and incubated at 20°C, 100 rpm under the different headspace compositions as specified above.

Analytical methods

Methane oxidation was monitored over time by measuring concentrations of methane, oxygen and carbon dioxide with a Compact GC (Global Analyzer Solutions, Belgium), equipped with one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. Values were converted to μM methane, oxygen and carbon dioxide by compensating for change in gas pressure (measured with an Infield 7 pressure meter, UMS, Germany) and taking the solubility of the gases into account. Grain size was determined by using laser diffraction, Malvern Mastersizer 2000. Statistical differences in methane oxidation rate (MOR) were assessed using factorial ANOVA after Levene's test for equality of variances followed by Least Significant Difference post-hoc testing in SPSS23 (n=2 for adhesion effect; n=3 for headspace composition).

2.3. RESULTS & DISCUSSION

The presence of mineral compounds, such as sediment, is assumed to exert a positive surface effect on bacterial productivity and substrate degradation [35, 36]. The addition of solid surfaces to which bacteria can adhere has already been shown to select for the enrichment of biofilm-forming and as-yet uncultured bacteria [37]. Here, the upper-cm of a sediment sample taken from the coastal station W04 enriched under a headspace of 20:80 v/v methane/air and actively oxidizing methane was homogenized and subcultured in various conditions to investigate the effect of adhesion material on the activity of methane-oxidizing bacteria. The addition of sterile natural *in situ* sediment had a clear effect on methane oxidation in *in situ* low nutrient conditions (fig. 2.1A). Sediments contain a large amount of minerals and nutrients that are in association with metals forming a solid phase. Despite vigorously washing the natural sediment, these mineral-bound metals have probably been retained and are readily available to the MOB. Under nutrient-limiting circumstances, microorganisms including MOB can colonize mineral surfaces and provide mineral dissolution [36, 38–41], leading to subsequent nutrient release and acquisition from the host mineral [39, 42, 43]. Therefore, the lack of activity in the absence of sterile sediment could result from nutrient limitation. To verify or refute that the observed effect of sterile sediment was merely due to adhesion, the experiment was repeated in nutrient rich medium (dNMS) (Fig. 2.1B) and with acid-washed silicium dioxide (Fig. 2.1C). Here, methane oxidation was observed without adhesion material added but the methane oxidation rate (MOR) was significantly lower than that with acid-washed silicium dioxide or sterile natural sediment ($MOR_{noaddition}$ = 0.346 ± 0.003 mM/day; $MOR_{silicium\ dioxide}$ = 0.629 ± 0.080 mM/day; $MOR_{sediment}$ = 1.549 ± 0.179 mM/day; $p < 0.004$). These data suggest that the observed positive effect on methane oxidation activity can be attributed to both adhesion, as acid-washed silicium dioxide does not contain any extra nutrients, and the nutrient release from natural sediment, since a clear but statistically not significant MOR difference between the addition of natural sediment and silicium dioxide was found. A likely group of nutrients that could explain this difference between both adhesion materials are the lanthanides. These compounds are readily present in marine ecosystems [44, 45], but are mainly incorporated in particulate material that form sedimentary deposits [44, 46]. These compounds have recently been discovered as an important cofactor for MDH [8] and were not added to the growth media used in this study.

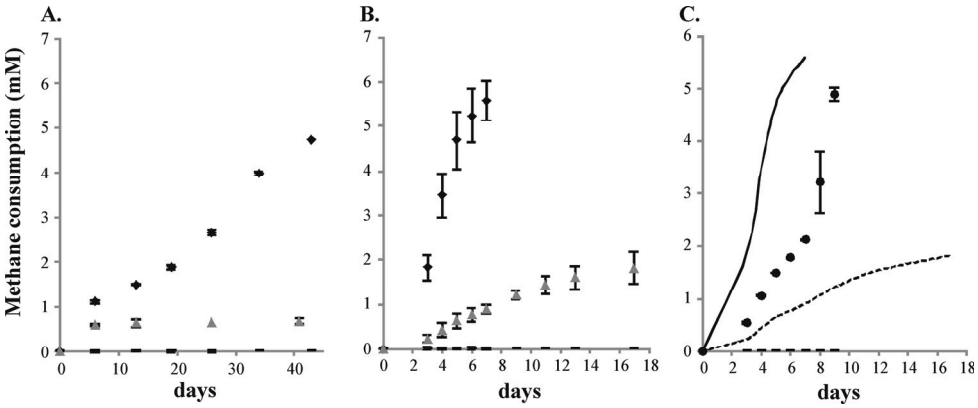

Figure 2.1. Effect of sediment addition on methane consumption of sample W04 over time in *in situ* low nutrient conditions (A), and dNMS (B & C). (A) Methane consumption is given for inoculated batch tests without (▲) and with sterile natural sediment (10 g; 130 to 330 µm) (♦) as well as for uninoculated negative control with sterile natural sediment (-) in *in situ* low nutrient conditions. (B) Methane consumption is given for inoculated batch tests without (▲) and with sterile natural sediment (♦) as well as for uninoculated negative control with sterile natural sediment (-) in dNMS. (C) Methane consumption is given for inoculated batch tests with acid-washed silicium dioxide (10 g; 400-500 µm) (●) as well as for uninoculated negative control (-) in dNMS. For comparison, solid and dashed line give methane consumption with and without sterile natural sediment respectively.

Next, the effect on methane oxidation activity of two different particle sizes of silicium dioxide, one larger (0.5-10 µm) and one smaller (0.01-0.02 µm; nanopowder) than the average bacterial cell, and different amounts thereof were tested (Table 2.1). The 0.5-10 µm silicium dioxide with a concentration of 1% (w/v) yielded the highest MOR, similar to the MOR observed for the 400-500 µm silicium dioxide. Lower concentrations of the 0.5-10 µm silicium dioxide, corresponding to a decreased sediment surface area, result in a lower MOR. The lowest concentration (0.01% w/v) had no effect and resulted in a MOR comparable to no addition. These data suggest that a minimum surface area is required for adhesion and subsequent growth and activity of the MOB. In contrast, a negative relationship between MOR and the amount of the added nanopowder was found, with the highest amounts resulting

in a lower MOR than without addition while the lowest amounts added had a similar positive effect on MOR than the highest concentration of the 0.5-10µm silicium dioxide (Table 2.1).

Table 2.1. Effect of size and amount of added sterile, acid-washed silicium dioxide on methane oxidation rate of sample W04. The main effect of size on MOR was significant ($p<0.005$), that of amount silicium dioxide added was not, although significant interaction effects were found ($p<0.05$) which are indicated per size group as different letters (combined letters are used to indicate non-significance for multiple variables).

Silicium dioxide		Methane oxidation rate (mM/day)
µm	% (w/v)	
0.5-10	0.01	0.331 ± 0.009^{A}
	0.1	0.516 ± 0.002^{B}
	1	0.608 ± 0.008^{BC}
0.01-0.02	0.01	0.546 ± 0.035^{a}
	0.1	0.336 ± 0.017^{b}
	1	0.281 ± 0.015^{c}

In aqueous environments adhesion is mediated by extracellular polymers formation (EPS) [47]. The involvement of EPS in bacterial attachment has been documented for both freshwater [48] and marine bacteria [49]. We observed a clear shift in the oxygen/methane ratio over time for the cultures grown with sterile natural sediment or silicium dioxide, from 1.25 (± 0.075) and 1.25 (± 0.044) respectively in the first cycle to 1.50 (± 0.038) in the second cycle. Remarkably, during the first methane cycle, the start of methane oxidation coincided with an immobilization of the adhesion material in the serum vial (Fig. 2.2). When methane is completely oxidized to CO_2 a ratio of 2 is theoretically expected, whereas a partial oxidation of methane to methanol would give a ratio of 1. As the ratio during the first methane cycle is closer to one, we assume that more methane is partially being oxidized and probably used for the production of EPS to establish a stable attachment to the sand particles. Oxygen concentration plays an important role in the regulation of methane oxidation and the microbial ecology of methanotrophs. At high oxygen tension intermediates or waste products of methane oxidation, such as methanol, formaldehyde and formate, have been detected in methanotrophic cultures that may reach detrimental concentrations [30, 31] The formation of EPS can create an oxygen diffusive barrier and thus lower oxygen zones in response to high oxygen concentrations [50]. But a minimum amount of substrate might be required to

bind/hold the formed EPS in place allowing the formation of a protective envelope resulting in a diffusion gradient. Earlier studies already reported on the effect of silicium dioxide nanopowder on the cell properties of *Methylomonas rubra* cells [51]. The cultivation of *M. rubra* cells in the presence of silicium dioxide resulted in a 30% increase of methane oxidation activity [52]. Interestingly transmission electron micrographs revealed the formation of a silicium dioxide complex forming a protective envelope around the bacterial cells. In that study this interaction resulted in an increasing negative charge of the cells. However the formation of this envelope might act as a diffusion gradient protecting the cells against harmful oxygen concentration. As a result, to high concentrations of silicium dioxide nanopowder might result in a cell envelope completely blocking the diffusion of oxygen to the cell, thus resulting in a lower MOR. A decreased activity and mobility of the cells was also observed with increasing silicium dioxide concentrations [51]. Further supporting the diffusion gradient protecting silicium dioxide envelope effect, is the absence of EPS formation in the dispersion medium after the interaction between *M. rubra* and the silicon dioxide [51].

Figure 2.2. Methane oxidation led to a immobilization of the sand particles, suggesting the formation of EPS during growth. The immobilization is visualized by turning the serum vials upside down. The sand pellet remained on the bottom of the serum vial in cultures demonstrating methane oxidation, whereas the sand moved towards the other side in control samples.

Adhesion to small-sized particles leads to EPS formation and immobilization which creates a stratified environment in which gas gradients can built up and various microniches are formed. We investigated the combined effect of low and high concentrations of methane (200 ppmv and 10,000 ppmv), oxygen (2% and 20 %) and carbon dioxide (400 ppmv and 50,000 ppmv) on methane oxidation in triplicate enrichments of the upper-cm of six different stations along a transect in the North Sea from estuarine to open sea sediments with acid-washed silicium dioxide (10 g; 400-500 μm) as adhesion material. As expected, methane concentration

significantly influenced MOR (Fig. 2.3) ($p<0.005$). Increased carbon dioxide concentration in the headspace only positively affected MOR under high methane concentrations ($p=0.00035$). A positive effect of lower oxygen concentration on MOR was limited to estuarine samples, was station-specific, dependent of carbon dioxide concentration, and only observed under high methane concentration: for LS01 at 400 ppmv carbon dioxide, and for LS02 and LS03 ($p<0.00001$) and W09 ($p=0.05$) at 5% carbon dioxide. These results demonstrate that concentrations of methane and carbon dioxide are crucial factors in the cultivation of marine MOB. Despite the importance of oxygen concentrations in the growth of methanotrophs, no clear uniform effect could be observed during this study. Perhaps the presence of adhesion material in the current set-up obscured the effect of the oxygen concentration by possible formation of a diffusive gradient through EPS production.

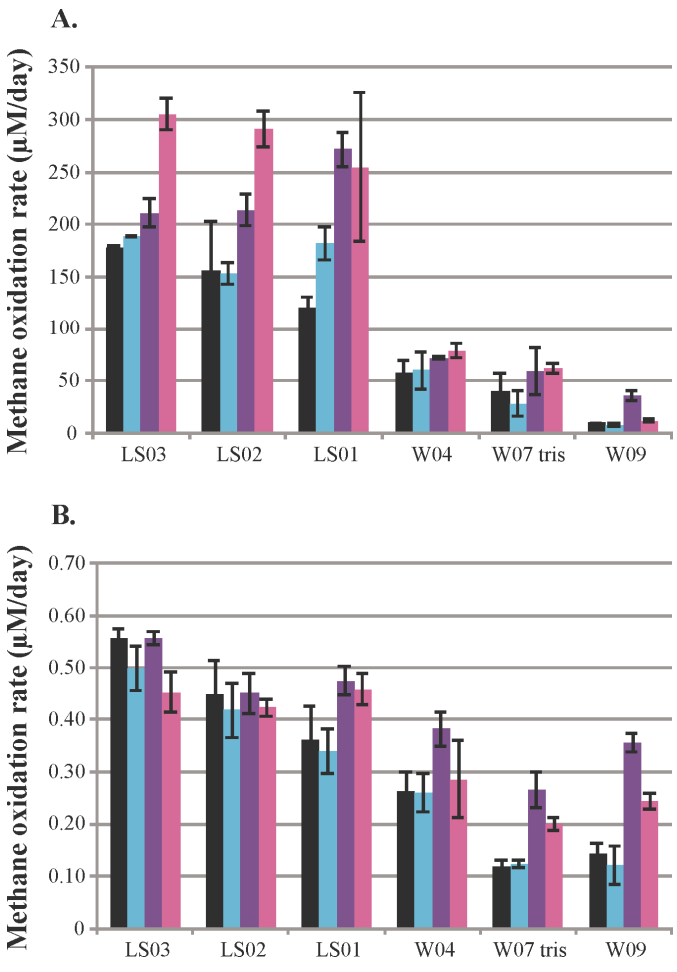

Figure 2.3. Effect of concentrations of methane, oxygen and carbon dioxide on methane oxidation rate over different stations for 10,000 ppmv (A) and 200 ppmv (B) methane. Combination of 2% oxygen and 400 ppmv carbon dioxide is given in black, 20% oxygen and 400 ppmv carbon dioxide in blue, 2% oxygen and 50,000 ppmv carbon dioxide in magenta and 20% oxygen and 50,000 ppmv carbon dioxide in pink. Standard deviations are given as error bars (n = 3).

In conclusion, the inclusion of adhesion material, preferably sterile natural sediment or alternatively and more conveniently silicium dioxide particles larger >0.5 µm, positively affects methane oxidation activity in enrichments of marine sediments and thus might facilitate the cultivation and subsequent enrichment of members of this functional guild. The exact mechanism of this positive effect needs further investigation but might be the facilitation of methane, carbon dioxide and oxygen gradients. The differential effect on MOB diversity and abundances still needs to be explored.

2.4. ACKNOWLEDGEMENTS

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Chapter 3

Methane oxidation and differential growth responses suggest extreme niche specialization in marine methylotrophic *Methyloceanibacter*

Redrafted from:

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Author's contributions:

B.V., P.D.V, P.V., N.B. and K.H. designed the research; B.V. performed research; B.V, G.C., F.M.K. and K.H. analyzed data; B.V., H.O.d.C. and K.H. interpreted the results and wrote the paper.

3.1. INTRODUCTION

Methylotrophs are key players in global marine carbon cycling [1–4]. They are capable of growth on methanol as well as other reduced methylated one-carbon (C_1) compounds such as methane, methylamine or formate [2, 5, 6]. Methanol is one of the major oxygenated volatile organic compounds in marine systems with major sources being atmospheric deposition, phytoplankton excretion and the turnover of the potent greenhouse gas methane emitted from the seafloor and organic rich sediments by methane-oxidizing methylotrophs (further denoted as methanotrophs or MOB). Ocean surface concentrations of methanol can reach up to 100 nM in the Pacific Ocean [7] and 300 nM in the Northern hemisphere [8], but it is unclear whether oceans are a methanol sink or source to the atmosphere [7, 9–12]. Methylotrophic bacteria, together with mixotrophic eukaryotes, represent an oceanic sink for methanol, mainly through its use as energy source and to a lesser extent as carbon source [13]. Using DNA-stable isotope probing *Methylophaga* spp. and other, novel *Gammaproteobacteria* were found as major consumers of methanol and methylamine, in surface seawater [14]. Cultivation-based studies have demonstrated that alphaproteobacterial SAR 11, which are some of the most abundant heterotrophs in the oceans, use one-carbon compounds like methanol for energy generation [15], while the betaproteobacterial OM43 clade is a dominant marine methylotroph [16, 17] in productive coastal waters, including in the North Sea [18].

Despite their crucial role in marine carbon cycling, only a handful of marine methanol oxidizers and even fewer methane oxidizers, the latter all belonging to the *Gammaproteobacteria* (designated as Type I MOB), have been isolated and physiologically characterized [16, 19–29]. However, cultures remain pivotal to unravel complex biological traits important to marine biogeochemistry, especially since evidence for wide ecotype variation among methylotrophs is accumulating. For example ecotypes have been identified among *Methylophilaceae* isolated from Lake Washington; these were characterized by differential responses to specific C1 stimuli [30], environmental adaptation strategies [31] and nitrate removal potential (assimilatory versus respiratory nitrate reduction) [30]. Noteworthy is that ecological population boundaries frequently occur at deep phylogenetic levels [32–35], which might preclude meta-omics approaches from uncovering essential trait differences among closely related strains with identical biomarker sequences. This deep-phylogenetic trait separation has already been demonstrated for the nitrogen metabolism of terrestrial methanotrophic *Methylomonas* strains [36]. Such niche specialization among congenerics (i.e.

belonging to the same genus) and even conspecifics (i.e. belonging to the same species) is a major confounding factor for linking biodiversity to ecosystem functions and further warrants the capture of a greater representation of marine methylotroph diversity into pure culture. These cultures should serve to expand understanding of the traits that influence their activity patterns in marine systems such as the capacity to use alternative carbon and energy sources or the interaction with nitrogen and sulfur cycling.

We now report an extreme example of niche specialization among marine methylotrophs discovered via shotgun sequencing of highly enriched methane-oxidizing cultures and subsequent isolation of the methylotrophs from these enrichments. Within the recently described methylotrophic genus *Methyloceanibacter*, we found representatives of four novel species. One of these new species is capable of methane oxidation, for which it uses solely the soluble methane monooxygenase (sMMO), making this the first alphaproteobacterial methanotroph (designated as type II MOB) isolated from a marine system, the third methanotrophic taxon only possessing sMMO and the first methanotroph within a non-methanotrophic genus. Furthermore, converging with the species boundaries, *Methyloceanibacter* spp. displayed a wide ecotypic variation related to growth kinetics on methanol, and preferences for nitrogen, pH, temperature and salt.

3.2. MATERIAL AND METHODS

Sampling, enrichment and isolation.

Marine surface sediments were collected in 2012 at two different stations in the Belgian North Sea, W04 (Vlakte van de Raan, N 51.449166 E3.237166) and W09 (Hinderbanken, N 51.75 E2.7), and used as inocula for enrichments. Initial enrichments were performed using media mimicking the *in-situ* nutrient conditions, based on *in-situ* concentrations measured periodically by the Belgian Marine Datacenter (http://www.mumm.ac.be/datacentre/) over the last 24 years. Phosphate concentrations were adjusted to the Redfield ratio (N/P: 16/1) [37] as data for phosphate was unavailable. Media composition was as follows: 25 µM KNO_3, 0.5 µM $NaNO_2$, 10 µM NH_4Cl, 4 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 30 µM FeNaEDTA for W04; 5 µM KNO_3, 0.3 µM $NaNO_2$, 3 µM NH_4Cl, 1.5 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 10 µM FeNaEDTA for W09. All media were prepared with aged seawater, collected at three meter depth from the sampling point. Enrichments were incubated at 20°C, shaking at 100 rpm and a headspace composition of 20:80 v/v methane/air.

After methane oxidation was observed and dense culture developed, the enrichments underwent an extinction culturing NaCl-dANMS media [38] supplemented with 1 mM KH_2PO_4, 500 µM NH_4Cl, 500 µM KNO_3, 1 µM $CuSO_4.5H_2O$, 40 µM FeNaEDTA, 100 nM of the lanthanides $LaCl_3$, $CeCl_3.7H_2O$, $NdCl_3.6H_2O$, $PrCl_3$ and 5 mM HEPES at a final pH of 7.8. Highest dilutions showing growth were transferred and underwent numerous subcultivations over a period of three years. Repeated subcultivations were not sufficient to purify the methane-oxidizing bacteria but resulted in a stable MOB-heterotroph community. Isolation of the strains was performed by plating on the growth medium solidified with Gelzan (1 % w/v) and supplemented with 0.5% methanol. After several months of incubation small white colonies formed on the highest dilution plates. These were picked and placed in 5 mL liquid medium containing 0.5% methanol as sole carbon source. Picked colonies were identified using partial 16S rRNA gene sequences, as described previously [39], and only isolates affiliated with *Methyloceanibacter* were retained. Purity was checked routinely through (i) microscopic evaluation and (ii) plating on 1/10 Trypticase Soy agar (TSA) under air atmosphere.

Genotyping and next generation sequencing.

Genomic DNA was obtained by using Guanidium-thiocyanate-EDTA-sarkosyl for the enrichment cultures [40] or hexadecyltrimethylammonium bromide (CTAB) for the pure cultures [41]. DNA quality and quantity were checked on agarose gel and with Qubit Fluorometric Quantitation.

Pure cultures were typed by performing $(GTG)_5$ and Box rep-PCR fingerprinting as previously described [42]. Random shotgun sequencing of pure and enrichment cultures were done using the IonTorrent PGM as described previously [43]. Analysis of reads was performed using SPAdes genome assembler v.3.5.0 [44] and CLC genomics workbench v7.0.4 (CLCbio, Denmark). The reads were length and quality score trimmed and used for *de novo* assembly. Contig binning of metagenomes from enrichment cultures was done by comparing GC content to sequencing depth and resulted in one and two draft genomes for enrichment W04 and W09 respectively. The Rapid Annotation Subsystem Technology (RAST) server was used for functional annotation and metabolic reconstruction of the (meta)genomes [45, 46]. The classic RAST annotation scheme was selected using RAST gene caller which allowed automatic error fixing, frameshift correction and the backfilling of gaps. Assigned functions were verified with PSI-BLAST [47]. Missing genes were searched for in the genome with pBLAST using homologous amino acid sequences from the closely related organisms.

Ecophysiology.

Growth of the different strains in different culture conditions was assessed in batch cultures (5 ml) by varying carbon source, nitrogen source, temperature, salinity and pH. All strains were grown at 28°C in NaCl-dANMS media [38], i.e. dANMS supplemented with 3% NaCl, containing a concentration of 1 µM $CuSO_4.5H_2O$, 1 mM KH_2PO_4, 2 mM KNO_3, 2 mM NH_4Cl, 40 µM FeNaEDTA, 100 nM of the lanthanides $LaCl_3$, $CeCl_3.7H_2O$, $NdCl_3.6H_2O$, $PrCl_3$ and 5 mM HEPES, unless indicated otherwise. Methanol (1%) was used as the sole carbon and energy source. Each growth conditions was tested in triplicate. Utilization of the following alternative carbon sources (0.03%) was evaluated by monitoring growth in media without methanol: tryptic soy broth, acetate, acetate, formate, methylamine, dimethyl sulfoxide, dimethylcarbonate, formamide, ethanol, and no carbon source as negative control. Utilization of nitrate, ammonium was tested at concentrations of 0.5 mM, 1 mM, 2 mM, 5 mM and 10 mM. Alternatively yeast extract (0.03% w/v) was added to test as nitrogen source.

The ability to fix nitrogen was assessed by evaluating growth in nitrogen free medium with 1% methanol under high oxygen tensions (20.94% O_2). Nitrous oxide production was tested for the different nitrate and ammonium concentrations by measuring headspace composition after 6 weeks growth. Salt tolerance (tested at 0%, 3%, 5% and 10% NaCl w/v spiked), pH range (tested at 5.8, 6.3, 6.8, 7.3, 7.8, 8.5, 9, 10 and 11) and temperature range (10°C, 18°C, 28°C, 37°C, 45°C and 52°C) were also determined.

Growth kinetics on methane and methanol.

Cells harvested from mid-exponentially grown cultures were used as inoculum to start methane and methanol growth curves. Cells were washed twice with fresh carbon free medium and inoculated to a final starting OD_{600} of 0.01. Methane oxidation was tested in 15 ml serum vials, containing 2.5 ml dANMS medium supplemented with 3% NaCl and 1% methanol. Vials were capped with grey butyl rubber stoppers at 28°C, shaking at 100 rpm and a headspace composition of either 20:80 v/v methane/air, 5:20:75 carbon dioxide/methane/air or 5:95 carbon dioxide/air. Per time point headspace composition analysis and biomass determination of three serum vials were analyzed, which were subsequently discarded from the experiment. Because of attached cell growth to the serum vials glass by strain R-67177, cell flocks were disrupted with a manual glass homogenizer before biomass determination.

Carbon source preference and sMMO activity assay.

Preferential use of methane or methanol as carbon source was tested using mid-exponentially grown cells as inoculum at a starting OD of 0.01 in 200 mL dANMS medium in 1L bottles. Medium was spiked with methanol to a final concentration of 1% under a headspace composition of 5:20:75 carbon dioxide/methane/air. Subsequently growth and methane were monitored over time in all six replicates. As negative control sterile medium was used and incubated under the same conditions. Qualitative sMMO expression was tested by the naphthalene oxidation assay in liquid cultures [48]. Napthol inoculated cultures and methane grown cultures were used as positive control. Quantitative sMMO activity assay was performed as described previously [49]. In short, 10^9 cells of mid-exponentially grown methanol fed cultures were harvested and washed twice in HEPES buffer (10 mM, pH 7.8), suspended in 1 ml HEPES buffer and placed into 60 mL glass vials sealed with grey butyl rubber stoppers and aluminum crimp seals. Methane and carbon dioxide were added to a final concentration of 5:20:75 carbon dioxide/methane/air, vials were incubated shaken (100 rpm) at 28°C and the headspace was sampled every hour during 5h and after 24h. Negative controls

consisted of heat killed cells incubated under identical conditions. Three replicates were assayed per sample per time point.

Analytical methods.

Growth was monitored over time by optical density at 600 nm using a Spectramax plus 384 spectrophotometer (Molecular devices). Mid-exponentially grown cells were used to correlate biomass to cell counts using live/dead flow cytometry as described previously [50]. Concentrations of methane, oxygen and carbon dioxide were measured with a Compact GC (Global Analyzer Solutions, Belgium), equipped with one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. Values were converted to µmol Liquid^{-1} for methane, oxygen and carbon dioxide by compensating for change in gas pressure (measured with an Infield 7 pressure meter, UMS, Germany) and taking the solubility of the gases into account. Methanol was analyzed using a 930 Compact IC Flex (Metrohm, Switzerland) ion chromatography system. Separation occurred at 35 °C on a Metrosep Carb 2 (250/4.0) column behind a Metrosep Trap 1 100/4.0 guard column. The eluent was 20 mM NaOH at a flow rate of 0.8 mL min^{-1}. An IC amperometric detector (cycle: 300 ms 0.05V, 50 ms 0.55 V, 200 ms -0.1V detection during 200-300 ms of each cycle) was used for detection of eluted components. The sample aspiration needle was cleaned with acetone between each analysis. The lower limit of quantification was 79.2 mg L^{-1}. Standards and controls were regularly used for calibration of the signals.

Data analyses.

A parametric fit of growth data for each replicate was made using non-linear least squares fitting with four different parametric models, i.e. the Gompertz, Exponential Gompertz, Richards and logistic growth curve models, using Grofit package v1.1.1-1 [51] in R 3.2.3. (data was ln(N/N0) transformed for specific growth rate fitting [52]. The model with the best Akaike Information Criterion is returned. Non-parametric fitting using lowess is used to perform initial parameter estimation. Generation time was calculated based on specific growth rate. For significance testing, one-way ANOVA with Tukey HSD contrasts was run if the data was homoscedastic (Modified Levene's test) and the residuals normally distributed (Shapiro-Wilks normality test). If the homoscedasticity assumption was not met, Weighted least squares was performed with Tukey simultaneous relative contrast effects for general linear hypotheses with a single-step method [53], while if the normality assumption was not met non-parametric multiple contrast effects were used for the multiple comparison [54].

ACCESSION NUMBERS.

The Whole Genome Shotgun projects of *Methyloceanibacter* sp. R-67174, R-67175, R-67176 and R-67177 have been deposited at DDBJ/EMBL/GenBank under the accession numbers LPWG00000000, LPWF00000000, LPWE00000000 and LPWD00000000 respectively. The versions described in this paper are the first versions. The reads of the metagenomes of both enrichment cultures are available in the SRA database (study accession number SRP068992).

3.3. RESULTS

Enrichment and isolation of four novel *Methyloceanibacter* species.

North Sea sediments from two sampling sites (W04, Vlakte van de Raan; W09, Hinderbanken) were incubated at 20°C with a headspace composition of 20:80 v/v methane/air under conditions mimicking the *in situ* nutrient availability. Methane oxidation was observed within one to two weeks, and after periodical headspace refreshments during two months, dense cultures were formed. Through extinction culturing under methane atmosphere, transfer of highest positive dilutions and numerous subcultivations over a period of three years, these two methane-oxidizing cultures were highly enriched. Subsequent metagenome sequencing revealed the presence of one highly enriched bacterium in sample W04 (representing 83.10% of the total reads) and two in sample W09 (together representing 87.04% of total reads). All three genomic bins were closely related with the recently described methylotrophic Alphaproteobacterium *Methyloceanibacter caenitepidi* Gela4^T, obtained from marine sediment collected near a hydrothermal vent in Japan [23]. Surprisingly, no *pmoA* genes encoding the particulate methane monooxygenase (pMMO) were detected in the metagenomes. However, one genomic bin in each metagenome contained a complete operon encoding a sMMO, making it very likely that these *Methyloceanibacter* spp. were responsible for the observed methane oxidation of the enrichment cultures. Furthermore, the second bin in sample W09 seemed to be originating from a methylotrophic *Methyloceanibacter* sp without sMMO encoding genes.

In a final effort to obtain pure cultures of *Methyloceanibacter*, methanol was used as carbon source and small white colonies were obtained on plate after two months. Forty-one *Methyloceanibacter* isolates (as verified by partial 16S rRNA gene sequencing) were picked up, genotyped with $(GTG)_5$ and Box rep-PCR fingerprinting and grouped into four separate clusters, all distinct from *M. caenitepidi* Gela4^T. One representative was chosen per cluster and designated with strain numbers R-67174, R-67175, R-67176, and R-67177 (Fig. S3.1). R-67174 was solely isolated from site W04, R-67175 and R-67177 solely from site W09, and R-67176 from both sites. Genomes of all four strains were sequenced (Table S3.1) and genomic taxonomy (Table S3.2) demonstrated that each strain represents a novel species within the genus *Methyloceanibacter* (Fig. S3.2). The strains clearly differed in growth morphology in liquid medium, with R-67174 forming small irregular flocs, R-67175 dense single cell cultures, R-67176 slightly dense single cell cultures with occasionally small flocs and R-

67177 attaching firmly to the side of the vials. *M. caenitepidi* Gela4^T also formed single cell cultures, similar to R-67175. All strains formed identical colonies (round, white and convex) on solid media and displayed similar rod like shaped cell morphology, similar to *M. caenitepidi* Gela4^T, with Gram-negative staining [23].

R-67174 showed a 100% 16S rRNA gene sequence similarity with both methanotrophic genomic bins of the W04 and W09 enrichments, although all isolates representing strain R-67174 originated from WE04. Its methane oxidation capacity was indeed confirmed physiologically and its genome contained a complete operon encoding a sMMO (see below).

C1 metabolism

Use of methane and methanol as sole source of carbon and energy was tested for all *Methyloceanibacter* strains including *M. caenitepidi* Gela4^T. Only R-67174 demonstrated growth coupled to methane and oxygen decrease and carbon dioxide increase over time (Fig. 3.1A). Methane oxidation by strain R-67174 was clearly inhibited partially or completely by black butyl rubber stoppers, but no growth inhibition was detected on methanol, indicating a possible inhibitory effect of black butyl rubber stoppers at the methane monooxygenase level (data not shown). The addition of extra carbon dioxide to the headspace had a growth-promoting effect by decreasing the lag phase, as could be seen from the methane oxidation profile (Fig. 3.1B). This effect was not linked to an acidification of the media as no significant pH shift was measured under increased carbon dioxide concentrations (data not shown). Furthermore cultures grown to stationary phase reached similar maximal cell densities (appr. 23 x 10^7 cells/mL) refuting autotrophic growth, which is supported by the lack of RubisCO genes necessary for fixation of the inorganic carbon dioxide into organic carbon. Thus, as R-67174 is a type II methanotroph, the effect of carbon dioxide is most likely linked to use of the serine pathway for carbon assimilation (Table S3.3), which requires one carbon dioxide for each formaldehyde [2]. As was expected from the metagenome data, the genome of R-67174 lacked *pmo* genes and contained a complete *mmoXYBZDC* operon for a sMMO (Fig. 3.1C). MmoX was most affiliated with that of *Methylocella silvestris* and *Methyloferulla stellata* (92.6 and 91.3% aa similarity) (Fig. 3.2), both type II methanotrophs with solely a sMMO. The genome environments of their sMMO operons were identical and mainly differed from other methanotrophs in the localization of adjacent *mmoR* and *mmoG* genes (Fig. S3.3).

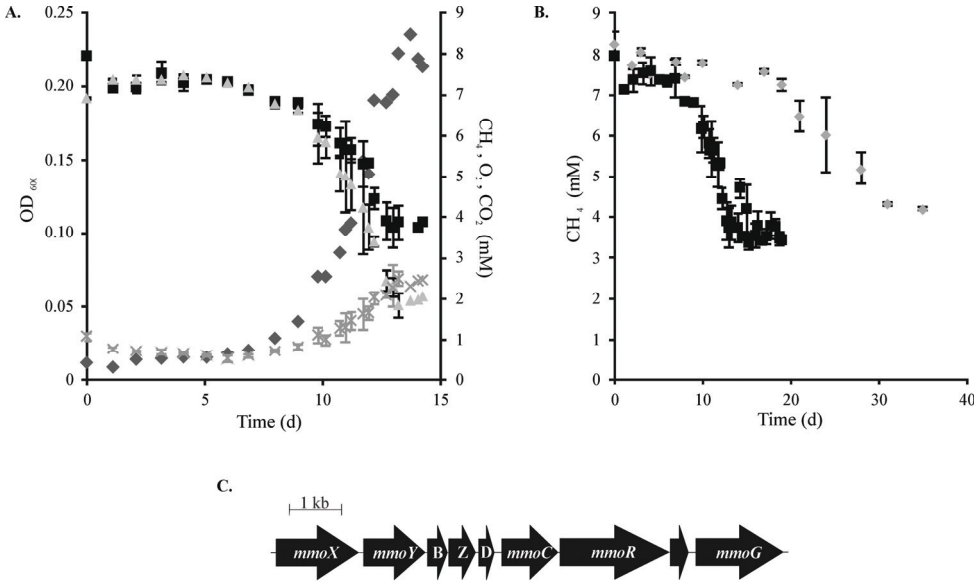

Figure 3.1 | Physiological (A, B) and genomic (C) evidence for methane oxidation by *Methyloceanibacter* sp. R-67174. (A) Growth under aerobic methane atmosphere amended with carbon dioxide was measured as OD_{600} (♦) (n=3). It coincided with methane (■) and oxygen (▲) consumption and carbon dioxide (X) production, indicative of methane oxidation activity. (B) methane oxidation activity under aerobic methane atmosphere with (■) and without (♦) amendment with carbon dioxide (n=3). Standard deviations are given as error bars but were omitted for OD_{600} for clarity. (C) A complete sMMO operon found in the genome supports the physiological observations. The open reading frames are drawn to scale and arrows show the direction of transcription.

R-67174 clearly demonstrated preferential growth on methanol compared to methane (Table 3.1), which is a common observation for pMMO lacking methanotrophs [55, 56], with a comparable effect of carbon dioxide addition as mentioned higher for methane (shortened lag phase without increased maximum cell density or specific growth rate). Also when both carbon sources were given at the same time, R-67174 preferred methanol as carbon and energy source: methane concentrations remained unaltered over time as growth progressed (Fig. S3.4), while lag phase, specific growth rate and maximal cell density did not differ significantly from those on methanol alone (Table 3.1). A naphthalene oxidation assay on mid-exponentially grown methanol-fed R-67174 cells further confirmed that the sMMO was not expressed (qualitative evaluation, data not shown).

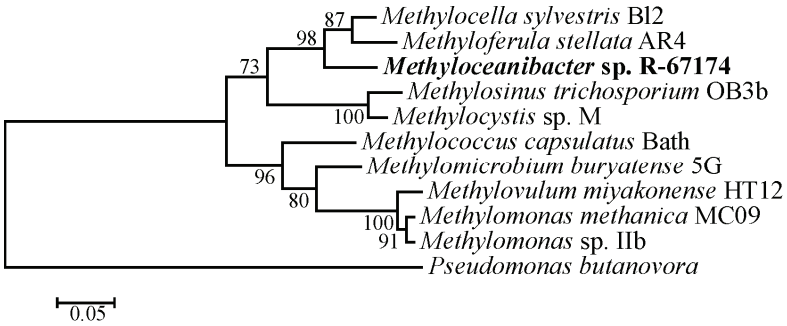

Figure 3.2 | Phylogenetic Maximum Likelihood tree showing the affiliation of the soluble methane monooxygenase. The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model [57] using 473 amino acid position. The tree with the highest log likelihood (-3557.4323) is shown. Bootstrap values (1000 replicates) higher than 70 are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5761)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Alignment and evolutionary analyses were conducted in MEGA6 [58]. Only complete sequences from genomes were included. Butane monooxygenase of *Pseudomonas butanovora* was used as outgroup.

All strains demonstrated growth with methanol as the sole carbon and energy source (Fig. 3.3). Strikingly, statistically significant strain-specific differences in kinetic parameters were observed (Fig. 3.3, Table 3.1). Gela4^T had the shortest lag phase and highest growth rate, while R-67175 needed more than eight days to start exponential growth but then reached the highest maximum cell density. In comparison, growth of R-67177 was more than five time slower and reached stationary phase at a cell density almost 1.5 order of magnitude lower. All the genomes contained a single gene cluster (*mxaFJGIRSACKLD*) encoding the canonical MxaFI methanol dehydrogenase (Table S3.3). MxaF sequences of *Methyloceanibacter* spp. were highly similar (from 100% between Gela4^T and R-67175 to 84.8% aa similarity between R-67174 and R-67177) and were most affiliated with MxaF from *Methyloligella solikamskensis* (Figure S3.5) (78.8 - 89.4% aa similarity). Other essential genes for the expression of the pyrroloquinoline quinone catalytic cofactor (*pqqABCDE*) were found (Table S3.3). The genomes also contained multiple *xoxF* genes [59–61], coding for the lanthanide dependent methanol dehydrogenase (Fig. S3.5; Table S3.3). Gela4^T, R-67174 and R-67176 had four *xoxF1* copies (two adjacent and two separated), R-67175 two adjacent *xoxF1*, and R-67177 two *xoxF1* copies (and two truncated genes located separately) as well as one complete

xoxF3. All XoxF1 grouped into three cluster (Fig. S3.5; clusters C1, C2, C3), with >90% aa similarities within each cluster. R-67174, R-67175 and R-67177 also encoded a PQQ-ADH type 9 quinoproteins (Fig. S3.5) [60]. Unfortunately, there is no clear link between the *xoxF* gene inventories and the kinetic parameters on methanol. But *xoxF* expression in methylotrophs containing calcium-dependent MxaF has already been shown in the marine environment for *Methylophaga* [62]. So, considering that lanthanides (specifically cerium, lanthanum, praseodymium and neodymium) were detected in the 0.1 to 10 nanomolar range in pore water of the sediment at each sampling site in this study and excess of these lanthanides were always added to the growth medium (throughout the enrichment procedure and during growth experiments), it is plausible that their differential expression contributed to the significant strain-dependent differences in growth on methanol.

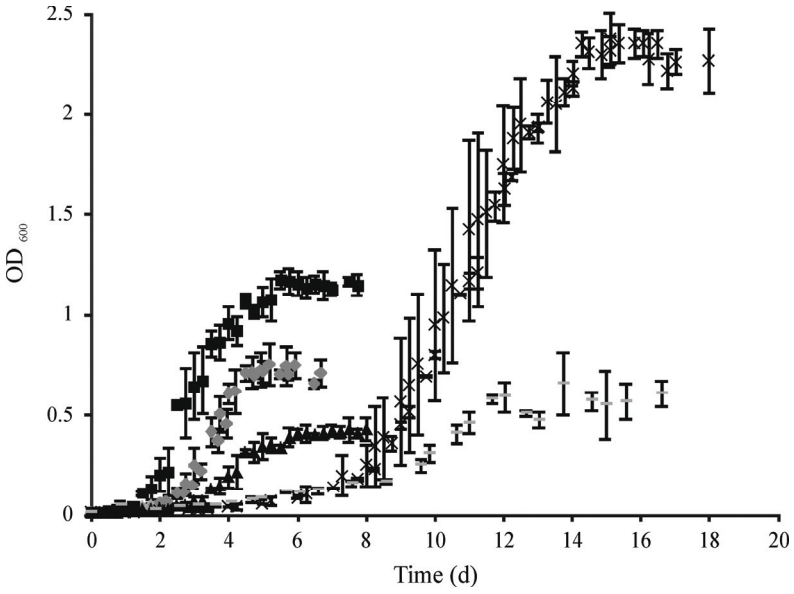

Figure 3.3 | Growth on 1% methanol for different *Methyloceanibacter* spp over time. R-67174 (▲), R-67175 (X), R-67176 (♦), R-67177 (–), Gela4T (■). All strains were grown till stationary phase (based on at least 3 replicate measurements) and demonstrated clear differences in lag phase, growth rate and maximum cell density (see Table 3.1).

Table 3.1 | Kinetic parameters for growth on 1% methanol for different *Methyloceanibacter* spp. Standard deviations are given between brackets (based on at least 3 replicate measurements). Significance ($p<0.01$) of between-strain differences for growth on methanol and within-strain differences per growth condition for R-67174 were determined via one-way ANOVA (see Materials and Methods for details) and are displayed as different upper- and lowercase letters respectively (combined letters are used to indicate non-significance for multiple variables).

Strain	Condition	Lag phase λ (d)	Specific growth rate (h^{-1})	Generation time (h)	Maximum cell density (10^8 cells mL^{-1})
R-67174	Methane + carbon dioxide	8.96^{a} (0.43)	0.017^{a} (0.001)	41.85^{a} (2.49)	2.32^{a} (0.06)
	Methanol	2.97^{bA} (0.25)	0.033^{bA} (0.002)	21.07^{bA} (1.44)	4.19^{bA} (0.14)
	Methanol + carbon dioxide	2.01^{c} (0.26)	0.033^{b} (0.002)	20.98^{b} (1.52)	4.62^{b} (0.27)
	Methane + methanol + carbon dioxide	1.76^{c} (0.34)	0.032^{b} (0.001)	21.40^{b} (0.94)	4.28^{b} (0.32)
R-67175	Methanol	8.23^{B} (0.15)	0.021^{B} (0.002)	33.06^{B} (2.63)	47.43^{B} (0.79)
R-67176	Methanol	2.72^{A} (0.14)	0.049^{C} (0.005)	14.14^{C} (1.44)	14.48^{C} (0.31)
R-67177	Methanol	6.11^{C} (0.59)	0.012^{D} (0.001)	57.67^{D} (2.43)	1.22^{D} (0.06)
Gela4^{T}	Methanol	1.57^{D} (0.09)	0.069^{E} (0.002)	10.02^{E} (0.31)	35.00^{E} (0.69)

Differential nitrogen preferences, alternative carbon source utilization and responses to environmental stimuli

Detailed physiological and genomic data revealed clear differences in the nitrogen metabolism between the *Methyloceanibacter* strains. All strains could transport and assimilate ammonium via glutamine synthetase – glutamate synthase (GS-GOGAT), while Gela4^{T}, R-67175 and R-67177 were also able to assimilate nitrate transported to the cytoplasm via an ABC-type nitrate transporter (Table S3.3). All strains indeed utilized ammonium as sole nitrogen source within a range of 0.5 to 10 mM (Fig. 3.4; Table S3.4), with mostly broad concentration range between 2 and 10 mM but with a well-defined preference at 10 mM for R-67176. For nitrate as sole nitrogen source, R-67175 and R-67177 showed highest cell density at 5 mM and Gela4^{T} at 10 mM (Fig. 3.4; Table S3.4). Genes for the dissimilatory membrane-bound cytoplasmic nitrate reductase NarG and one or two NarK-type nitrate/nitrite antiporters were found in all genomes except that of the methanotroph R-67174 (Table S3.3). NarG aa similarity ranged between 82.8-99.8% and its phylogeny agreed with that of the 16S rRNA gene (Fig. 3.2) and MxaF (Fig. S3.5), suggesting that R-67174 might have lost the *nar* operon quite recently as it still contained a *narK*. Although dissimilatory nitrate reduction was not experimentally verified, nitrous oxide production by Gela4^{T}, R-67175 and R-67177 grown

on nitrate was observed at end exponential/early stationary phase (at oxygen concentration between 100-300 μM), suggesting that dissimilatory nitrate reduction proceeds once oxygen is depleted. The amount of produced nitrous oxide appeared correlated with the initial nitrate concentration and 4 to 8% of initial nitrate was converted to nitrous oxide (data not shown). This low conversion rate combined with the absence of denitrification genes in the genomes, the presence of several genes for nitrosative stress response regulators (NsrR, NnrR, NnrS and NnrU) and nitrous oxide production being restricted to strains able to assimilate nitrate led us to hypothesize that nitrous oxide is produced via a nitrite detoxification pathway. Similar to nitrate-reducing *Enterobacteriaceae* [63–65], high - potentially toxic - levels of produced nitrite could be converted to nitric oxide via the dual action of Nar and/or NirB. However, genes encoding the usual suspects for the further reduction to nitrous oxide, namely dedicated nitric oxide reductases like qNorB and Cu_ANor or flavohemoglobin Hmp were absent from the genomes. Lastly, the genomes of R-67174 and R-67176 also contained a complete gene inventory for nitrogen fixation (Table S3.3), with gene organization similar to that of *Methylocella silvestris* BL2 and a NifH aa sequence similarity of 95.9%. Although it was not experimentally verified under low oxygen tension, nitrogen fixation could compensate for the inability to assimilate nitrate under ammonium limitation or provide a competitive benefit in absence of (in)organic nitrogen sources.

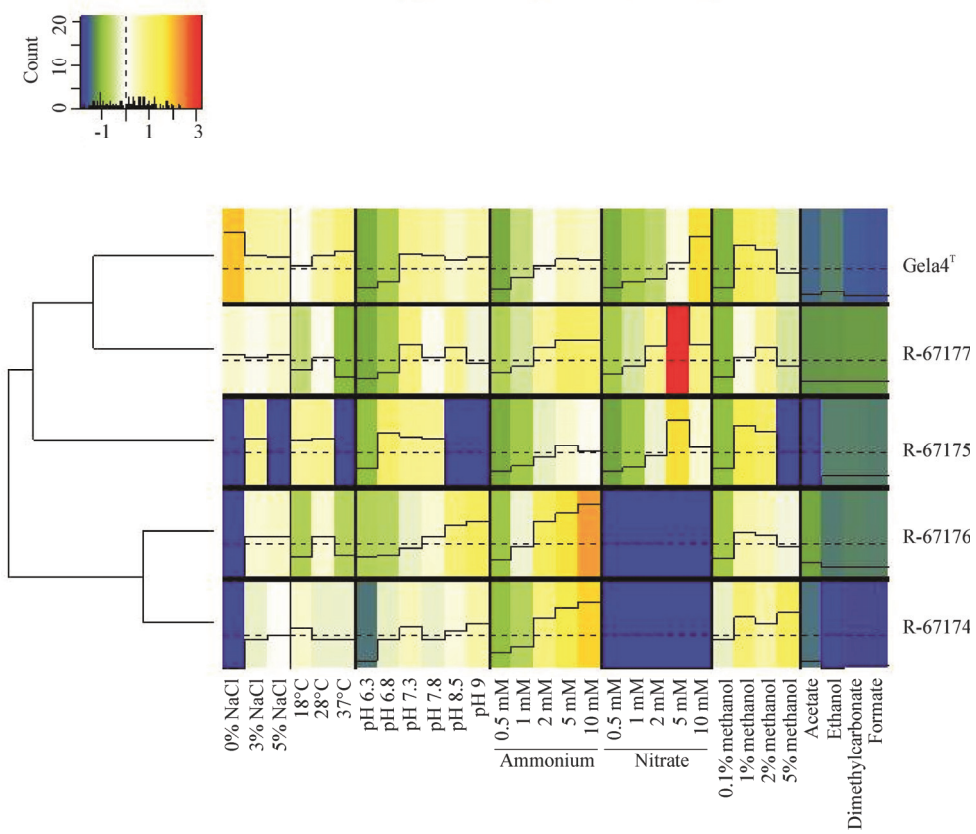

Figure 3.4 | Heat map of differential growth responses (OD_{600}) of the *Methyloceanibacter* spp (see Table S3.4). Colors and full lines represent normalized OD_{600} values. Values on the x-axis represent normalized OD_{600} values. These values are represented by the colors, where blue indicates no growth and red indicates highest growth. Counts on the y-axis represent the amount of times a certain value was reported. Rows of the matrix are clustered using complete hierarchical clustering.

Significant strain dependency of growth kinetics on methanol were already described above (Table 3.1), but, as was observed for nitrogen, different life strategies became apparent in relation to methanol concentrations, alternative carbon source utilization and responses to environmental stimuli (Fig. 3.4; Table S3.4). All strains except R-67175 grew with 0.1 to 5% methanol. R-67174 displayed a similar growth over this concentration range, while the others clearly preferred 1-2%. They were also all facultative methylotrophs able to use dimethylcarbonate and formate. In addition, only R-67175 could not utilize acetate, while

only R-67174 was unable to grow on ethanol. However, maximal cell densities on these alternative carbon and energy sources were low compared to methanol, except for Gela4^T on ethanol and R-67176 on acetate. Both compounds are realistic alternative carbon sources *in situ* reaching up to 2–33 nM for ethanol [66] and from 1 to 10 µM for acetate [67–69] in marine sediments. R-67177 seemed to have adapted to a lifestyle of a generalist, without a clear preference for either carbon source. Next to carbon metabolism, variation in growth ranges for salinity, temperature and pH further underlined strain-dependent physiologies of the *Methyloceanibacter* strains (Fig. 3.4, Table S3.4). R-67177 and Gela4^T demonstrated the broadest salinity range supporting growth, with no differences in maximum cell density for the former while the latter had an unexpected optimum at 0% salinity (given *in situ* salinity of 33-35 psu). In contrast, R-67174 and R-67176 needed salt to grow, and R-67175 was limited to a salinity of 3%. Also the temperature range of R-67175 (18-28°C) was narrower than that of the others (18-37°C). Again it was strain-dependent whether maximal cell densities varied with temperature. A similar observation was made for pH, with R-67175 being restricted to pH 6.3 to 7.8.

3.4. DISCUSSION

Marine methylotrophic *Methyloceanibacter* consist of five ecotypes that coincide with five species, four of which were novel. Surprisingly, one ecotype (represented by R-67174) was capable of methane oxidation, making this the first report on a methanotroph within a methylotrophic genus. Some claims were made in the past [70–72], but could never be independently verified [73]. Furthermore, its sole use of a sMMO and its marine origin as type II methanotroph makes strain R-67174 a unique discovery. *Methyloceanibacter* is the first genus with pMMO lacking methanotrophs retrieved from marine systems and only the third overall, next to *Methylocella* and *Methyloferrula* [56, 74], also phylogenetically the most closely related methanotrophs (Fig. 3.2; Fig. S3.2). Given the scattered phylogenetic distribution of the sMMOs among methanotrophs [75], its soluble nature requiring fewer genes than the particulate form and its promiscuity [76], it is very plausible that horizontal gene transfer (HGT) is responsible for the spread of the trait. Trait acquisition should enable recipients to colonize a wider range of habitats than possible with the pMMO alone [75] or without any MMO. Nevertheless, to date no hard evidence or experimental data for HGT of sMMO has been reported. We found a deviation in GC content of *mmoXYBZCRG* (56%) compared to the average GC content of the genome (64%) in R-67174, which might point to a recent acquisition, but the gene cluster was completely flanked by hypothetical proteins and its immediate genome environment lacked actual remaining traces of HGT. As the sMMO machinery is the sole distinctive factor separating methanotrophs from other methylotrophs, the occurrence of methanotrophs within strict methylotrophic clades is most likely widespread but problematic to observe. Liquid extinction cultures under methane headspace, as in this study, might consist of a population of genotypically distinct congenerics or conspecifics not easily differentiated at morphological, physiological or genomic levels. Once transferred to solid media under methane headspace, the non-methane oxidizing methylotrophs are lost. All four pMMO lacking methanotrophs (*Methylocella sylvestris, M. tundrae, M. palustris and Methyloferulla stellata*) belong to the *Alphaproteobacteria*, with R-67174 being the first isolated from a marine environment. Its isolation is not trivial as currently only eight marine methanotrophs are available in culture, *Methylomicrobium pelagicum* [21], *Methylobacter marinus* [20], *Methylomicrobium japanense* [22], *Methylomonas methanica* [26], *Methylomarinum vadi* [19], *Methylocaldum marinum* [24], *Methyloprofundis sedimenti* [25] and *Methylomarinovum caldicuralii* [27], all of which are type I MOB belonging to the *Gammaproteobacteria*. Only three of them contain sMMO in addition to the pMMO [22, 24,

26]. The availability of marine methanotrophs *ex situ* clearly reflects the *in situ* situation, where type II have a widespread environmental distribution [77, 78] but type I are numerically dominant in marine systems.

Although very closely related, the five *Methyloceanibacter* ecotypes occupy distinct ecological niches and/or adhere to different life strategies, as evidenced by their differential kinetics of the methanol metabolism, nitrogen preferences, alternative carbon source utilization and optima for temperature, salt and pH. Taken together, R-67175 is a clear k-strategist, with high maximal cell densities but limited metabolic spectrum, while Gela4^T and R-67177 seem r-strategists, and R-67174 and R-67176 probably invest more in resource usage. One major driving force behind niche specialization (and subsequent speciation) among sympatric highly-related microorganisms is resource competition, even in, nutrient sufficient environments like the North Sea. Competition rather than cooperation for nutritional resources seems to be the norm among microbial species [79, 80]. All *Methyloceanibacter* strains preferentially use methanol as their sole source of carbon and energy, albeit at different growth rates and efficiencies, and assimilated ammonium for nitrogen. As such, competition for carbon and nitrogen could have enforced niche separation. The discriminatory features ethanol or acetate utilization are beneficial traits when methanol is not readily available. Similarly, acquisition of the methane oxidation capacity gives R-67174 a competitive advantage under methanol limitation. Like *Methylocella* and *Methyloferrula* [56, 81], it also preferred growth on methanol over methane (evidenced by higher growth rate and shorter lag phase), which now seems characteristic for pMMO-lacking methanotrophs. Therefore, R-67174 might behave as a methylotroph *in situ*, as stable isotope probing already revealed *Methylocella* spp. cross-feeding on the excreted products of other methanotrophs rather than oxidizing methane as a primary carbon source (C. Murrell, personal communication). In addition, methane oxidation can bestow R-67174 with the benefit of receiving secondary metabolites like vitamins or growth promotors from co-existing microorganisms in return for 'leaking' methane-derived carbon compounds [82]. Likewise, nitrogen fixation or nitrate assimilation, which were mutually exclusive in the *Methyloceanibacter* strains, would ensure assimilation of alternative nitrogen sources under ammonium limitation. Notably, all *Methyloceanibacter* strains contained the genomic potential to produce bacteriocin, which are a large group of antimicrobial peptides that are mostly used to target conspecifics or closely related species [79]. These proteinaceous toxins might have led to mutual inhibition and further excluded the closely related organisms to occupy the same niche under resource

limitation, as has been demonstrated for *Paenibacillus dendritiformis* [83]. Fundamental for ecotype formation is the niche space landscape of the environment, which is very large in stratified marine sediments. Sediment microheterogeneity, both vertically and horizontally, at scales less than a cm create an infinite amount of available microniches ranging from 1 µm to several millimeters or centimeters [84]. Exclusion of closely related organisms from the same niche might have driven niche displacement of *Methyloceanibacter* in North Sea sediments and further adaptation led to speciation, as previously suggested for the *Methylococcaceae* spp. and *Methylophilaceae* spp [85].

The physiological and genomic data presented here helped to define ecotype variation within methylotrophic bacteria and to disentangle the phylogenetic levels at which environmental variables influence marine methanotrophy and methylotrophy. Besides carbon metabolism, nitrogen cycling is confirmed to play a fundamental role in shaping methylotrophic populations and salinity preferences will determine the localization of specific *Methyloceanibacter* ecotypes in more coastal areas. The diverse metabolic features demonstrated here among only a small set of closely related methylotrophs, relative to the great genetic diversity revealed by ongoing metagenomic surveys, underlines the need for ongoing efforts in obtaining marine representatives *ex situ*. A change in isolation strategy targeting marine methanotrophs to broad screening of methanol-grown cultures will also aid to find more pMMO-lacking methanotrophs.

Description of *Methyloceanibacter methanicus* sp. nov.

Type strain: R-67174, LMG 29429

Etymology. N. L. neut. n. *methanum*, methane; L. masc. suff. *-icus*, adjective forming suffix used with the sense of pertaining to; N.L. masc. adj. *methanicus*, related to or associated with methane

Locality. Isolated from marine sediment in the North Sea at the station 'Vlakte van de Raan' (N51.449166 E3.237166)

Properties. Gram-negative, non-motile rods, liquid cultures form small microcolonies. Cells grow optimally at 18-37°C and pH 6.3-9 but requires NaCl at 3-5%. Grows aerobically on methane using a soluble methane monooxygenase but prefers methanol. Grows better with carbon dioxide added to headspace. Also grows using alternative carbon sources acetate, formate and dimethylcarbonate. Requires ammonium as inorganic nitrogen source. The G+C content of the type strain is 64.0 mol%.

Description of *Methyloceanibacter superfactus* sp. nov.

Type strain: R-67175, LMG 29430

Etymology. L. prep. *super*, above, on top; N.L. v. *facere*, to produce by growth; *superfactus*, referring to high maximal cell densities.

Locality. Isolated from marine sediment in the North Sea at the station Hinderbanken (N51.75 E2.7)

Properties. Gram-negative, non-motile rods. Cells grow optimally at 18-28°C and pH 6.3-7.8 but requires 3% NaCl. Grows aerobically on methanol. Also grows using alternative carbon sources ethanol, formate and dimethylcarbonate. Capable of using ammonium and nitrate as inorganic nitrogen sources. The G+C content of the type strain is 64.5 mol%.

Description of *Methyloceanibacter stevinii* sp. nov.

Type strain: R-67176, LMG 29431

Etymology. *stevinii*, of Stevin, referred to the Belgian scientific research vessel 'Simon Stevin', used to collect the samples at the North Sea.

Locality. Isolated from marine sediment in the North Sea at the station Vlakte van de Raan (N51.449166 E3.237166) and at the station Hinderbanken (N51.75 E2.7)

Properties. Gram-negative, non-motile rods, liquid cultures are single cells with occasional flock formation. Cells grow at 18-37°C and pH 6.3-9 but requires NaCl at 3-5%. Grows aerobically on methanol. Also grows using alternative carbon sources ethanol, acetate, formate and dimethylcarbonate. Requires ammonium as inorganic nitrogen source. The G+C content of the type strain is 63.1 mol%.

Description of *Methyloceanibacter marginalis* sp. nov.

Type strain: R-67177, LMG 29432

Etymology. L. n. *margo*, edge, margin; L. masc. suff. *-alis*, suffix denoting pertaining to; N.L. masc. adj. *marginalis*, referring to cells stick to the edge of the recipient in liquid culture.

Locality. Isolated from marine sediment in the North Sea at the station Hinderbanken (N51.75 E2.7)

Properties. Gram-negative, non-motile rods, liquid cultures grow attached to the side of the recipient and form flocks. Cells grow at 18-37°C, pH 6.3-9 and 0-5% NaCl. Grows aerobically on methanol. Also grows using alternative carbon sources ethanol, acetate, formate and dimethylcarbonate. Capable of using ammonium and nitrate as inorganic nitrogen sources. The G+C content of the type strain is 63.5 mol%.

3.5. ACKNOWLEDGEMENTS

The authors wish to thank André Cattrijsse of the Flanders Marine Institute (VLIZ) and the captain and crew of the research vessel Zeeleeuw and Simon Stevin for the logistic and practical support during the different sampling surveys. Special thanks to C. Dumolin for assistance with flow cytometry analysis, B. Tytgat for his assistance with R, D. Speth for his help with metagenome data analyses and J. Arends for methanol analyses. We also thank C. Dumolin, F. Beeckman, J. Wille and H. Decleyre for their much appreciated help in maintaining enrichment and pure cultures and performing the sampling campaigns.

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3.6. SUPPLEMENTARY MATERIALS

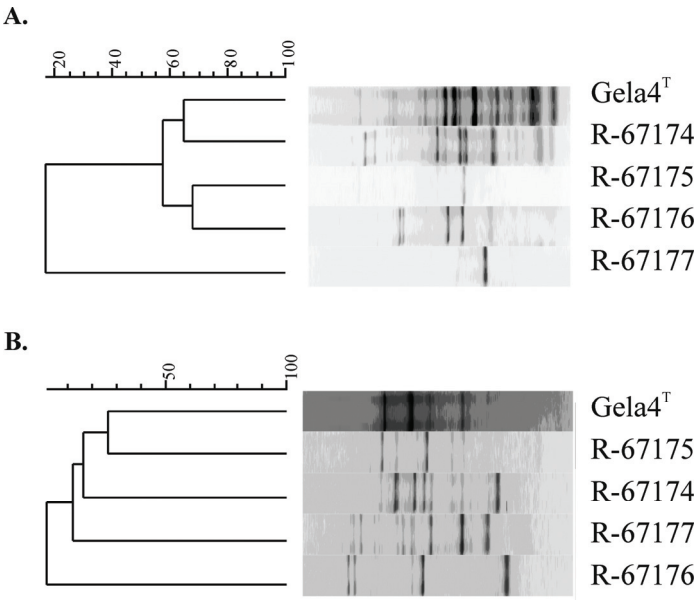

Figure S3.1 | Representatives of each *Methyloceanibacter* genotype based on $(GTG)_5$ (A) and Box (B) repetitive sequence based (Rep) PCR fingerprinting. Forty-one isolates assigned to the genus *Methyloceanibacter* based on partial 16S rRNA gene sequence similarity were grouped into four clusters, all distinct from *Methyloceanibacter caenitepidi* Gela4^T, with both techniques. Dendrograms were created using Pearson product moment correlation coefficient and UPGMA using BioNumerics 7.5.

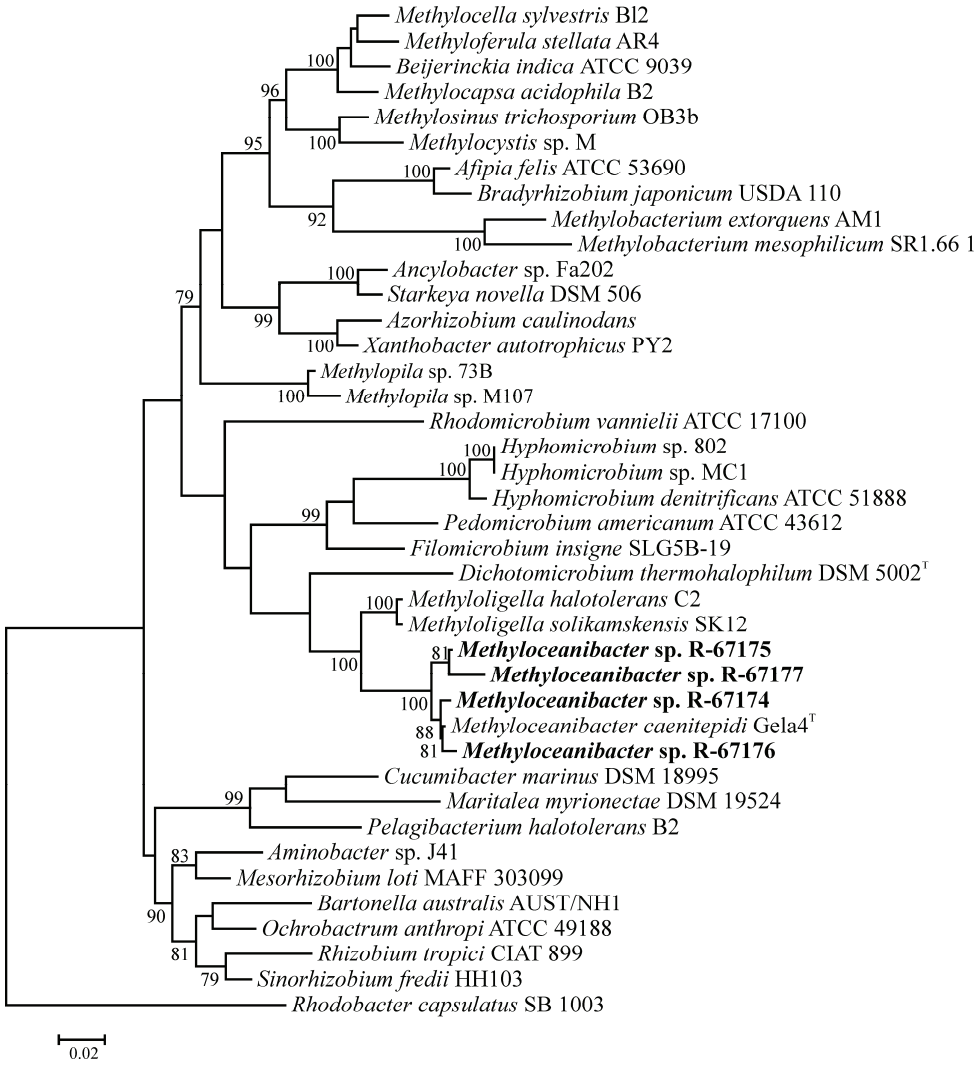

Figure S3.2 | Phylogenetic Maximum Likelihood showing the affiliation of the novel *Methyloceanibacter* spp. based on the 16S rRNA gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [86] using 1322 nucleotide positions. The tree with the highest log likelihood (-9625.9027) is shown. Bootstrap values (1000 replicates) higher than 70 are shown next to the branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5937)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Alignment was performed using SILVA Incremental Aligner [87] and evolutionary analyses were conducted in MEGA6 [58].

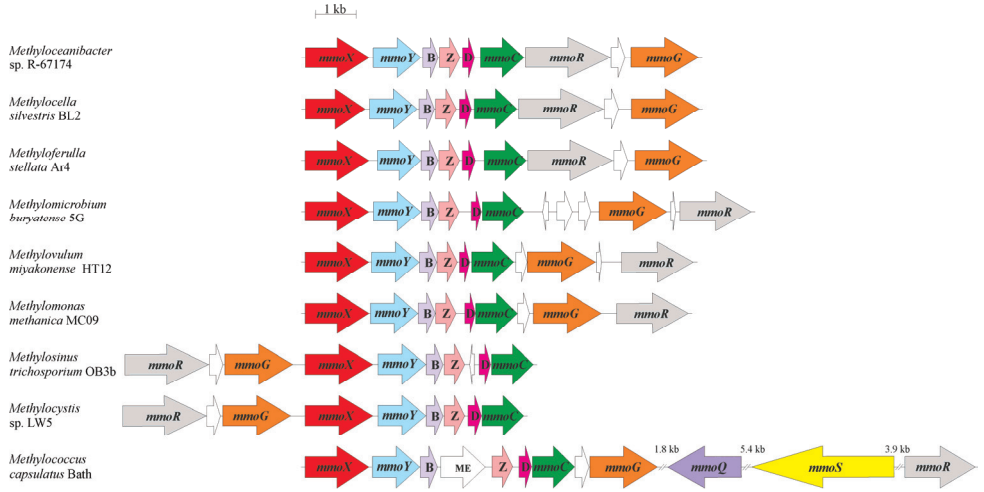

Figure S3.3 | Physical map of genome environment of sMMO operon in R-67174, closely related and other methanotrophs. Homologs are depicted in identical colors. ORF of hypothetical proteins are given as white arrows. ME, ORF for mobile element. Drawn to scale.

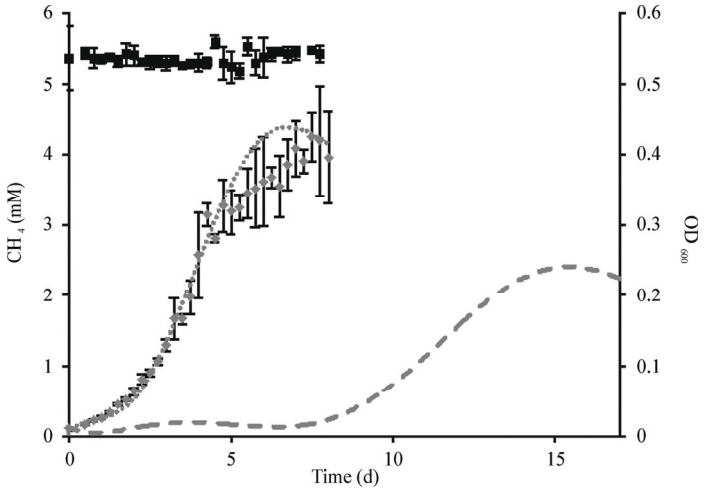

Figure S3.4 | Growth (♦) and methane oxidation (■) by R-67174 over time when both methane and methanol are provided as carbon source (n = 6). No methane was consumed and lag phase, specific growth rate and maximal cell density was not different from that on methanol alone (see Table 3.1). Growth on methanol (1%) or methane (5.4 mM) as sole carbon source are given for comparison as grey dotted and dashed lines respectively. All growth experiments were performed with addition of carbon dioxide.

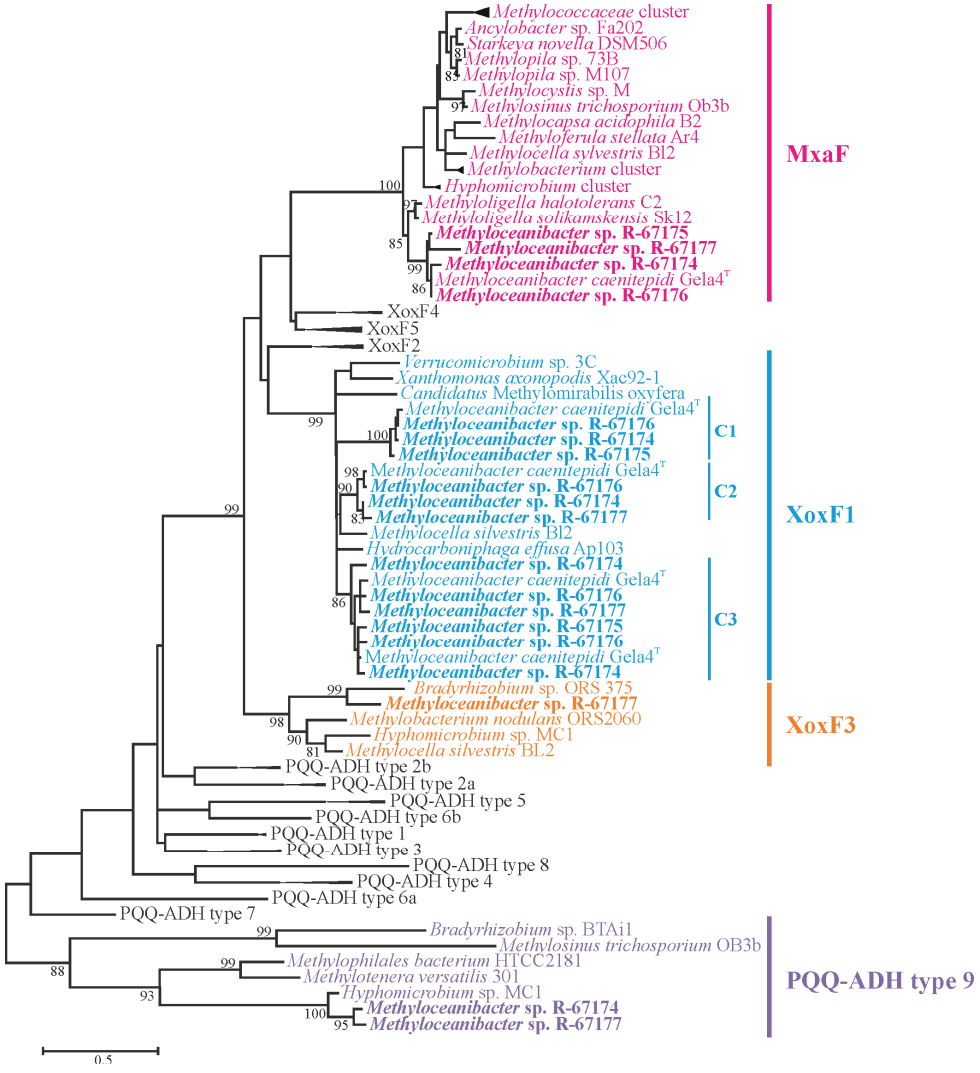

Figure S3.5 | Phylogenetic Maximum Likelihood showing the affiliation of the methanol dehydrogenases. Representatives of MxaF, five Xox types, and nine clades of other type I and II alcohol dehydrogenase quinoproteins were chosen based on Keltjens *et al* (2014) [60]. Sequences from this study are given in bold. For further explanation on subgrouping of XoxF1, see text.

The evolutionary history was inferred by using the Maximum Likelihood method based on Le_Gascuel_2008 model [57] using 132 amino acid positions. The tree with the highest log likelihood (-9399,9841) is shown. Bootstrap values (1000 replicates) higher than 70 are shown next to the branches but are omitted for collapsed branches. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 1,9073)). The rate variation model allowed for some sites to be evolutionarily

invariable ([+I], 1,4692% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA6 [58].

Table S3.1 | Genome statistics. Details on genome of Gela4^T are included for convenience of comparison. Number of CDS and RNA taken from RAST annotation.

Strain	Genome size (bp)	# Contigs	Coverage	CDS ; RNA	%GC	Accession number
Gela4^T	3,424,964	1	-	3318 ;48	63.9	NZ_AP014648.1
R-67174	3,139,022	14	33.6x	3279 ; 49	64.0	LPWG00000000
R-67175	3,100,099	38	27.6x	3403 ; 49	64.5	LPWF00000000
R-67176	3,216,104	14	26.6x	3380 ; 49	63.1	LPWE00000000
R-67177	2,997,425	470	5.3x	3407 ; 43	63.5	LPWD00000000

Table S3.2 | Genomic taxonomy of the *Methyloceanibacter* spp. Using cut-off values of 95% Average Nucleotide Identity (ANI), 70% *in silico* Genome-to-Genome Hybridization similarity (GGDH) and 97% 16S rRNA gene sequence similarity [88], the four new *Methyloceanibacter* strains represent four novel species distinct from *M. caenitepidi* Gela4^T. ANI was calculated via http://enve-omics.ce.gatech.edu/, GGDH via http://ggdc.dsmz.de/distcalc2.php .

	R-67174	R-67175	R-67176	R-67177	Gela4^T
16S rRNA gene sequence similarity (%) to					
R-67174	100.0				
R-67175	97.7	100.0			
R-67176	98.7	98.0	100.0		
R-67177	96.5	98.5	97.0	100.0	
Gela4^T	99.0	98.3	99.1	97.0	100.0
ANI (%) to					
R-67174	100.0	100.0			
R-67175	78.6	100.0			
R-67176	82.2	78.2	100.0		
R-67177	78.9	80.6	78.7	100.0	
Gela4^T	81.9	78.2	88.4	78.6	100.0
GGDH (%) to					
R-67174	100.0				
R-67175	19.7	100.0			
R-67176	24.2	19.2	100.0		
R-67177	20.6	22.6	20.1	100.0	
Gela4^T	23.7	19.4	37.4	20.2	100.0

Table S3.3 | Genome inventory associated with C1 and nitrogen metabolism.

function	gene	gene product	Locus tag			
			R-67174	R-67175	R-67176	R-67177
Methane oxidation	*mmoX*	Mmo component A alpha chain	AUC68_02950	-	-	-
	mmoY	Mmmo component A beta chain	AUC68_02955	-	-	-
	mmoB	Mmo regulatory protein B	AUC68_02960	-	-	-
	mmoZ	Mmo component A gamma chain	AUC68_02965	-	-	-
	mmoD	Mmo component A D subunit	AUC68_02970	-	-	-
	mmoC	Iron-sulfur flavoprotein of sMMO	AUC68_02975	-	-	-
	mmoR	Sigma 54 dependent transcriptional regulator	AUC68_02980	-	-	-
	mmoG	Heat shock protein 60 family chaperone GroEL	AUC68_02990	-	-	-
methanol dehydrogenase	*mxaF*	Methanol dehydrogenase large subunit protein	AUC68_10545	AUC69_10415	AUC70_02345	AUC71_12615
	mxaJ	MxaJ, protein involved in methanol oxidation	AUC68_10550	AUC69_10410	AUC70_02340	AUC71_12620
	mxaG	Cytochrome c-L precursor	AUC68_10555	AUC69_10405	AUC70_02335	AUC71_12625
	mxaI	Methanol dehydrogenase, small subunit	AUC68_10560	AUC69_10400	AUC70_02330	AUC71_12630
	mxaR	Methanol dehydrogenase regulatory protein	AUC68_10565	AUC69_10395	AUC70_02325	AUC71_15715
	mxaS	MxaS, protein involved in methanol oxidation	AUC68_10570	AUC69_10390	AUC70_02320	AUC71_15720
	mxaA	MxaA protein	AUC68_10580	AUC69_10386	AUC70_02315	AUC71_15725; AUC71_09725
	mxaC	Protein involved in Ca2+ insertion into methanol dehydrogenase	AUC68_10585	AUC69_10385	AUC70_02310	AUC71_09730
	mxaK	MxaK	AUC68_10590	AUC69_10381	AUC70_02305	AUC71_09735
	mxaL	MxaL protein	AUC68_10595	AUC69_10380	AUC70_02300	AUC71_09740
	mxaD	MxaD protein	AUC68_10545	AUC69_10375	AUC70_02295	AUC71_09745
	xoxF1	Methanol dehydrogenase large subunit protein	AUC68_06085	AUC69_09565	AUC70_02260	AUC71_01560
			AUC68_09730	AUC69_11085	AUC70_04360	AUC71_02155
			AUC68_10955	AUC69_11090	AUC70_05315	AUC71_09470
			AUC68_10960	-	AUC70_05320	AUC71_01155
	xoxF3	Methanol dehydrogenase large subunit protein	-	-	-	AUC71_05000
			-	-	-	AUC71_05270
	adh	PQQ-ADH type9	AUC68_00330	AUC69_15290	-	AUC71_09200
Formaldehyde activating enzyme	*fae*	Formaldehyde activating enzyme	AUC68_06870	AUC69_1003	AUC70_10390	AUC71_05755
			-	AUC69_13030	-	-
Methenyl H4MPT pathway	*mtdB*	Methylene tetrahydromethanopterin dehydrogenase	AUC68_05780	AUC69_13005	pAUC70_10415	AUC71_11895
			AUC68_06895	AUC69_07035	AUC70_15310	-
	mch	N(5),N(10)-methenyltetrahydromethanopterin cyclohydrolase	AUC68_06885	AUC69_13015	AUC70_10405	AUC71_05770

Table S3.3 | *Continued*

function	gene	gene product	Locus tag			
			R-67174	R-67175	R-67176	R-67177
methylene H4F pathway	*fhcB*	Formylmethanofuran dehydrogenase subunit B	AUC68_06915	AUC69_12980	AUC70_10435	-
			AUC68_05310	-	-	-
	fhcA	Formylmethanofuran dehydrogenase subunit A	AUC68_06920	AUC69_12975	AUC70_10440	-
			AUC68_05305	-	-	-
	fhcD	Formylmethanofuran--tetrahydromethanopterin N-formyltransferase	AUC68_06925	AUC69_12970	AUC70_10445	-
	fhcC	Formylmethanofuran dehydrogenase subunit C	AUC68_06930	AUC69_12965	AUC70_10450	-
	folD	Methenyltetrahydrofolate	AUC68_05775	AUC69_07040	-	AUC71_11900
	fhs	Formate--tetrahydrofolate ligas	AUC68_05795	AUC69_07025	AUC70_15295	AUC71_11880
	fdhD	Formate dehydrogenase chain	AUC68_00285	AUC69_15380	AUC70_12905	AUC71_08935
formate dehydrogenase	*fdsD*	NAD-dependent formate dehydrogenase delta subunit	AUC68_10995	AUC69_11150	AUC70_05350	-
	fdsA	NAD-dependent formate dehydrogenase alpha subunit	AUC68_11000	AUC69_11155	AUC70_05355	-
	fdsB	NAD-dependent formate dehydrogenase beta subunit	AUC68_11005	AUC69_11160	AUC70_05360	-
	fdsG	NAD-dependent formate dehydrogenase gamma subunit	AUC68_11010	AUC69_11165	AUC70_05365	-
serine pathway	*sga*	Serine--glyoxylate aminotransferase	AUC68_05790	AUC69_07025	AUC70_15300	AUC71_11885
	glyA	Serine hydroxymethyltransferase	AUC68_07830	AUC69_03685	AUC70_11295	AUC71_05945
ammonium assimilation	*amtB*	Ammonium transporter	AUC68_12330	AUC69_01150	AUC70_02740	AUC71_13475
			-	AUC69_11805	AUC70_00720	AUC71_05820
			-	AUC69_04905	AUC70_14040	AUC71_09905
			-	-	-	AUC71_13475
	glnA	Glutamine synthetase type I	AUC68_02425	AUC69_01140	AUC70_00700	AUC71_05835
			AUC68_06805	AUC69_04885	AUC70_00715	AUC71_10150
			-	AUC69_04910	AUC70_00725	AUC71_12365
			-	AUC69_04900	-	AUC71_09910
	gltB	Glutamate synthase [NADPH] large chain	AUC68_02430	AUC69_01135	AUC70_00695	AUC71_05840
			AUC68_04155	AUC69_07475	AUC70_14025	-
			-	-	AUC70_14690	-
	gltD	Glutamate synthase [NADPH] putative GlxC chain	AUC68_02435	AUC69_10730	AUC70_00690	AUC71_05845
			AUC68_04160	AUC69_01130	AUC70_14020	AUC71_12355
			-	-	AUC70_14685	-
	glsF	Ferredoxin-dependent glutamate synthase	AUC68_10790	-	-	-

Table S3.3 | *Continued*

function	gene	gene product	Locus tag			
			R-67174	R-67175	R-67176	R-67177
nitrate assimilation	*nasC*	Assimilatory nitrate reductase large subunit	-	AUC69_09280	-	AUC71_03460
			-	AUC69_09285	-	AUC71_03465
	nirA	Ferredoxin--nitrite reductase	-	AUC69_09290	-	AUC71_01065
	nirB	Nitrite reductase [NAD(P)H] large subunit	-	AUC69_09295	-	AUC71_01060
	ntrA	Nitrate ABC transporter, ATP-binding protein	-	AUC69_09300	-	AUC71_01055
	ntrB	Nitrate ABC transporter, permease protein	-	AUC69_09305	-	AUC71_01050
	ntrC	Nitrate ABC transporter, nitrate-binding protein	-	AUC69_09310	-	AUC71_01045
dinitrogen fixation	*nifA*	Nitrogenase (molybdenum-iron)-specific transcriptional regulator	AUC68_10280	-	AUC70_04730	-
	nifB	Nitrogenase FeMo-cofactor synthesis FeS core scaffold/assembly protein	AUC68_10310	-	AUC70_04765	-
	nifZ	NifZ protein	AUC68_10335	-	AUC70_04790	-
			AUC68_10340	-	AUC70_04795	-
	nifT	NifT protein	AUC68_10345	-	AUC70_04800	-
	nifH	Nitrogenase iron protein polypeptide	AUC68_10390	-	AUC70_04850	-
	nifD	Nitrogenase molybdenum-iron protein alpha chain	AUC68_10395	-	AUC70_04855	-
	nifK	Nitrogenase molybdenum-iron protein beta chain	AUC68_10400	-	AUC70_04860	-
	nifE	Nitrogenase FeMo-cofactor scaffold and assembly protein	AUC68_10405	-	AUC70_04865	-
	nifN	Nitrogenase FeMo-cofactor scaffold and assembly protein	AUC68_10410	-	AUC70_04870	-
	nifX	Nitrogenase FeMo-cofactor carrier protein	AUC68_10415	-	AUC70_04875	-
	nifX2	NifX-associated protein	AUC68_10420	-	AUC70_04880	-
	nifQ	Nitrogenase FeMo-cofactor synthesis molybdenum delivery protein	AUC68_10435	-	AUC70_04895	-
	nifU	Iron-sulfur cluster assembly scaffold protein	AUC68_10450	-	AUC70_04910	-
	nifS	Cysteine desulfurase	AUC68_10455	-	AUC70_04915	-
	nifV	Homocitrate synthase	AUC68_10460	-	AUC70_04920	-
	nifP	Serine acetyltransferase	AUC68_10465	-	AUC70_04925	-
	nifW	Nitrogenase stabilizing/protective protein	AUC68_10470	-	AUC70_04930	-
	nifO	Nitrogenase-associated protein	AUC68_00240	--	AUC70_12865	-
dissimilatory nitrate reduction	*narG*	Respiratory nitrate reductase alpha chain	-	AUC69_09325	AUC70_04305	AUC71_05195
	narH	Respiratory nitrate reductase beta chain	-	AUC69_09330	AUC70_04310	AUC71_05200
	narJ	Respiratory nitrate reductase delta chain	-	AUC69_09340	AUC70_04315	AUC71_05205
	narI	Respiratory nitrate reductase gamma chain	-	-	AUC70_04320	AUC71_05210
	narK	Nitrate/nitrite transporter	AUC68_11610	AUC69_06070	AUC70_01925	AUC71_12050
			-	AUC69_07450	AUC70_04350	-

Table S3.4 | Differential growth responses (OD_{600}) of the *Methyloceanibacter* spp. Standard deviations are given between brackets (n=3). The following conditions did not support growth for any of the strains: pH 5.8, 10, 11; 10% salt; 10°C, 45°C, 52°C; 0.03% yeast extract or N_2 as sole nitrogen source; methylamine, TSB, formamide or DMSO as sole carbon source; methanol concentrations of 0.001%, 0.01% and 0.1%. Significant within-strain differences per parameter were determined via one-way ANOVA (see Materials and Methods for details) and are displayed as different lowercase letters (combined lower letters are used to indicate non-significance for multiple variables). Significance levels for between-strain differences per parameter were not determined because of bias introduced by the inherent significant difference in maximal cell density between the strains (see Table 3.1).

All included values were significantly different ($p<0.01$) from negative controls incubated without carbon source (n=3).

-, no growth.

Parameter	Variable	R-67174	R-67175	R-67176	R-67177	Gela4^T
Ammonium	0.5 mM	0.178 (0.010)	0.277^a (0.015)	0.242^a (0.010)	0.337^a (0.320)	0.276^a (0.008)
	1 mM	0.235 (0.052)	0.531^b (0.051)	0.576^b (0.072)	0.541^a (0.040)	0.620^b (0.030)
	2 mM	0.544 (0.047)	1.093^c (0.063)	1.228^c (0.097)	1.244^b (0.193)	1.002^c (0.054)
	5 mM	0.660 (0.137)	1.676^d (0.007)	1.449^c (0.023)	1.511^b (0.221)	1.226^d (0.024)
	10 mM	0.711 (0.252)	1.419^d (0.152)	1.717^d (0.098)	1.477^b (0.118)	1.143^d (0.030)
Nitrate	0.5 mM	-	0.222^a (0.027)	-	0.282^a (0.024)	0.288^a (0.016)
	1 mM	-	0.511^b (0.050)	-	0.550^a (0.115)	0.478^b (0.064)
	2 mM	-	1.147^c (0.047)	-	1.292^{ab} (0.439)	0.586^c (0.023)
	5 mM	-	3.020^d (0.162)	-	2.841^c (2.013)	1.054^d (0.370)
	10 mM	-	1.620^c (0.913)	-	1.349^b (0.479)	1.917^c (0.573)
Methanol	0.1%	0.316^a (0.020)	0.386^a (0.031)	0.277^a (0.09)	0.147^a (0.009)	0.305^a (0,095)
	1%	0.554^b (0.123)	2.740^b (0.248)	0.940^b (0.834)	0.907^b (0.139)	1.642^b (0.009)
	2%	0.481^b (0.111)	2.430^b (0.189)	0.879^b (0.439)	1.265^c (0.111)	1.529^b (0.044)
	5%	0.594^b (0.130)	-	0.592^{ab} (0.948)	0.545^a (0.915)	0.748^c (0.047)
Alternative carbon source	acetate	0,093^a (0,006)	-	0,130^a (0,010)	0,016 (0,008)	0,075^a (0,002)
	ethanol	-	0,035^a (0,014)	0,026^b (0,004)	0,022 (0,019)	0,150^b (0,006)
	dimethylcarbonate	0,027^b (0,003)	0,049^b (0,004)	0,044^c (0,001)	0,033 (0,002)	0,035^c (0,001)
	formate	0,030^b (0,002)	0,030^a (0,002)	0,034^{bc} (0,003)	0,031 (0,001)	0,015^d (0,002)
Salinity	0%	-	-	-	1.003 (0.043)	2.054^a (0.127)
	3%	0.318 (0.009)	2.096 (0.456)	0.816 (0.044)	0.851 (0.217)	1.301^b (0.106)
	5%	0.365 (0.088)	-	0.846 (0.065)	1.019 (0.206)	1.266^b (0.022)
Temperature	18°C	0.429 (0.045)	2.000 (0.121)	0.319^a (0.022)	0.440^a (0.050)	0.959^a (0.017)
	28°C	0.319 (0.009)	2.096 (0.456)	0.816^b (0.044)	0.851^b (0.217)	1.301^a (0.106)
	37°C	0.322 (0.167)	-	0.349^a (0.065)	0.15^a (0.117)	1.492^b (0.113)
pH	6.3	0.097 (0.024)	0.411^a (0.024)	0.333^a (0.024)	0.097^a (0.024)	0.286^a (0.011)
	6.8	0.314 (0.066)	2.383^b (0.005)	0.360^a (0.005)	0.297^a (0.084)	0.489^a (0.037)
	7.3	0.449 (0.040)	2.126^b (0.204)	0.551^a (0.019)	1.358^b (0.879)	1.367^b (0.237)
	7.8	0.319 (0.009)	2.096^b (0.456)	0.816^{ab} (0.044)	0.851^b (0.217)	1.301^b (0.106)
	8.5	0.411 (0.105)	-	1.124^b (0.286)	1.271^b (0.478)	1.140^b (0.052)
	9	0.477 (0.378)	-	1.228^b (0.106)	0.677^c (0.239)	1.273^b (0.074)

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Chapter 4

Genome characteristics of two novel Type I methanotrophs enriched from North Sea sediments containing exclusively a lanthanide-dependent XoxF5-type methanol dehydrogenase

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Author's contributions:

B.V., P.D.V and K.H. designed the research ; B.V., J.W., D.S. and G.C. performed research and analyzed data ; B.V., H.O.d.C. and K.H. interpreted the results and wrote the paper.

4.1. INTRODUCTION

Marine environments harbor diverse methane sources and can yearly emit up to 48 Tg methane to the atmosphere, i.e. approximately 8% of the global methane budget [1, 2]. Methanotrophs, capable of methane oxidation to carbon dioxide, are major players in the global carbon cycle and can serve as a biological filter preventing the escape of methane to the atmosphere. Aerobic methanotrophs are phylogenetically affiliated with *Alpha-* and *Gammaproteobacteria* as well as *Verrucomicrobia*. The latter, although mostly limited to acidic habitats [3, 4] have recently been found in marine sediments [5]. A fundamental understanding of the localized marine sources and sinks of methane is critical for understanding the transfer of methane from the ocean to the atmosphere. Availability of methanotrophs *ex situ* is necessary to investigate kinetics, ecophysiology, inhibitory or stimulating interactions. But to date most isolated cultures are derived from terrestrial and freshwater environments. Cultures of marine methanotrophic are scarce and despite the advances in culturing techniques, thus far only eight have been isolated, all *Gammaproteobacteria* [6–14], and only four genomes sequenced [6, 15]. Their inability to form colonies on conventional solid media [16] and mutualistic relationships with other bacteria [17] are drawbacks for their isolation [12, 13]. As such, a large diversity of marine methanotrophs (based on *pmoA* gene phylogenies) have evaded isolation so far.

Here we describe two novel type I methanotrophs representing thus far uncultured lineages. Both cultures were enriched from North Sea sandy sediment (inoculum for E33 was sampled at Station W04: 51.44N, 3.23E; D14 at Station W07tris: 51.53N, 2.87E) and used methane as sole carbon and energy source **(Chapter 5)**. They only grew when supplied with 250 µM NO_3^-/250 µM NH_4^+ and a high (20%; D14) or low (5%; E33) oxygen tension. Despite serial subcultivation and multiple extinction culturing events over a period of three years, no pure cultures were obtained. Because of the inability to amplify *pmoA* or *mmoX* genes using the currently available primers **(Chapter 5)** despite active methane oxidation being observed, metagenome sequencing was performed to allow phylogenomics and determine potential ecological niche of these enriched methanotrophs.

4.2. MATERIAL AND METHODS

After DNA extraction [18], sequencing was done using the IonTorrent PGM as described previously [19]. The reads were length (>100bp) and quality score trimmed and used for *de novo* assembly with CLC genomics workbench v7.0.4 (CLCbio, Denmark). Binning of the resulting contigs was done using GC content and sequencing depth. One genomic bin from each enrichment culture contained the *pmoCAB* operon encoding the particulate methane monooxygenase. The *pmoA* genes of both cultures were 100% identical, while the genomes' average nucleotide identity (ANI; calculated via http://enve-omics.ce.gatech.edu/) was >95% suggesting that both cultures represented different strains of the same species. This was confirmed by *in silico* DNA:DNA values (GGDH; calculated via http://ggdc.dsmz.de/distcalc2.php) and average amino acid values (AAI; calculated via http://enve-omics.ce.gatech.edu/). Genome features are given in Table 4.1. The closest neighbor based on 16S rRNA gene sequence analyses was *Methylomonas methanica* MC09, with 95% gene sequence similarity, sufficient to putatively group the cultures in a novel genus within the *Methylococcaceae*. Phylogenetic analysis of the PmoA assigned both cultures to the deep-sea cluster 2 (Fig. 4.1), so far only containing one isolated representative *Methylomarinum vadi* [9].

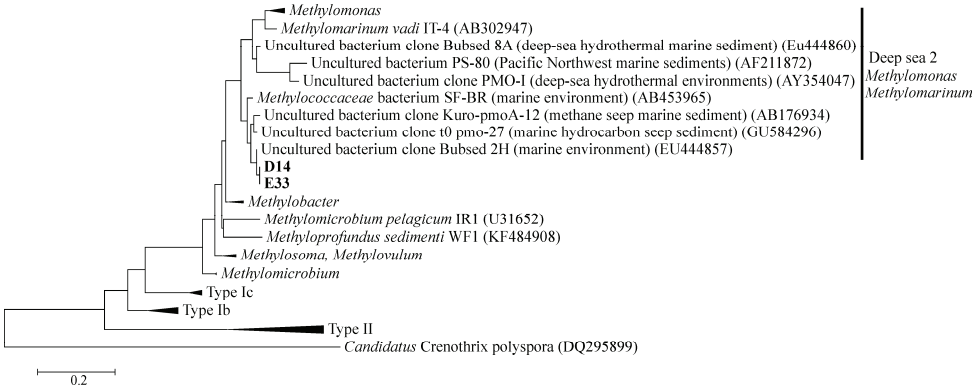

Figure 4.1. Phylogenetic Neighbor Joining tree showing the affiliation of the particulate methane monooxygenase. Representative sequences of type strains, marine cultures and marine environmental sequences were included. The evolutionary history was inferred using 135 amino acid positions and evolutionary distances are given as number of amino acid differences per site. Accession numbers are given between brackets.

Table 4.1. Genome features of binned genomes from both marine methanotrophic enrichments. The completeness of both draft genomes was assessed using checkM [20]. The Rapid Annotation Subsystem Technology (RAST) server was used for functional annotation and metabolic reconstruction of the (draft)genomes [19, 20]. Classic RAST annotation scheme was selected using the RAST gene caller, which allowed automatic error fixing, frameshift correction and the backfilling of gaps. Assigned functions were verified with PSI-BLAST [21]. Missing genes were searched for in the genome with pBLAST using homologous amino acid sequences from the closely related organisms. The reads of the metagenomes of both enrichment cultures are available in the SRA database (study accession number SRP068992).

	D14	E33
Genome size	4.05 Mb	3.91 Mb
Number of contigs	249	255
G+C content	37.7	37.7
Number of CDS	4109	3832
Number of RNAs	37	41
Completeness of draft genomes	>98%	>96%

4.3. RESULTS

Genes for the soluble methane monooxygenase (sMMO) as well as *pxmABC*, a homologue of *pmoCAB* found in many methanotrophs [20, 21], were missing from both genomes. The lack of sMMO is not surprising as these genes are not so widely spread among the type I and are more restricted to the type X and type II methanotrophs [22]. The classic calcium dependent methanol dehydrogenase, encoded by the gene operon *mxaFJGIRSACKLD* is up till now found in all methanotrophs except in the *Verrucomicrobia*, which contain a lanthanide-dependent XoxF-type methanol dehydrogenase [23, 24]. Both E33 and D14 genomes solely encoded a lanthanide-dependent XoxF5 homologue of the methanol dehydrogenase (87% aa similarity with *Methylomicrobium buryatense*) and a PQQ-ADH type 9 alcohol dehydrogenase (53% aa similarity with *Burkholderia* sp.) (Fig. 4.2). The XoxF5 proteins form the largest group methanol dehydrogenase homologs from Alpha-, Beta-Gammaproteobacterial methylotrophs [23]. Phylogenetically, both XoxF5 from D14 and E33 were placed within the *Methylococcales* subgroup [23, 25]. A *xoxJ* gene, encoding the periplasmic solute binding protein, was found adjacent to *xoxF* in both genomes. This is the first report of proteobacterial methanotrophs exclusively dependent on XoxF5. The absence of MxaF might suggest that some Gammaproteobacterial methanotrophs are undergoing genome streamlining through gene loss as observed for *Methylophilaceae* spp. [26, 27].

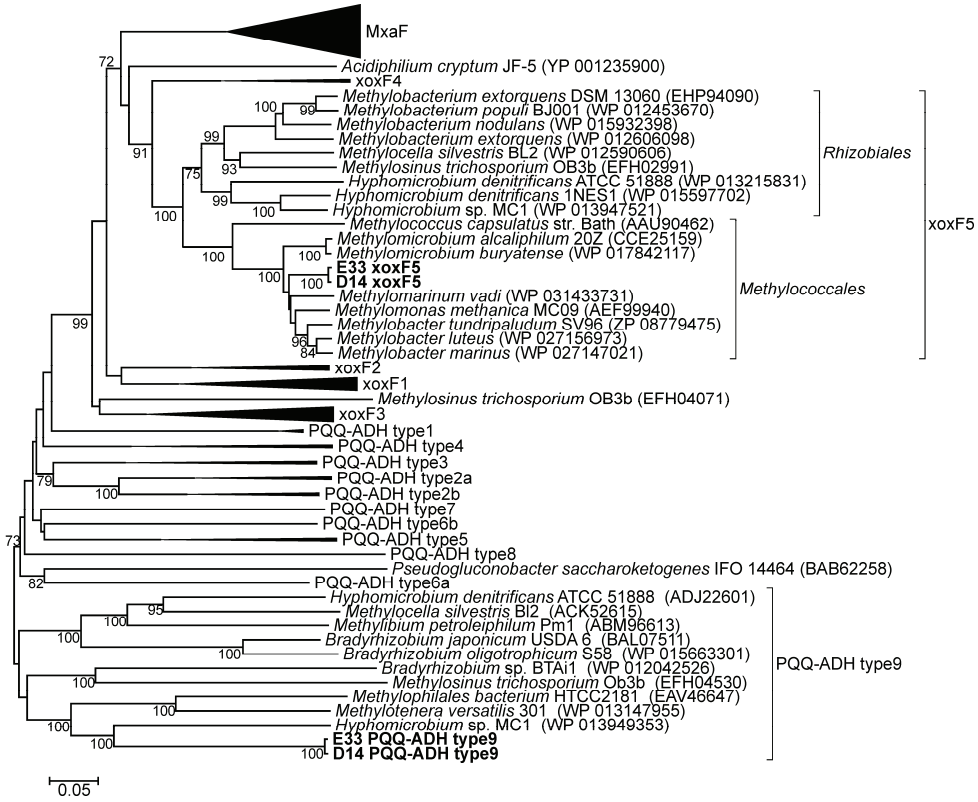

Figure 4.2. Neighbor joining tree showing the affiliation of the methanol dehydrogenases. Representatives of MxaF, five XoxF types, and nine clades of other type I and II alcohol dehydrogenase quinoproteins were chosen based on Keltjens et al. (2014) [24]. Sequences from this study are shown in bold. The evolutionary history was inferred using the Neighbor-Joining method [28] using 386 positions. The optimal tree with the sum of branch length = 13.82 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [29]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [30] and are in the units of the number of amino acid differences per site. Evolutionary analyses were conducted in MEGA6 [31].

Other genes typical for type I methanotrophs were also found (Fig. 4.3), including genes encoding pyrroloquinoline quinone catalytic cofactor (*pqqABCDE*), genes for tetrahydromethanopterin (H4MPT)- and tetrahydrofolate (H4F)-dependent C1-transfer pathways, genes of the ribulose monophosphate pathway and genes encoding a complete

tricarboxylic acid cycle and a partial serine cycle. Furthermore, the genomes contained genes for methylamine utilization (*mauG*), glycogen synthesis (*glgA*, *glgB* and *glgC*) and degradation (*glgP*, *glgX*, *gdb* and *pgm*). Key genes involved in the polyhydroxybutyrate (PHB) synthesis were absent, consistent with the previous observation that synthesis of PHB as storage material seems to be restricted to the type II MOB [28]. Genes for ectoine production (*ectA*, *ectB* and *ectC*), frequently described for halotolerant and halophilic bacteria [29], were present. Carotenoid pigment synthesis, type IV pili, flagella and chemotaxis were also encoded in both the genomes.

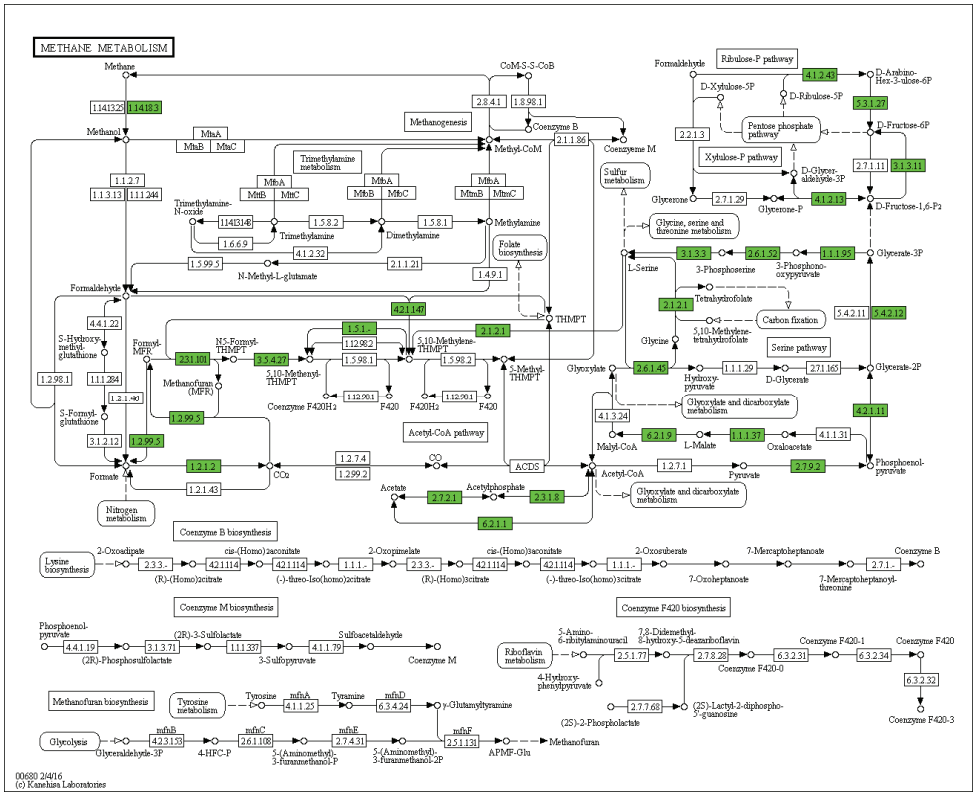

Figure 4.3. KEGG pathway of methane metabolism. Genes related to the methane metabolism as detected in the draftgenomes. The full KEGG pathway for methane metabolism is shown with genes detected indicated in green. Detection of genes was done using BlastKOALA (http://www.kegg.jp/blastkoala/) and completed through a manual curation by searching for missing genes with pBLAST using homologous amino acid sequences from the closely related organisms.

Methane and ammonia monoxygenases are evolutionary closely related [30], and pMMO can oxidize ammonia to hydroxylamine. The conversion of hydroxylamine directly to nitric oxide or indirectly via nitrite is well studied and limited to the activity of hydroxylamine oxidoreductase [31–33], which was encoded in both genomes. Interestingly, like recently reported for several methanotrophs belonging to the genus *Methylomonas* [34, 35], both cultures have the (genomic) capability to reduce nitrite to nitric oxide, with genes for both the copper-dependent nitrite reductase NirK and the cytochrome cd_1-dependent nitrite reductase NirS, until recently thought to be mutually exclusive [36] (Fig. 4.4). Surprisingly they lacked the genes for the NarG-type cytoplasmic or the Nap-type periplasmic nitrate reductases, suggesting a distinct role for nitrite reductase in nitrosative stress response rather than those of denitrification pathways. This is further supported by the lack of a dedicated nitric oxide reductase and the presence of a gene for flavohemoglobin Hmp, capable of nitric oxide reduction to nitrous oxide, a potent greenhouse gas. Furthermore, genes necessary for import and assimilation of ammonium (*amtB*, *glnA*, *gltBD*), urea (*urtABCDE* and *ureABCDEFG*) and nitrate/nitrite (*nrtABC*, *nirC*, *narK*, *nasA*, *nirBD*) were present. The presence of *nifHDK* together with many accessory *nif* genes points towards the ability to fix nitrogen. Although initially mostly type II and type Ib were thought to possess this trait [37], some Type Ia and verrucomicrobial methanotrophs can also fix nitrogen [38–40]. As nitrogen fixation is an energetically costly process, it is thought to function as a survival strategy when nitrogen is limited in the dynamic marine sediments.

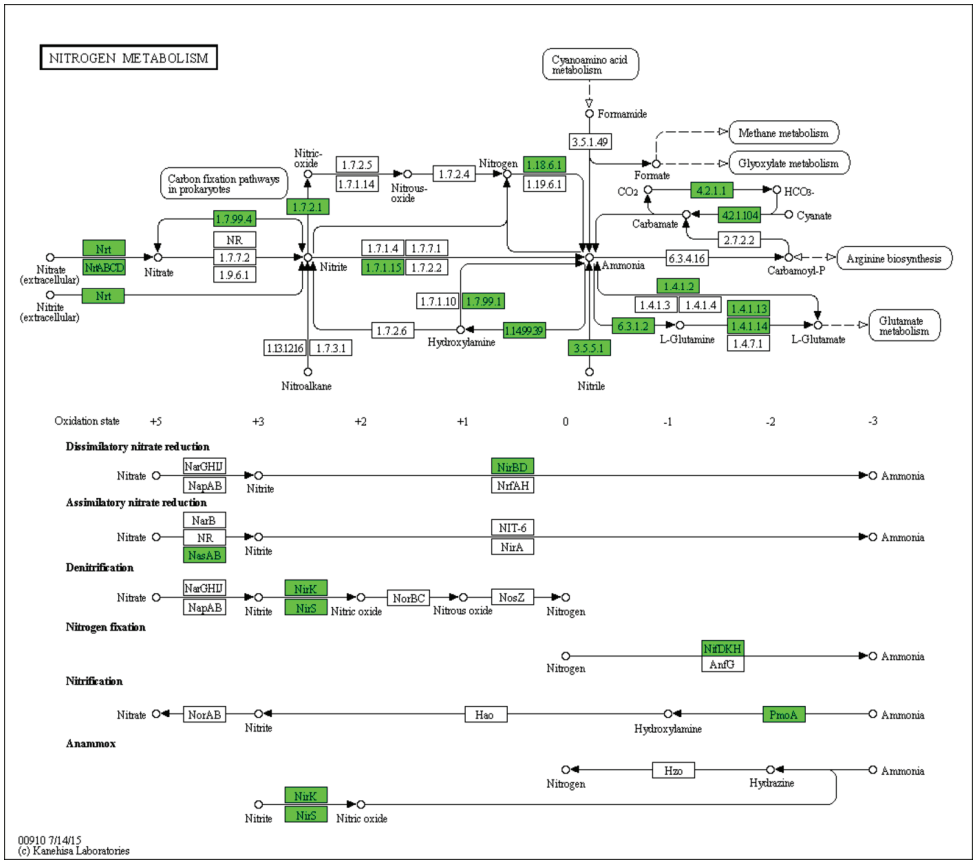

Figure 4.4. KEGG pathway of nitrogen metabolism. Genes related to the nitrogen metabolism as detected in the draftgenomes. The full KEGG pathway for nitrogen metabolism is shown, with genes detected indicated in green. Detection of genes was done using BlastKOALA (http://www.kegg.jp/blastkoala/) and completed through a manual curation by searching for missing genes with pBLAST using homologous amino acid sequences from the closely related organisms.

In conclusion, we present two draft genomes derived from marine methanotrophic enrichments and affiliated with deep-sea cluster 2 of which only few cultures are available. Their most distinctive feature is their putative use of a lanthanide-dependent XoxF5 as sole methanol dehydrogenase, which thus far has not been reported.

4.4. ACKNOWLEDGEMENTS

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Chapter 5

Cultivability and detectability of marine methanotrophs in North Sea sediments decrease from estuarine to open sea areas

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Author's contributions:

Bram Vekeman (B.V.), Paul De Vos (P.D.V), Nico Boon (N.B.) and Kim Heylen (K.H.) designed the research; B.V, Charles Dumolin (C.D.) and (Fabian Beeckman) F. B. performed research; B.V, (Carl Van Colen) C.V.C. and K.H. analyzed data; B.V. and K.H. interpreted the results and wrote the paper.

5.1. INTRODUCTION

Methane is a potent greenhouse gas that occurs in enormous amounts in marine sediments. Despite the apparent ubiquity of methanogenesis in marine systems in combination with about 70% of the world surface covered by oceans, marine methane emissions only account for approximately 3% of the global budget, i.e. 5-20 Tg yr^{-1} [1, 2]. Marine consortia of archaea and bacteria that perform anaerobic oxidation of methane act as natural gatekeepers of these methane reservoirs and consume about 80% of the sedimentary methane [2]. Part of the methane that bypassed this anaerobic microbial filter gets oxidized by aerobic methane-oxidizing bacteria at the sediment surface and/or in the water column and the remainder is emitted to the atmosphere. Estuarine and shelf areas [3] are responsible for up to 75% of the marine emissions of methane [4, 5]. Typically high methane concentrations are detected in estuaries. In the Western Scheldt estuary, reaching the North Sea between Breskens and Vlissingen in the Netherlands, methane concentrations reach 500-5000 nmol L^{-1} [6, 7], decreasing further offshore due to microbial oxidation, mixing with methane poor water and degassing [3, 8]. Specifically in these areas, aerobic methane oxidation might well be an underestimated methane sink [3].

Aerobic methane-oxidizing bacteria (MOB) use a methane monooxygenase enzyme, either the soluble cytoplasmic form (sMMO) and/or the particulate membrane bound form (pMMO), to oxidize methane. Both MMO-encoding genes *mmoX* and *pmoA*, showing congruence at the genus level with the 16S rRNA phylogeny [9, 10], are widely used functional biomarkers for assessing methanotrophic diversity in the environment [11, 12]. In marine environments, surveys focused on high methane-loaded systems like mud volcanoes [13], vent fields [14], Red sea brine pools [15] and methane gas seeps [16–18] revealed the dominance of gammaproteobacterial methanotrophs (Type I MOB) affiliated to the *Methylococcaceae* as the most abundant aerobic methanotrophs, with only occasional reports on the detection of alphaproteobacterial methanotrophs (Type II MOB) [13, 14, 17, 19, 20]. Furthermore, *pmoA* amplicon sequencing demonstrated that > 80% of detected sequences were attributed to novel species of as yet uncultivated methanotrophs [15, 16, 18]. Several of these *pmoA* clades, termed marine deep-sea cluster 1 to 5, seem largely restricted to marine environments [21] and are considered as important methane oxidizers [11, 12, 21, 22]. Indeed, only few marine methanotrophs have been brought into culture and consequently, we know very little of the ecophysiology of marine methanotrophs and the environmental conditions influencing

methane oxidation activity as only few have been cultured. The problematic cultivation of (marine) methanotrophs is mainly caused by their inability to form colonies on conventional solid media [23] and their mutualistic relationships with other bacteria [24] making it very difficult and laborious to obtain axenic cultures.

Here we report on an cultivation-based study to retrieve methanotrophs from marine sediments collected at six stations located along an inshore-offshore transect from the brackish part of the Western Scheldt estuary up to the Southern Bight of the North Sea (Fig. S5.1). The main source of methane in the North Sea is sedimentary biological methane production in the nutrient-rich estuarine tidal flats and salt marshes as well as by riverine input, [8, 25–27]. The Western Scheldt estuary is a well-mixed, turbid macrotidal, eutrophied estuary with methane concentrations of 500-5000 nmol L^{-1} [6, 7] and a nitrogen load of 5 x 10^9 mol N yr^{-1} [28]. It brings methane-loaded water to the Southern Bight where it is mixed is with Atlantic methane-poor water flowing in through the Strait of Dover. We are unaware of research on methanotrophs in the Western Scheldt estuary and the Southern Bight of the North Sea, but the few available biomarker-based studies on other estuaries and areas of the North Sea either report negative *pmoA* amplification results [29] or low abundances of *pmoA*-containing MOB. In addition, *mmoX*-containing MOB were limited to *Methylomonas* spp [30] although also facultative MOB *Methylocella* have already been detected via nested qPCR [31]. We obtained >200 active, highly enriched marine methanotrophic cultures and investigated their ecophysiology. Cultured representatives of the as-yet uncultured deep-sea cluster 3 and novel groups within deep-sea cluster 2 were found. We observed that the sampled transect from estuary to open sea coincided with a decreasing trend in cultivability and molecular detectability of marine MOB. Amplification of biomarker genes was unsuccessful for almost half of the actively methane-oxidizing cultures, probably due to single nucleotide polymorphisms in the primer regions, pointing to a potential gross underestimation of marine methane oxidizers using biomarker-based approaches.

5.2. MATERIAL AND METHODS

Sediment and seawater sampling

The North Sea (including estuaries and fjords) covers a surface area of about 750.000 km² and contains a volume of about 94.000 km³. The Southern Bight of the North Sea is a well-mixed area of about 39.000 km² with an average depth of 28 m. The Western Scheldt estuary is a well-mixed, turbid macrotidal estuary covering an area of 370 km². The yearly average fluvial freshwater discharge amounts to 104 m³/S [32] and salinity ranges from approximately 12 at the estuary head to 32 PSU at the mouth. Sediment samples from six stations covering a gradient from brackish estuarine (Stations LS03, LS02, and LS01) to fully marine offshore waters (Station W04, W07tris and W09) were collected mid-September 2012 (Table S5.1; Fig. S5.1). These stations are part of a routinely analyzed set of stations by the MUMM (Management Unit of the North Sea Mathematical Models and the Scheldt estuary) and reported by the Belgian Marine Datacenter. A large set of different physicochemical parameters, measured over the last 25 years, are freely accessible via www.mumm.ac.be/datacentre/ and were used to select the stations based on their presence along an inshore-offshore gradient of increasing salinity and decreasing inorganic nitrogen (both parameters were systematically monitored in the water column). Sampling was carried out with the RV Simon Stevin (http://www.vliz.be). Sediment samples were collected by means of a Reineck box-core at all stations, not disturbing the vertical sediment profiles. Sub-samples were taken from each sediment core with a single Plexiglas tube (internal diameter Ø 6.2cm; H: 25cm) and stored at 4°C till further processing in the lab. Sediment cores used for nutrient and metal analyses were immediately sliced per 2 cm and stored at -20°C. Surface seawater at 3m depth was collected per station with Niskin bottles [33]. Seawater was stored in polycarbonate containers for at least several months at 4°C and filtered before use (further referred to as “aged seawater”).

Sediment oxygen penetration depth was quantified in a temperature controlled room at 15°C (which is in accordance with the in-situ temperature recorded via a CTD Seabird 19plusV2 type). Intact sediment cores were immediately aerated to maintain the oxygen saturation in the water. After one day of acclimatization, depth profiles were measured with oxygen microsensors (100 µm tip size, Unisense) in vertical increments of 250 µm, starting just above the sediment surface downwards, until oxygen was depleted. Oxygen electrodes were connected to a picometer and output was displayed on an online PC using SensorTracePro

software (Unisense). Several cores from the same station were profiled (Fig. S5.2). Next, cores were sliced in steps of two centimeters, retaining only oxygenated sediments for further processing and enrichment of methane oxidizing bacteria. Biological replicates (n=2) were included for each station, resulting in 24 samples (depth 0-2 cm for stations LS03, LS01 and W04; depths 0-2 cm and 2-4 cm for LS02 and W07tris; depths 0-2 cm, 2-4 cm, 4-6 cm, 6-8 cm, 8-10 cm, for station W09, based on an extrapolation of the oxygen slope decrease.

Methane-oxidizing enrichments of marine sediments

Ten gram of each sediment sample was used as inoculum for enrichments under a headspace of 20:80 v/v methane/air. Initial enrichments were performed in duplicate in 60 ml serum bottles sealed with grey butyl rubber stoppers, using media mimicking the *in-situ* nutrient conditions, as reported by the Belgian Marine Datacenter over the last 25 years. All media were prepared with aged seawater and supplemented with 400 µM KNO_3, 5 µM $NaNO_2$, 100 µM NH_4Cl, 50 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 100 µM FeNaEDTA for LS03; 200 µM KNO_3, 3 µM $NaNO_2$, 80 µM NH_4Cl, 30 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 70 µM FeNaEDTA for LS02; 50 µM KNO_3, 1.5 µM $NaNO_2$, 50 µM NH_4Cl, 10 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 50 µM FeNaEDTA for LS01; 25 µM KNO_3, 0.5 µM $NaNO_2$, 10 µM NH_4Cl, 4 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 30 µM FeNaEDTA for the station W04; 15 µM KNO_3, 0.5 µM $NaNO_2$, 5 µM NH_4Cl, 2.5 µM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 20 µM FeNaEDTA for W07tris; and 5 µM KNO_3, 0.3 µM $NaNO_2$, 3 µM NH_4Cl, 1.5 µM KH_2PO_4, 1µM $CuSO_4.5H_2O$, 10µM FeNaEDTA for W09. All media was supplemented with 0.01% acid-washed silicium dioxide 10-20 nm particles (Sigma Aldrich, CAS 7631-86-9) **(Chapter 2)**. Enrichments were incubated at 20°C shaken orbital at 100 rpm.

Dense cultures were observed after two months and several methane and nutrient replenishment cycles. These enrichments were subjected to a miniaturized extinction culturing in 96-well plates [34, 35]. To maximize the retrieval of methanotrophic diversity, different realistic potential niches were mimicked by varying the concentration of inorganic nitrogen in the medium (50µM/50µM NO_3^-/NH_4^+, 250µM/250µM NO_3^-/NH_4^+ and 500µM/500µM NO_3^-/NH_4^+) and oxygen concentration in headspace (5% and 20%). Media were supplemented with 1 mM KH_2PO_4, 40µM FeNaEDTA, a salt solution and a trace element solution, both according to the standard dNMS medium [36], to avoid limitation. Extinction culturing of each enrichment was performed in duplicate for each condition yielding a total of 288 incubations. After a two-month incubation period, the highest dilutions showing growth were

transferred to 15-mL serum vials and screened for methanotrophic activity. Repeated subcultivations over a three year period of the remaining 204 methane-oxidizing enrichments nor passages through gellan gum solidified media were sufficient to obtain axenic MOB. Enrichments were cryopreserved with 10% dimethyl sulfoxide in liquid nitrogen as previously described **(Chapter 6 & 7)** [37, 38].

Amplification and phylogenetic analyses of *pmoA* and *mmoX*

DNA from enriched cultures was extracted via alkaline lysis. After washing harvested cells with a solution of 0.15 M NaCl and 0.01M EDTA to remove the majority of exopolysaccharides, 20 µl of lysis buffer (2.5ml 10% SDS; 5ml 1M NaOH; 92.5ml MilliQ water) was added and left at 95°C for 15 min. Lysates were centrifuged, 180 µl MilliQ was added were stored at -20°C.

Primers sets pmoA189 [39] - mb661r [40], wcpmoA189f-wcpmoA661r [41] and V170f-V613b [42] were used for amplification of *pmoA*. Primers sets 534f-1393r [43] and 206f-886r [44] were used for amplification of *mmoX*. PCR reagent mixture and temperature conditions were as originally described, except 100 µM bovine serum albumin was added to avoid aspecific amplification. Obtained amplification products, showing single bands of the expected size on a 1% agarose gel, were purified using a Nucleofast 96PCR cleanup membrane system (Macherey-Nagel) and Tecan Workstation 200 (Tecan) as described elsewhere [45]. Purified PCR products were sequenced with the corresponding forward and reverse primers used for amplification. The ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems) was used for sequencing and BioNumerics 7.5 software for assembly. The resulting sequence was translated to amino acids, checked for conserved domains using InterProScan and used for phylogenetic analyses in MEGA 6 [46]. Binning of sequences into OTUs was done manually using an amino acid dissimilarity cut-off of approximately 7% for PmoA [21, 47] and 4-5% for MmoX [11, 48]. Both cut-off criteria were shown to correspond with the 3% 16S rRNA gene sequence similarity level, and were used to delineate methanotrophic species. Neighbor joining trees with evolutionary distances computed using the p-distance method were constructed.

Analytical methods

Methane oxidation activity of enrichment cultures was monitored over time by measuring concentrations of methane, oxygen and carbon dioxide with a Compact GC (Global Analyzer

Solutions, Belgium), equipped with one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. Values were converted to µmol methane, oxygen and carbon dioxide Liquid^{-1} by compensating for change in gas pressure (measured with an Infield 7 pressure meter, UMS, Germany) and taking the solubility of the gases into account.

Several physicochemical parameters were determined from the water and sediment samples. Nutrient and metal composition was determined for sediment pore water. Sediment slices were stored in sterile petri dishes at -20°C till further processing. Pore water was extracted by gas pressure using nitrogen gas for nutrient analyses. The pore water was forced over a GF/C^{TM} glass microfiber filter (Whatman) and further filtered over a 0.2 µm filter. Three independently collected sediment cores per station were used as biological replicates to determine nitrate, nitrite, ammonium, silicate, and phosphate pore water concentrations (SAN^{plus} segmented flow analyzer, SKALAR). Two biological replicates were used to determine metal composition. To avoid metal contamination, pore water extraction for metal determination was performed through centrifugation using Centrifugal Nylon filters (0.45µm) (Capital analytical). Samples were centrifuged at 4°C during 30 min at 2.500 g [49]. Subsequently, the pore water was filtered through a 0.2 µm nylon filter and acidified to a final concentration of 1% nitric acid. Ca, K, Mg, Na were determined by ICP-OES (with a detection limit of 0.02 mg/L) and Cd (0.1µg/L), Ce (0.02µg/L), Cu (5µg/L), Fe (5µg/L), La (0.02µg/L), Li (5µg/L), Mn (5µg/L), Nd (0.02µg/L), Ni (0.5µg/L), Pr (0.02µg/L) and Zn (20µg/L) were determined by ICP-MS. Furthermore, total organic matter (TOM) (loss of mass after incineration at 500°C for 5h), total organic carbon (TOC) and nitrogen (TON) (Organic Elemental Analyzer Flash 2000, Interscience) and grain size (using laser diffraction, Malvern Mastersizer 2000) were determined for each sample.

Significant differences in environmental properties between stations and between depths were detected using a non-parametric Kruskal-Wallis test. Relationships among sediment properties and between sediment properties and the number of methanotrophic and/or identified cultures were determined using Spearman-rank correlations in SPSS23.

5.3. RESULTS AND DISCUSSION

Decreased cultivability of marine MOB from estuary to open sea

We conducted the first cultivation-based study to retrieve and characterize aerobic MOB from six stations that were selected along an increasing salinity and decreasing nitrogen gradient across the Western Scheldt estuary up to an offshore area in the Southern Bight of the North Sea (Fig. S5.1; Table S5.1). This inshore-offshore transect coincided with a coarsening of the sediment grain size gradient (Table S5.2), and was further correlated to several other sediment properties such as concentrations of total organic carbon, total organic matter, total nitrogen and sediment pore water nitrite (Table S5.3). The aerobic zone (based on oxygen penetration depth; see Fig. S5.2) of duplicate sediment cores from each station was sliced per two centimeters, resulting in 24 samples that were used to start enrichments under a headspace of 20:80 v/v methane/air. To maximize the retrieval of methanotrophic diversity, different realistic niches were mimicked, by varying oxygen, nitrate and ammonium within the concentration range observed *in situ*. As such, a total of 144 unique enrichments (in duplicate) were obtained. We anticipated that the different sediment pore water nutrient and metal composition between the original samples (Table S5.1; Table S5.2) would yield a different outcomes of the cultivable methanotrophic diversity.

After more than three years of enrichments, extinction culturing, multiple subcultivations and failed attempts to obtain axenic cultures via passages on solidified media, 204 out of 288 enrichment cultures were found positive for methane oxidation. Despite retrieving active MOB from each stations and depths, a clear decreasing trend of cultivability, i.e. the ability to grow *ex situ* in the applied growth conditions, along the transect from estuarine to open sea was observed. This was supported by the significant, negative correlation between number of methanotrophic cultures and median grain size coinciding with the inshore-offshore gradient (Fig. 5.1). Significant, but less pronounced, correlations were also found between number of methanotrophic cultures and nitrite and copper concentration (Fig. 5.1; Table S5.4). A higher copper concentration (63.5 µg/L) during the enrichment than *in situ* might have disadvantaged pMMO-lacking MOB, possibly leading to an underrepresentation of MOB only containing sMMO [50, 51]. Still, it seems more likely that both copper and nitrite are also indicative of the chosen gradient across the stations (Table S5.3), making it difficult to link one specific environmental property to the decreasing cultivability. Lower cultivability of MOB from open sea sediments compared to estuarine sediments has not been reported previously. Furthermore,

it is generally acknowledged that marine microorganisms are more problematic to grow *ex situ* than their terrestrial counterparts. Marine sediments harbor highly diverse microbial communities [52]. In these complex communities of co-occuring bacteria the species adapt in their use of resources compared with the same species in monocultures and evolved to use waste products generated by other species [53, 54]. Possibly this results in an obligate interdependence for growing MOB [24, 55, 56] with negative affect on the cultivability and isolation because the conditions for the growth of accompanying microbiota were not met in this study. These strong cooperative interactions can become more pronounced in nutrient poor environments [57], which could very well explain the lower cultivability in enrichments of open sea sediments. Although custom-made media were designed based on local aged sea water for each station and nutrients were added according to *in situ* concentrations, the existence of a still unknown determining growth parameter making the growth conditions less suitable for cultivation of MOB from open sea sediments compared to those from estuarine sediments is also possible.

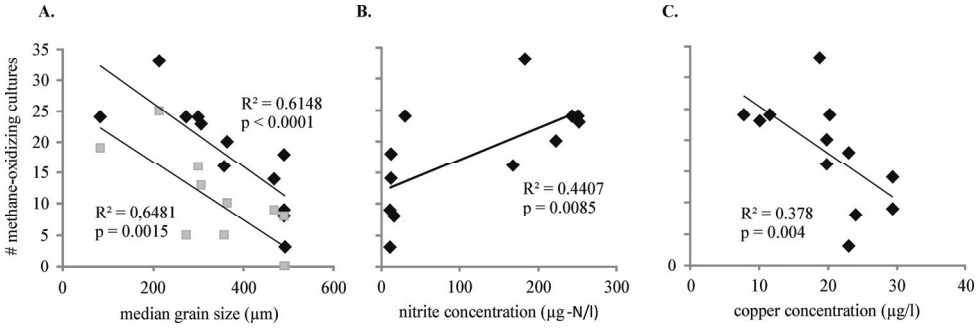

Figure 5.1. Correlations between environmental characteristics of sampling sites and number of methane-oxidizing cultures that were obtained (♦) or number of methane-oxidizing cultures that could be identified (■). Only significant correlations with sufficient spread of data points are given. Correlations with all environmental characteristics determined in this study are given in Supplementary Table 5.3.

Identification and habitat preferences of known and new marine MOB clades

As no axenic MOB cultures were obtained, methane-oxidizing enrichment cultures were identified via *pmoA* and *mmoX* sequence analyses. Surprisingly, only 51% of the cultures

rendered an amplicon for either biomarker. If gene amplification was successful, it resulted always in a high quality sequence without ambiguities referring to a single MOB being the result of several extinction culturing events, subsequent enrichments and multiple subcultivations over a three-year period. Retrieved sequences were translated to amino acids and binned into ten different PmoA-based OTUs (Fig. 5.2) and two MmoX-based OTUs (Fig. 5.3). Cut-off criteria for binning were chosen to correspond to the 3% 16S rRNA similarity distance level for species, as described previously [11, 21, 47, 48]. Representatives of deep-sea cluster 3 were brought into culture as well as two novel groups within deep-sea cluster 2 [21] (Fig. 5.2). Deep-sea cluster 2 consists of 27 OTUs [22] of which several have already been identified as endosymbionts or epibionts of marine animals [58–60]. PmoA_OTU 2 (90% aa similarity with *Methylobacter* sp) and pmoA_OTU 4 (92% aa similarity with *Methylomarinum* sp.) fell into clusters distinct from *Methylomonas* and *Methylomarinum* and lacking cultured representatives (Fig. 5.2). Furthermore, two OTUs (pmoA_OTU 9, 85% aa similarity with *Methylosoma* sp. and pmoA_OTU 10, 82% with *Methylomicrobium* sp.) were placed in the deep-sea cluster 3 (Fig. 5.2), and appeared to be the first successful cultivation, albeit non-axenic, of representatives of this clade. Most sequences belonging to this clade have been exclusively found in the marine environment, although some of them were reported from aquatic environments, mud volcanoes and landfill cover soil [22]. Other MOB belonged to major lineages known from environmental studies in methane rich ocean sediments [13, 14, 17, 18] and isolated pure cultures (Fig. 5.2) like *Methyloprofundus* (pmoA_OTU 3, 94% aa similarity) [61], *Methylobacter* (pmoA_OTU 5 & 8 with 90-100% aa similarity) [62], *Methylomicrobium* (pmoA_OTU 6 with 99% aa similarity) [63], *Methylocaldum* (pmoA_OTU 7, 99% aa similarity) [64], and *Methylomonas* (pmoA_OTU1 & mmoX_OTU2, 99-100% aa similarity) [65]. MmoX_OTU2 was only detected in enrichments also yielding a PmoA_OTU1, with identical phylogenetic affiliation, while some cultures only rendered PmoA_OTU1. This suggests that PmoA_OTU 1 comprised of different strains, of which some contain only the particulate methane monooxygenase and others contain both forms for the oxidation of methane. The presence of sMMO in in a MOB population/community enlarges the metabolic versatility in a changing environment or under acute environmental stress. These results further support the generally accepted dominance of type I MOB in marine systems, but, we did obtain a marine type II MOB. MmoX_OTU 1 represented the recently isolated marine *Methyloceanibacter methanicum* **(Chapter 3)**. This organism was isolated from several methane-oxidizing enrichments of W04, using a targeted isolation approach using metagenomics, floating filters [66], previously successful for the isolation of

methane-oxidizing *Verrucomicrobia* [67] unable to grow on solid media, with methanol as sole carbon source.

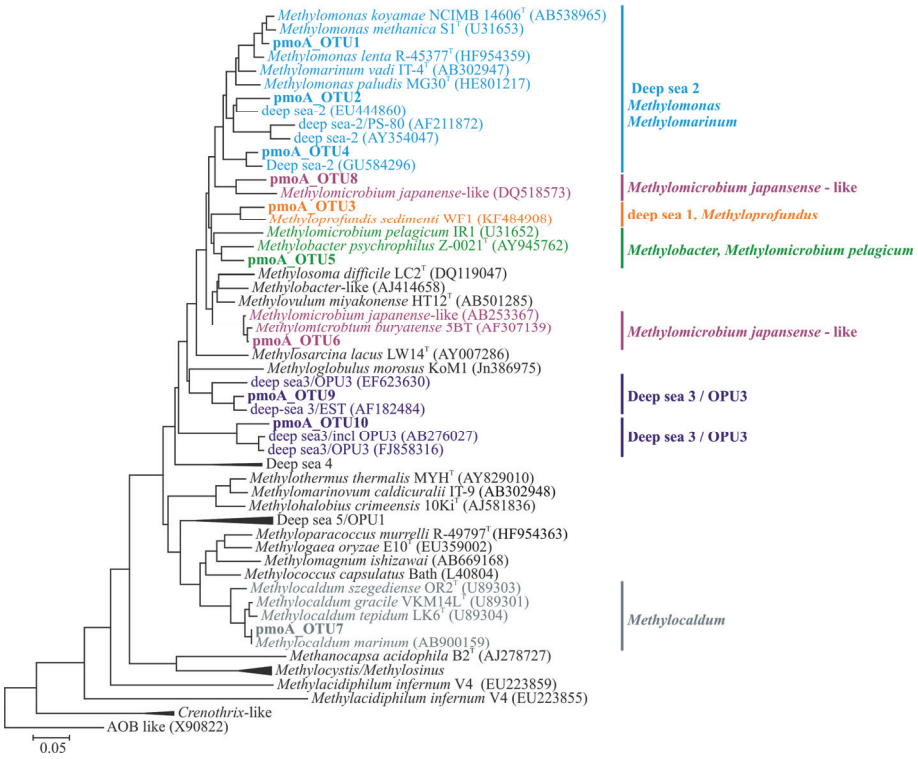

Figure 5.2. Phylogenetic Neighbor Joining tree showing the affiliation of the particulate methane monooxygenase. Representative sequences of type strains, marine cultures and marine environmental sequences were included. OTUs were delineation using a cut-off of 7% amino acid dissimilarity. The evolutionary history was inferred using 135 amino acid positions and evolutionary distances are given as number of amino acid differences per site. Accession numbers are given between brackets.

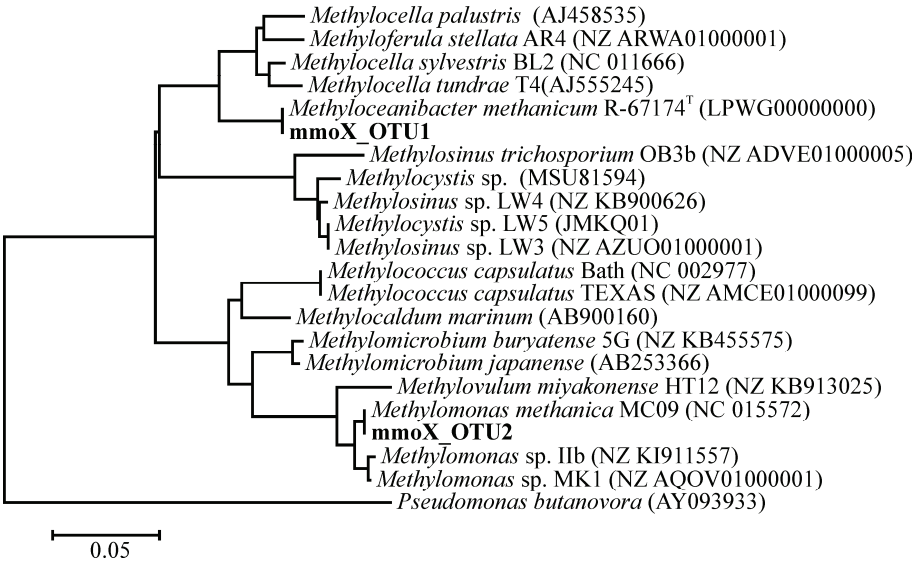

Figure 5.3. Phylogenetic Neighbor Joining tree showing the affiliation of the soluble methane monooxygenase. Representative sequences of type strains, marine cultures and marine environmental sequences were included. OTUs were delineation using a cut-off of 4-5% amino acid dissimilarity. The evolutionary history was inferred using 271 amino acid positions and evolutionary distances are given as number of amino acid differences per site. Accession numbers are given between brackets.

Furthermore, it is also known that occurrence and activity of MOB in different ecosystems is influenced by methane and oxygen concentration, nutrient and metal availability, pH, temperature, salinity and water availability [68], of which methane concentration, nitrogen and the role of copper have been studied most [68–70]. We visualized the membership of each OTU in each sample (Fig. 5.4) to get more insight into the ecophysiology and habitat preference of the cultivated marine MOB. *Methylomonas* sp. (pmoA_OTU 1) dominated station LS03, positioned closest to the Scheldt river mouth and characterized by the lowest water salinity. Its occurrence further shows a decreasing trend along the inshore-offshore gradient (Fig. 5.4). This fits with the general assumption that *Methylomonas* spp, prevalent in freshwater, marine coastal and estuarine systems [20, 30, 65, 71, 72], only occur in marine environments because of their salt tolerance and not because of adaptation to a more versatile lifestyle. Also *Methylobacter* sp (pmoA_OTU 5), *Methylomicrobium* sp (pmoA_OTU 6) seemed to be restricted to low salinity environments. *Methylomicrobium* sp (pmoA_OTU 8)

and *Methylocaldum* sp. (pmoA_OTU 7) were only found in the open sea stations with highest salinity. In addition, the latter MOB was only cultivated in media with lower nitrogen concentration (50 and 250 µM nitrate/ammonium). Interestingly, one of the deep-sea cluster 3 (pmoA_OTU 9) was restricted to 50 µM nitrate/ammonium and 5% oxygen, while this was not the case for the other representative of the clade (pmoA_OTU 10). A similar observation was made for the two novel groups of deep-sea cluster 2, with pmoA_OTU 4 being restricted to 250 µM nitrate/ammonium and pmoA_OTU 2 having no specific preference for concentration of nitrogen although it was restricted to low saline environments. These data suggest that nitrogen, oxygen and salinity can drive niche specialization in deep-sea cluster 2 and 3.

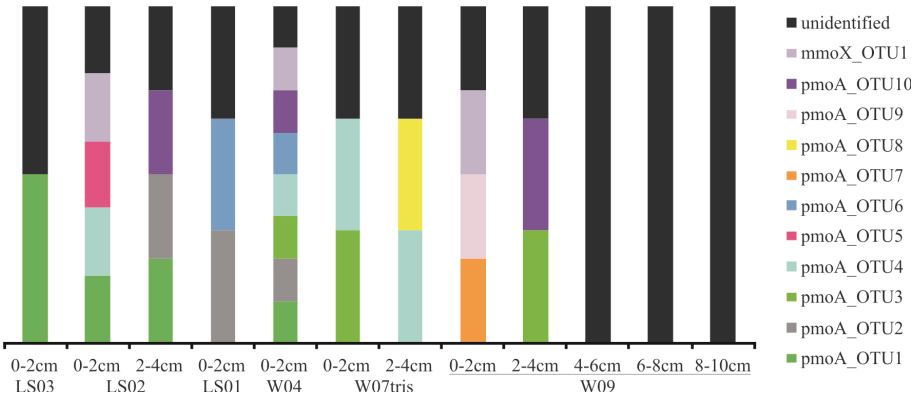

Figure 5.4. Membership (presence/absence) of different MOBic species in enrichments over sampling sites and depths. Identification deduced from on PmoA and MmoX phylogeny. Mmox_OTU2 is phylogenetically identical to pmoA_OTU1 (see text) and is therefore not included.

The highest number of OTUs were retrieved from station W04 (Fig. 5.4). This station is located in the plume of the Western Scheldt estuary (Fig. S5.1), with higher nitrogen concentrations than the further offshore stations and higher salinity than the estuary stations. The combination of high salinity and high nitrogen content, both determining factors for microbial diversity [52], might create a unique environment in station W04 resulting in a higher bacterial, and presumably higher methanotrophic diversity.

Decreased detectability of marine MOB from estuary to open sea

Failure to amplify biomarker genes for MOB in environmental samples are often consistent with low microbial turnover rates of methane and linked to their low abundance [29]. However, we failed in this study to amplify *pmoA* and *mmoX* in almost half enrichments that actively oxidized methane, leaving nearly half of the cultivated MOB unidentified. The number of unidentified MOB were significantly correlated with median grain size (Fig. 5.1A), demonstrating a decreased detectability of these biomarker genes along the sampled inshore-offshore transect in the North Sea (Fig. 5.4). Comparison of the amplification success rate of the different primer sets per sample (Table 5.1) revealed the highest efficiency was observed for the stations located in the Western Scheldt estuary (79% amplification success rate, i.e. 19 out of 24 cultures), while no amplicons were detected from the deeper layers of the open sea sediment samples. Albeit active methane oxidation was observed for all 20 enrichments originating from these three samples. None of the applied primers had a 100% amplification efficiency for detecting the *pmoA* sequences from the enrichment cultures (Table 5.1). Clearly the *pmoA* primers wcpmoA189f-wcpmoA661r more specific for marine systems [41] had higher amplification success than the more broadly used pmoA189f-mb661r [39, 73], which are supposed to provide the widest coverage of the known *pmoA* diversity [11, 21]. The primer set V170f-V613b [42], selective for amplification for various verrucomicrobial *pmoA* sequences, did not render any amplicon, suggesting that no MOB related to the *Verrucomicrobia* were present in our enrichment samples. A similar efficiency difference between the two applied *mmoX* primer sets was observed. Primer set mmoX534f-1393r [43] detected 94% of all amplified *mmoX* compared to only 13% with mmoX206f-886r [74]. It is difficult to speculate about the efficiency of *mmoX* primers in this experimental set-up because the sMMO is not obligatory for methane oxidation and might not be present in the cultures. Still, the superiority of the mmoX534f-1393r primer set [43] for the detection of *mmoX* genes in marine environments is evident.

Table 5.1. Amplification success rate of genes encoding for particulate and soluble methane monooxygenase (*pmoA* and *mmoX* respectively), expressed as percentage of methane-oxidizing enrichment cultures (n=204). Amplification success unique to single primer set is given between brackets. Primers V170f-V613b [42], selective for amplification for divers verrucomicrobial pmoA sequences, did not rendered any amplicon.

Sample		% *pmoA* amplification			% *mmoX* amplification		
Station	Depth	pmoA189f mb661r	wcpmoA189f-wcpmoA661r	Total	mmoX534f-1393r	mmoX206f-886r	Total
LS03	0-2cm	71 (8)	71 (8)	79	25 (21)	4 (0)	25
LS02	0-2cm	39 (0)	57 (17)	57	4 (4)	4 (4)	9
	2-4cm	54 (4)	75 (25)	79	29 (25)	4 (0)	29
LS01	0-2cm	22 (0)	35 (13)	35	0 (0)	0 (0)	0
W04	0-2cm	33 (3)	45 (15)	48	24 (24)	0 (0)	24
W07tris	0-2cm	25 (6)	25 (6)	31	19 (19)	0 (0)	19
	2-4cm	20 (0)	45 (25)	45	0 (0)	0 (0)	0
W09	0-2cm	17 (0)	22 (6)	22	28 (28)	6 (6)	33
	2-4cm	64 (0)	71 (7)	71	0 (0)	0 (0)	0
	4-6cm	0 (0)	0 (0)	0	0 (0)	0 (0)	0
	6-8cm	0 (0)	0 (0)	0	0 (0)	0 (0)	0
	8-10cm	0 (0)	0 (0)	0	0 (0)	0 (0)	0

Unfortunately, the low amplification success rates of the biomarker genes most probably resulted in an important number of unidentified MOB, potentially containing more divergent *pmoA* and *mmoX* sequences or representing novel lineages. We randomly selected two enrichment cultures, D14 from station W07tris and E33 from station W04, both failing to yield a *pmoA* or *mmoX* amplicon, for shotgun sequencing (**Chapter 4**). Metagenomic analyses demonstrate the presence of one methanotroph in each enrichment culture and both distantly related to the *Methylococcacea,* representing 52% and 28% of the total reads respectively. One complete pMMO operon was obtained from both metagenomes and no sMMO operon could be found. The *pmoA* sequences were extracted from the genome and appeared identical, indicating a closely related MOB was present in both samples. Alignment with representative sequences and primers (Fig. 5.5) revealed that the metagenome-derived *pmoA* sequence had one mismatch to both forward and reverse *pmoA* primers used in this study. A mismatch in the 3' region of the forward primer, being crucial in the correct binding of the primer to the DNA strand and being very sensitive to mismatches and degenerations, might have led to negative amplification results mentioned above. Originally, pmoA189f-

mb661r primers were designed based on pmoA and amoA sequences solely derived from terrestrial systems and may therefore not have been optimized for marine settings [39, 41]. Primer improvements to substantially enhance the sensitivity for pmoA screens in marine environments resulted in the primers wcpmoA189f and wcpmoA661r [41]. However based on the here presented results these primers could still be further improved by introducing an extra degenerate position to increase amplification specificity in marine systems.

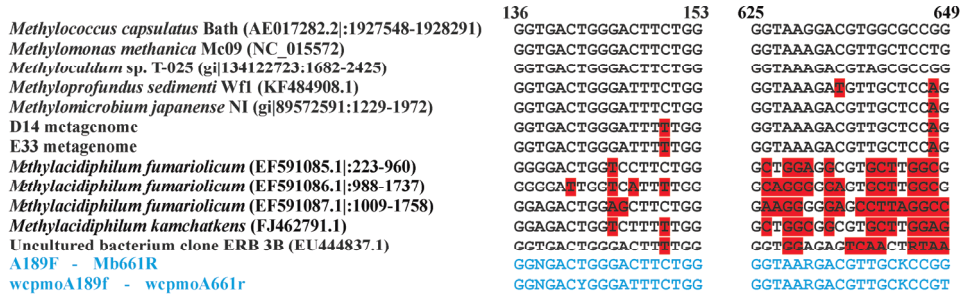

Figure 5.5. Alignment of mismatches of the *pmoA* genes retrieved from metagenome D14 and E33 with primers used in this study. Sequences of different model or marine MOBs are included. Accession numbers are given between brackets. Mismatches are shaded in red. Primers are given in blue. Reverse primers are shown as reverse complementary sequences. Nucleotide positions are relative to *M. capsulatus* Bath.

The metagenome-derived PmoA sequence was highly similar (100% aa similarity) to three sequences belonging to PmoA_OTU 4 picked up by the wcpmoA189f-wcpmoA661r primer set, grouped within the deep-sea cluster 2. In contrast to our initial expectations, and based on 2 out of more than 90 unidentified cultures, we suggest that that the undetected methanotrophic diversity can be closely related to the detected diversity. Furthermore the presence of highly novel MOBs in our culture collection seems supported by the number of unidentified cultures which did not correlate with any of the environmental parameters (Table S5.4). This suggests that the unidentified cultures at least consist of two different species, having different correlations with the different factors, outbalancing the correlation effect. Similar observations of limited molecular detection have already been made for other functional genes such as denitrification genes [75]. Be it known or novel diversity, it is clear that current widely applied primers highly underestimate the *in situ* diversity of marine

methane oxidizers, which needs to be thoroughly considered in PCR-based molecular surveys [41, 76–78].

5.4. ACKNOWLEDGEMENTS

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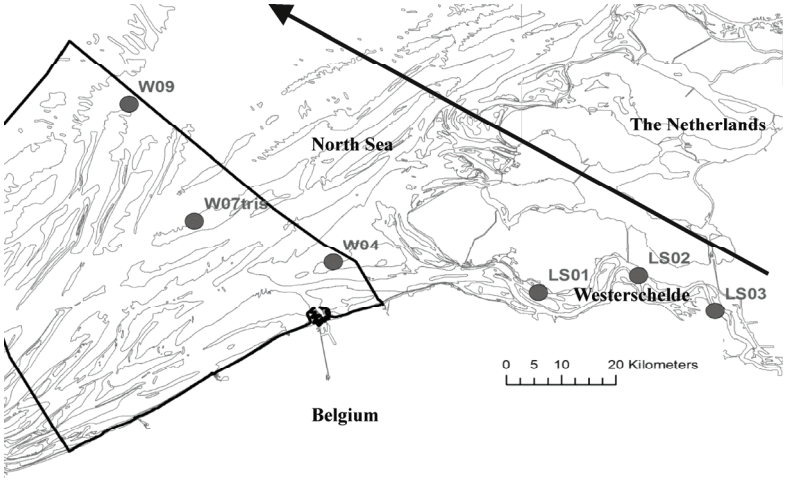
5.5. SUPPLEMENTARY MATERIALS

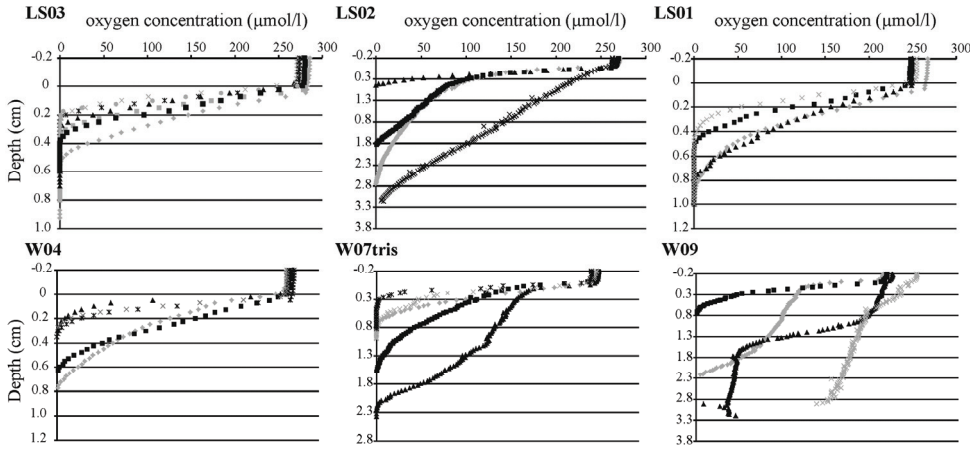


Supplementary Figure 5.1. Geographical location of sampling sites in the Western Scheldt estuary and North Sea. Names and exact coordinates are given in Table 5.1. The arrow indicates an increasing salinity and decreasing nitrogen gradient based on data from a > 25 year-long monitoring program (Belgian Marine Datacenter) of water column concentrations at these stations. Black line demarcates the Belgian Continental shelf.

Supplementary Table 5.1. Details on sampling sites.

Station	Location	Coordinates	Median grain size (µm) *	Salinity (psu)$^{\Phi}$	Depth (m)$^{\lambda}$
LS03	(near) Bath	N 51.35116 E 4.239333	84.06 (±7.67)	13.47 (±0.45)	13.4-13.6
LS02	(near) Hansweert	N 51.41983 E 4.0395	308.40 (±6.27)	23.52 (±0.01)	13.1-13.2
LS01	(near) Borsele	N 51.38983 E 3.776	274.81 (±18.10)	28.93 (±0.47)	16.2-17.6
W04	Vlakte van de Raan	N 51.449166 E 3.237166	213.65 (±1.46)	32.92 (±0.04)	6.07-6.58
W07tris	Thornton bank	N 51.528668 E 2.874000	359.10 (±20.10)	34.42 (±0.03)	20.76-22.17
W09	Hinderbanken	N 51.75 E 2.7	490.22 (±51.47)	35.02 (±0.16)	34.75-35.27

* Median grain size depicted for the upper two centimeter of the sediment
$^{\Phi}$ Average salinity is depicted for the whole water column
$^{\lambda}$ Depth represents the distance from water surface till sediment surface measured at arrival and departure at time of sampling



Supplementary Figure 5.2. Oxygen profiles of samples taken at six different stations. Different symbols indicate different replicate measurements. These data were used to delineate the aerobic zone and determined sampling depths used as inoculum for enrichment cultures.

Supplementary Table 5.2. Sediment properties measured for different depths for each site within the aerobic zone. Averages and standard deviations are based on triplicate measurements for nutrients and duplicate measurements for metals. Significant differences in distribution of characteristics between stations (*) and between depths (°) were detected using a non-parametric Kruskal-Wallis test ($p < 0.05$). TN, total nitrogen; TOC, total organic carbon; TOM, total organic matter; MGS, median grain size.

	LS03	**LS02**		**LS01**	**W04**	**W07tris**		**W09**				
	0-2 cm	**0-2 cm**	**2-4 cm**	**0-2 cm**	**0-2 cm**	**0-2 cm**	**2-4 cm**	**0-2 cm**	**2-4 cm**	**4-6 cm**	**6-8 cm**	**8-10 cm**
Nitrite*,°	30.80 ±	252.6 ±	251.41 ±	243.93 ±	183.5 ±	168.03 ±	222.87 ±	12.87 ±	12.99 ±	11.80 ±	12.42 ±	17.10 ±
(µg-N/L)	8.39	55.29	81.82	103.56	61.82	20.24	37.39	3.65	3.15	2.79	0.65	8.70
Silicate*	2.85 ±	0.86 ±	0.84 ±	1.45 ±	0.92 ±	0.36 ±	0.51 ±	0.38 ±	0.35 ±	0.37 ±	0.36 ±	0.40 ±
(mg/L)	0.92	0.32	0.63	0.56	0.24	0.04	0.17	0.03	0.08	0.06	0.06	0.07
Ammonia*	1.50 ±	0.23 ±	0.39 ±	3.67 ±	0.72 ±	0.99 ±	1.02 ±	0.29 ±	0.84 ±	1.60 ±	0.45 ±	0.26 ±
(mg-N/L)	0.89	0.22	0.37	4.49	0.18	0.14	0.26	0.11	0.97	2.34	0.38	0.04
Phosphate*	0.560 ±	0.15 ±	0.13 ±	0.22 ±	0.55 ±	1.11 ±	1.10 ±	0.38 ±	0.50 ±	0.81 ±	0.40 ±	0.35 ±
(mg/L)	0.22	0.04	0.03	0.06	0.05	0.28	0.57	0.17	0.08	0.42	0.17	0.23
Nitrate*,°	81.20 ±	620.70 ±	606.90 ±	258.85 ±	339.80 ±	289.78 ±	328.93 ±	177.27 ±	147.67 ±	115.42 ±	97.35 ±	121.91 ±
(µg-N/L)	44.27	17.39	41. 98	148.50	149.48	26.51	80.94	48.19	37.90	39.01	51.30	78.82
	55.66 ±	7.53 ±	18.63 ±	14.75 ±	11.80 ±	7.87 ±	6.30 ±	5.12 ±	5.44 ±	9.36 ±	4.30 ±	3.78 ±
‰ TN*	5.80	1.65	17.56	4.64	0.19	1.16	3.10	0.63	2.88	6.5	4.30	4.48
	0.84 ±	0.12 ±	0.12 ±	0.21 ±	0.12 ±	0.10 ±	0.10 ±	0.10 ±	0.08 ±	0.10 ±	0.06 ±	0.07 ±
% TOC*,°	0.15	0.05	0.035	0.12	0.01	0.2	0.01	0.02	0.02	0.03	0.02	0.04
	3.03 ±	0.51 ±	0.40 ±	1.03 ±	0.53 ±	0.45 ±	0.47 ±	0.31 ±	0.45 ±	0.35 ±	0.29 ±	0.24 ±
% TOM*,°	0.71	0.21	0.02	0.39	0.01	0.15	0.12	0.02	0.33	0.09	0.05	0.02
MGS*,°	84.056 ±	308.39 ±	301.37 ±	274.81 ±	213.64 ±	359.08 ±	364.32 ±	490.21 ±	468.23 ±	491.29 ±	494.01 ±	492.28 ±
(µm)	7.67	6.27	6.82	18.10	1.46	20.86	28.71	51.47	8.5	21.89	81.99	36.16
Ca*	309.75 ±	351.75 ±	367.50 ±	472.50 ±	399.00 ±	451.50 ±	477.75 ±	462.00 ±	498.75 ±	446.25 ±	446.25 ±	493.50 ±
(mg/L)	7.42	22.27	29.70	14.85	29.70	14.85	7.42	59.40	37.12	22.27	7.42	44.55
K*	262.50 ±	425.25 ±	409.50 ±	651.00 ±	840.00 ±	866.25 ±	876.75 ±	834.75 ±	929.25 ±	892.50 ±	845.25 ±	866.25 ±
(mg/L)	29.70	7.42	14.85	44.55	59.40	22.27	7.42	22.27	81.67	14.85	7.42	7.42

Supplementary Table 5.2.*Continued*

	LS03	LS02		LS01	W04	W07tris		W09				
	0-2 cm	0-2 cm	2-4 cm	0-2 cm	0-2 cm	0-2 cm	2-4 cm	0-2 cm	2-4 cm	4-6 cm	6-8 cm	8-10 cm
Mg*	0.60 ±	0.91 ±	0.92 ±	1.26 ±	1.36 ±	1.36 ±	1.41 ±	1.36 ±	1.36 ±	1.42 ±	1.31 ±	1.36 ±
(g/L)	0.03	0.07	0.04	0	0	0	0.07	0.15	0	0.074	0.07	0
Na*	3.10 ±	5.30 ±	5.35 ±	7.51 ±	9.66 ±	8.98 ±	9.29 ±	9.03 ±	9.24 ±	9.40 ±	8.82 ±	9.03 ±
(g/L)	0.22	0.52	0.15	0.07	1.19	0.22	0.22	1.04	0.15	0.22	0	0.15
Cd*	0.35 ±	0.83 ±	0.72 ±	0.34125 ±	0.38 ±	4.88 ±	1.36 ±	1.44 ±	0.10 ±	0.77 ±	0.67 ±	0.78 ±
(µg/L)	0.21	0.07	0.18	0.21	0.12	1.41	0.15	0.63	37	0.10	0	0.04
Ce	0.13 ±	<0.02	<0.02	<0.02	<0.02	<0.02	0.06 ±	<0.02	0.08 ±	0.05 ±	<0.02	0.02 ±
(µg/L)	0.10						0.04		0.04	0.01		0
Cu*	7.77 ±	10.13 ±	20.32 ±	11.55 ±	18.90 ±	19.95 ±	19.95 ±	23.10 ±	29.40 ±	29.40 ±	23.10 ±	24.15 ±
(µg/L)	1.93	2.00	17.30	0	2.97	2.97	1.48	1.48	10.39	8.91	4.45	7.42
Fe	897.75 ±	31.71 ±	64.57 ±	35.17 ±	20.05 ±	11.76 ±	53.55 ±	14.70 ±	83.47 ±	68.25 ±	134.98 ±	84.52 ±
(µg/L)	957. 78	33.86	72.02	18.56	14.70	5.64	38.61	2.97	74.99	66.82	180.34	58.65
La	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	0.04 ±	<0.02	0.06 ±	0.05 ±	0.07 ±	<0.02
(µg/L)							0.01		0.04	0.01	0.06	
Li*	59.85 ±	87.15 ±	84.00 ±	120.75 ±	136.50 ±	162.75 ±	162.75 ±	173.25 ±	162.75 ±	173.25 ±	162.75 ±	168.00 ±
(µg/L)	1.48	1.48	4.45	7.42	14.85	7.42	7.42	7.42	7.42	7.42	7.42	0
Mn*	525 ±	<0.02	<0.02	55.65 ±	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
(µg/L)	193.04			56.43								
Nd	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
(µg/L)												
Ni	4.3575 ±	3.6225 ±	2.52 ±	4.67 ±	5.41 ±	11.23 ±	4.15 ±	7.56 ±	15.80 ±	17.01 ±	6.25 ±	5.51 ±
(µg/L)	0.67	1.11	0.45	2.45	0.7	7.87	1.26	2.82	16.26	17.52	3.04	1.86
Pr	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
(µg/L)												
Zn	40.42 ±	31.50 ±	27.82 ±	32.55 ±	31.50 ±	38.85 ±	25.20 ±	27.30 ±	43.57 ±	45.15 ±	49.35 ±	61.95 ±
(µg/L)	6.68	2.97	3.71	7.42	0	10.39	2.97	5.94	25.99	20.79	34.15	5.94

Supplementary Table 5.3. Spearman rank correlation among sediment properties.

TN. total nitrogen; TOC. total organic carbon; TOM. total organic matter; MGS. median grain size.

*, correlation is significant at the 0.05 level (2-tailed); **, correlation is significant at the 0.01 level (2-tailed).

	Nitrite	Silicate	Ammonia	Nitrate	Phosphate	‰ TN	% TOC	% TOM	MGS	Ca	K	Mg	Na+
Nitrite	1	0.608*	-0.126	-0.392	0.811**	0.441	0.594*	0.573	-0.685*	-0.277	-0.525	-0.458	-0.413
Silicate	0.608*	1	0.126	-0.315	0.238	0.629*	0.776**	0.678*	-0.762**	-0.515	-0.757**	-0.548	-0.396
Ammonia	-0.126	0.126	1	0.559	-0.336	0.469	0.308	0.476	-0.294	0.091	0.196	0.203	0.151
Nitrate	-0.392	-0.315	0.559	1	-0.308	0.105	-0.126	0.119	0.049	0.126	0.539	0.585*	0.487
Phosphate	0.811**	0.238	-0.336	-0.308	1	0.280	0.315	0.266	-0.406	-0.144	-0.217	-0.065	0
‰ TN	0.441	0.629*	0.469	0.105	0.280	1	0.839**	0.727**	-0.867**	-0.616*	-0.469	-0.309	-0.126
% TOC	0.594*	0.776**	0.308	-0.126	0.315	0.839**	1	0.811**	-0.902**	-0.634*	-0.704*	-0.574	-0.466
% TOM	0.573	0.678*	0.476	0.119	0.266	0.727**	0.811**	1	-0.888**	-0.343	-0.403	-0.392	-0.245
MGS	-0.685*	-0.762**	-0.294	0.049	-0.406	-0.867**	-0.902**	-0.888**	1	0.511	0.634*	0.519	0.350
Ca	-0.277	-0.515	0.091	0.126	-0.144	-0.616*	-0.634*	-0.343	0.511	1	0.716**	0.617*	0.502
K	-0.525	-0.757**	0.196	0.539	-0.217	-0.469	-0.704*	-0.403	0.634*	0.716**	1	0.862**	0.774**
Mg	-0.458	-0.548	0.203	0.585*	-0.065	-0.309	-0.574	-0.392	0.519	0.617*	0.862**	1	0.913**
Na	-0.413	-0.396	0.151	0.487	0	-0.126	-0.466	-0.245	0.350	0.502	0.774**	0.913**	1
Cd	-0.126	-0.657*	-0.329	0.301	0.238	-0.531	-0.455	-0.406	0.434	0.413	0.487	0.509	0.280
Ce	-0.312	-0.047	0.398	0.452	-0.522	-0.039	-0.125	0.172	0.039	0.223	0.359	0.223	0.164
Cu	-0.703*	-0.794**	-0.130	0.067	-0.355	-0.580*	-0.808**	-0.794**	0.815**	0.574	0.727**	0.621*	0.551
Fe	-0.371	0.014	0.119	-0.056	-0.699*	-0.175	-0.266	-0.140	0.238	-0.025	0.039	-0.218	-0.210
La	-0.574	-0.612*	0.233	0.325	-0.429	-0.404	-0.649*	-0.312	0.603*	0.321	0.667*	0.419	0.379
Li	-0.727**	-0.692*	-0.068	0.317	-0.332	-0.645*	-0.720**	-0.720**	0.820**	0.614*	0.738**	0.815**	0.677*
Mn	0.172	0.650*	0.570	-0.032	-0.355	0.462	0.650*	0.650*	-0.591*	-0.237	-0.533	-0.553	-0.533
Ni	-0.832**	-0.748**	0.224	0.476	-0.538	-0.343	-0.476	-0.434	0.566	0.448	0.704*	0.585*	0.539
Zn	-0.588*	-0.354	0.081	0.091	-0.722**	-0.308	-0.354	-0.361	0.459	0.151	0.337	0.004	0

Supplementary Table 5.3. *Continued*

	Cd	Ce	Cu	Fe	La	Li	Mn	Ni	Zn
Nitrite	-0.126	-0.312	-.703*	-0.371	-0.574	-0.727**	0.172	-0.832**	-0.588*
Silicate	-0.657*	-0.047	-.794**	0.014	-0.612*	-0.692*	0.650*	-0.748**	-0.354
Ammonia	-0.329	0.398	-0.130	0.119	0.233	-0.068	0.570	0.224	0.081
Nitrate	0.301	0.452	0.067	-0.056	0.325	0.317	-0.032	0.476	0.091
Phosphate	0.238	-0.522	-0.355	-0.699*	-0.429	-0.332	-0.355	-0.538	-0.722**
‰ TN	-0.531	-0.039	-0.580*	-0.175	-0.404	-0.645*	0.462	-0.343	-0.308
% TOC	-0.455	-0.125	-0.808**	-0.266	-0.649*	-0.720**	0.650*	-0.476	-0.354
% TOM	-0.406	0.172	-0.794**	-0.140	-0.312	-0.720**	0.650*	-0.434	-0.361
MGS	0.434	0.039	0.815**	0.238	0.603*	0.820**	-0.591*	0.566	0.459
Ca	0.413	0.223	0.574	-0.025	0.321	0.614*	-0.237	0.448	0.151
K	0.487	0.359	0.727**	0.039	0.667*	0.738**	-0.533	0.704*	0.337
Mg	0.509	0.223	0.621*	-0.218	0.419	0.815**	-0.553	0.585*	0.004
Na	0.280	0.164	0.551	-0.210	0.379	0.677*	-0.533	0.539	0
Cd	1	0.016	0.366	-0.455	0.087	0.542	-0.640*	0.322	-0.249
Ce	0.016	1	0.188	0.624*	0.371	0.064	0.264	0.156	0.281
Cu	0.366	0.188	1	0.236	0.590*	0.778**	-0.594*	0.685*	0.423
Fe	-0.455	0.624*	0.236	1	0.466	-0.118	0.296	-0.014	0.634*
La	0.087	0.371	0.590*	0.466	1	0.390	-0.307	0.458	0.371
Li	0.542	0.064	0.778**	-0.118	0.390	1	-0.543	0.760**	0.250
Mn	-0.640*	0.264	-0.594*	0.296	-0.307	-0.543	1	-0.263	0.075
Ni	0.322	0.156	0.685*	-0.014	0.458	0.760**	-0.263	1	0.532
Zn	-0.249	0.281	0.423	0.634*	0.371	0.250	0.075	0.532	1

Supplementary Table 5.4. Spearman rank correlation between sediment properties and MOBs. #MOB, total number of methane-oxidizing cultures; # ID MOB, number of identified methane-oxidizing cultures; TN. total nitrogen; TOC. total organic carbon; TOM. total organic matter; MGS. median grain size. Mmox_OTU2 is identical to pmoA_OTU1(see text) and is therefore not included.

*, correlation is significant at the 0.05 level (2-tailed); **, correlation is significant at the 0.01 level (2-tailed).

	pmoA OTU1	pmoA OTU2	pmoA OTU3	pmoA OTU4	pmoA OTU5	pmoA OTU6	pmoA OTU7	pmoA OTU8	pmoA OTU9	pmoA OTU10	mmoX OTU1	unidentified	#MOB	# ID MOB
nitrite	0.520	0.496	-0.064	0.504	0.480	0.312	-0.306	0.218	-0.306	0.195	0.156	0.426	0.718**	0.589*
silicate	0.670*	0.542	-0.441	0.101	0.218	0.511	-0.131	0.044	-0.131	-0.028	0.257	0.139	0.810**	0.589*
ammonia	-0.187	0.156	0.092	-0.050	-0.480	0.290	-0.306	0.218	-0.306	-0.139	-0.477	0.188	0.169	-0.078
phosphate	-0.291	-0.349	0.404	0.399	-0.393	-0.102	-0.131	0.393	-0.131	-0.195	-0.193	0.028	-0.183	-0.067
nitrate	0.283	0.459	0.147	0.643*	0.480	0.274	-0.044	0.218	-0.044	0.362	0.450	0.505	0.542	0.497
TN	0.653*	0.652*	0.119	0.160	-0.044	0.532	-0.306	-0.131	-0.306	0.362	0.138	0.103	0.824**	0.649*
TOC	0.678*	0.477	-0.083	0.143	0.306	0.441	-0.131	-0.218	-0.131	0.028	0.239	0.345	0.838**	0.600*
TOM	0.508	0.404	0.165	0.349	0.218	0.511	-0.306	0.131	-0.306	0.139	0.147	0.203	0.796**	0.688*
MGS	-0.716**	-0.597*	-0.138	-0.269	-0.131	-0.521	0.218	0.044	0.218	-0.307	-0.220	-0.188	-0.944**	-0.808**
Ca	-0.834**	-0.184	0.267	-0.080	-0.394	-0.027	0.131	0.306	0.131	-0.028	-0.285	0.174	-0.480	-0.509
K	-0.767**	-0.377	0.441	0.128	-0.306	-0.183	-0.131	0.306	-0.131	0.028	-0.276	-0.032	-0.660*	-0.521
Mg	-0.709**	-0.210	0.257	0.255	-0.408	-0.045	0.136	0.454	0.136	-0.029	-0.029	0.188	-0.432	-0.388
Na	-0.533	0.055	0.386	0.244	-0.394	0.237	0.087	0.306	0.087	0.251	0.202	0.014	-0.233	-0.170
Cd	-0.424	-0.597*	0.303	0.382	0.131	-0.570	0.393	0.306	0.393	-0.139	0.092	0.330	-0.416	-0.180
Ce	0.000	-0.461	0.041	-0.141	-0.243	-0.360	-0.243	0.341	-0.243	-0.031	-0.461	-0.384	-0.173	0.063
Cu	-0.619*	-0.332	0.152	-0.435	-0.395	-0.384	0.176	-0.088	0.176	0.168	-0.258	-0.295	-0.758**	-0.612*
Fe	0.125	-0.285	-0.303	-0.563	-0.218	-0.333	-0.393	-0.044	-0.393	-0.028	-0.587*	-0.771**	-0.345	-0.176
La	-0.475	-0.393	0.104	-0.143	-0.208	-0.307	-0.208	0.260	-0.208	0.033	-0.393	-0.450	-0.624*	-0.407
Li	-0.819**	-0.449	0.051	-0.124	-0.312	-0.258	0.445	0.089	0.445	-0.284	0.019	0.114	-0.733**	-0.703*
Mn	0.317	0.212	-0.254	-0.310	-0.134	0.302	-0.134	-0.134	-0.134	-0.257	-0.254	0.046	0.455	0.198
Ni	-0.678*	-0.367	0.459	-0.290	-0.393	-0.124	0.218	-0.306	0.218	-0.084	-0.101	-0.082	-0.648*	-0.610*
Zn	-0.263	-0.317	0.097	-0.509	-0.175	-0.170	-0.394	-0.481	-0.394	-0.168	-0.469	-0.495	-0.649*	-0.615*

5.6. REFERENCES

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Part II:

Preservation of microbiomes

Chapter 6

A generally applicable cryopreservation method of nitrite-oxidizing bacteria

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Author's contributions:

B.V., P.D.V, S.H. and K.H. designed the research; E.S. provided the NOB strains, B.V performed research; B.V, S.H. and K.H. analyzed data; B.V.and K.H. interpreted the results and wrote the paper, E.S., S.H. and P.D.V. commented on the manuscript.

6.1. INTRODUCTION

Nitrite-oxidizing bacteria (NOB) are obligate or facultative chemolithoautotrophic bacteria assimilating carbon dioxide as main carbon source and oxidizing nitrite as sole energy source. NOB perform the second step of the nitrification process, the conversion of nitrite to nitrate. By doing so they remove the toxic nitrite from the environment, which is harmful for living organisms [1], and convert it to nitrate that acts as a major nitrogen source for many organisms. Despite their ecological relevance, the handling is restricted to a few research groups, in comparison to aerobic ammonia-oxidizing bacteria / archaea or anaerobic ammonia-oxidizers. NOB are fastidious and slow growing microorganisms, and consequently their isolation and cultivation is a time-consuming process. As a result, only few representatives of this functional guild have been validly described. These are phylogenetically affiliated to the *Alpha*-, *Beta*-, *Gammaproteobacteria* and the deep-branching phyla *Nitrospirae* and *Nitrospinae* [2-5]. Recently a new member was added to this guild belonging to the widespread phylum *Chloroflexi* [6].

The conventional way to preserve and safeguard NOB is the continuous subcultivation and storage at a temperature of 17°C. However, this approach increases the risk of contamination and loss of authenticity over time, requires periodic maintenance, time and physical space, and is therefore impractical for individual researchers as well as large-scale culture collections [7]. The lack of a simple, high quality preservation protocol for members of this functional guild increases the possibility of strains being lost for future research and application [8]. In addition, the description of novel species is also hampered, since this requires the deposition of the type strain in two different public culture collections [9], which is mostly not possible because their standard preservation methods do not apply for these fastidious bacteria. Well-defined freezing protocols for nitrite-oxidizing bacteria - freezing in liquid nitrogen with hatefi buffer - have been proposed in the past [3], but were not picked up by the scientific community. This has led to the current situation of only few NOB species being publically available (http://www.wfcc.info). A fast, simple, high-quality but cheap method (without the necessity of costly equipment) would provide significant benefits for NOB maintenance, enabling better availability of the biological material, which will undoubtedly stimulate further research. A recent study on a wide diversity of methane-oxidizing bacteria demonstrated the effectiveness of a simple cryopreservation protocol, applying (i) the innate cryoprotective effects of carbon-rich media, which are often overseen in preservation

procedures of oligotrophic microorganisms, and (ii) the use of dimethyl sulfoxide (DMSO) that outperformed the widely used glycerol as a cryoprotectant (CPA) [10]. This easy method subsequently proved successful for the preservation of other oligotrophic microorganisms such as aerobic and anaerobic ammonia oxidizers [11, 12] and nitrite-dependent anaerobic methane oxidizers (K. Heylen, K. Ettwig, M. Jetten, B. Kartal, unpublished data). However, thus far only one carbon-rich medium (i.e. ten-fold diluted trypticase soy broth supplemented with 1% trehalose) was tested in combination with one fixed concentration of DMSO (5%) [10]. The cryoprotective effects of this condition on other oligotrophic bacteria, of other organic carbon sources and/or different DMSO concentrations remain unknown.

Because preservation success cannot be extrapolated with acceptable level of certainty, even within closely related strains, it should be experimentally demonstrated for each organism of interest. Therefore we here aimed at assessing the above-mentioned "established" cryopreservation protocol for an additional functional guild, namely the nitrite-oxidizing bacteria. In addition, we wanted to evaluate the influence of different carbon-rich media and various concentrations of DMSO on the preservation of NOB and ideally determine suitable preservation conditions for all (tested) members of this functional group.

6.2. MATERIALS & METHODS

Nitrite oxidizing strains.

Six different NOB strains representing six different species and five different genera were included in this study. Three non-marine strains, *Nitrobacter vulgaris* AB1 from sewage, *Candidatus* Nitrospira defluvii A17 from activated sludge and *Candidatus* Nitrotoga arctica 6680 from permafrost soil, and three marine strains, *Nitrococcus mobilis* 231, *Nitrospina gracilis* 3/211 and *Nitrospira marina*-like bacterium strain Ecomares 2.1, were cultivated in mineral salt medium and marine medium respectively [13]. These were all pure cultures, except for *Candidatus* Nitrotoga artica 6680, which was highly enriched. All strains were grown at a pH of 7.4-7.6, a temperature of 28°C and a nitrite concentration of 3 mM, with exception of the strain *Candidatus* Nitrotoga artica 6680, which was grown at a temperature of 15°C and a nitrite concentration of 0.3 mM. Nitrite was added periodically to maintain these concentrations.

Parameters for success rate evaluation.

Colorimetric methods were used for monitoring of nitrite oxidation [14] and nitrate production [15] in the different media. Both activity measures correlated well in accordance with the stoichiometry of the nitrite oxidation reaction (data not shown) and therefore only nitrite oxidation data is shown further. Growth was monitored via optical density at 600 nm, while ATP values obtained with BacTiter-GloTM Microbial Cell Viability assay (Promega) were used as a measure for viability. Specific activity measured as nitrite oxidation was demonstrated to be a reliable proxy for both growth and viability for all cultures as data from simultaneous monitoring of all three parameters showed strong positive Pearson correlation values (Table S6.1). Based on these data and because of high doubling times of NOB, only nitrite oxidation activity values were used for preservation evaluation.

The lag phase induced by preservation defined as the time point at which significant nitrite oxidation occurred (determined as the point when nitrite oxidation was at least five times the standard deviation of the technical error on the nitrite determination assay) was also determined (=0.52 mM NO_2^-). Technical error was determined on a data set of more than 10,000 measurements. Purity checks were performed periodically by plating on trypticase soy agar. To further ensure the purity and authenticity of each culture, 16S rRNA gene sequence

analysis was performed periodically before and after preservation, yielding always identical sequences for each strain.

Growth on carbon-rich media

All NOB strains were screened for growth on different carbon-rich media. Screening was miniaturized in microtiter plates, to allow a high throughput testing of a large number of carbon-rich media. Following media were investigated: (1) trypticase soy broth (TSB), (2) ten-fold diluted TSB, (3) ten-fold diluted TSB supplemented with 1% trehalose (TT), (4) 1% yeast extract, (5) 0.5% yeast extract, (6) 1% malt extract, (7) 0.5% malt extract, (8) 1% skimmed milk, (9) 1% trehalose, (10) 5% trehalose and (11) 1% sucrose. All carbon-rich media were prepared by adding the carbon compounds to the standard (mineral salt or marine) medium [13]. For each strain the standard medium was included as a growth control. Also, a blank was incorporated for each carbon medium to compensate for NO_2^- fluctuations due to evaporation.

Pre-preservation growth, cryopreservation and resuscitation

Prior to preservation, NOB strains were grown in standard medium and three carbon-rich media (i.e. ten-fold diluted TSB, TT, and 1% sucrose). Starting with an OD value of 0.001, cultures were grown for two months, spiking nitrite periodically and eventually starved via depletion of nitrite to enter stationary phase. Biomass was concentrated via centrifugation (8000 rpm, 15min) to a final volume of 1 ml, and washed two times with fresh growth medium. Samples were taken from each culture to determine biomass using the BCA assay. Total protein content was measured with the bicinchoninic acid (BCA) assay (Pierce, US), according to manufacturer's instructions and using bovine serum albumin as a standard. A total of 29 preservation conditions were applied per strain by varying the pre-preservation growth medium (standard vs carbon-rich), the preservation medium (standard vs carbon-rich) and the type and concentration of cryoprotective agent (CPA) (0, 1, 5, 10% DMSO & 100% Hatefi). Specifically, all six strains were grown in four different media (standard vs three different carbon rich) in combination with the addition of five different CPA's (0%, 1%, 5%, 10% DMSO & Hatefi) resulting in 20 different preservation conditions. Additionally cultures grown in standard medium were also subjected to nine extra preservation conditions by preserving in a carbon rich media (Ten-fold diluted TSB, TT, and 1% sucrose) in combination with 1%, 5% & 10% DMSO. Hatefi (consisting of 100 mL distilled water, 0.6 g TRIS, 22.6

sucrose, 0.015 g histidine, pH 7.5) was used as a 100% solution, so without addition of preservation medium [13].

Addition of DMSO was performed at 4°C to decrease toxicity, after which cultures were immediately preserved. Because of the laborious nature of the experiments each preservation condition was only prepared in duplicate. Subsequently all six NOB strains were subjected to long-term cryopreservation in liquid nitrogen (-196°C). Cultures were stored in Cryotube™ vials (Nunc, Denmark) and were placed in the gas phase just above the liquid phase. The cryopreserved NOB strains were resuscitated after a preservation period of eight months. After preservation biomass was thawed quickly at 37°C in an incubator and upon thawing, immediately transferred to eppendorf tubes and centrifuged (8000rpm, 15 min) at 4°C to minimize the toxic effect of DMSO. NOB biomass was washed twice with fresh standard medium (without nitrite) to ensure the removal of DMSO residues. Subsequently, the biomass was resuspended in a volume of 1 ml standard medium after which sodium nitrite was added. Nitrite was periodically spiked to maintain a concentration of approximately 20 mM for *N. vulgaris* AB1 & *N. mobilis* 231, 10 mM for *Candidatus* N. defluvii A17, *N. gracilis* 3/211 and *Nitrospira marina*-like bacterium strain Ecomares 2.1.and 1 mM for *Candidatus* N. arctica 6680

6.3. RESULTS AND DISCUSSION

High-quality and stable long-term cryopreservation of nitrite-oxidizing bacteria during eight months was successful for all six tested strains representing five different genera. A total of twenty-nine different conditions were tested on both non-marine (*Nitrobacter vulgaris* AB1, *Candidatus* Nitrospira defluvii A17 and *Candidatus* Nitrotoga arctica 6680) and marine strains (*Nitrococcus mobilis* 231, *Nitrospina gracilis* 3/211 and *Nitrospira marina*-like bacterium strain Ecomares 2.1. further designated as *Nitrospira* sp. Ecomares 2.1.), as we intended to determine a generally applicable protocol for all nitrite oxidizers regardless of phylogenetic affiliation and/or origin. Suitable cryopreservation conditions were strain-dependent. But, in general, most strains resuscitated surprisingly well, with only short lag phases of a couple of days, and showed mainly small, non-significant differences in their specific activity recovery after preservation in different conditions (used as a proxy for survival rate of biomass; compared to unpreserved control samples) (Table S6.3). Only cryopreservation of *Nitrospira* sp. Ecomares 2.1. and *Nitrospina gracilis* 3/211, most notably two marine strains, proved to be more challenging, although efficient cryopreservation still was successful in several of the applied conditions (Fig. 6.2 & 6.3, Table S6.3). Possibly, freshwater strains can more easily be preserved than marine NOB strains, due to the increasing salt stress during freezing in marine growth media for the latter.

Cryopreservation of NOB without the addition of organic carbon

The only previously described cryoprotective agent used for nitrite oxidizers, hatefi buffer consisting of sucrose and histidine [3], was only tested and proven successful on a limited number of strains from the genus *Nitrobacter* and *Nitrospira* and has thus far not been widely applied. Therefore, it was compared to the golden standard among cryoprotective agents dimethyl sulfoxide (DMSO), used in various concentrations. The choice of CPA, hatefi versus DMSO, or the concentration of DMSO made little to no significant difference in the specific activity recovery of five out of six strains (Fig. 6.1A) while within strain variation of lag phase duration was also limited (Table S6.3). Although between-strain results are tricky to compare due to differences in growth rates and final biomass obtained, it was clear that *Nitrospira* sp. Ecomares 2.1. recovered similarly from all applied preservation conditions but with a significantly lower recovered specific activity than the four other strains. *N. gracilis* 3/211 only resuscitated successfully when preserved with 10% DMSO, albeit with a lag phase of almost 16 days.

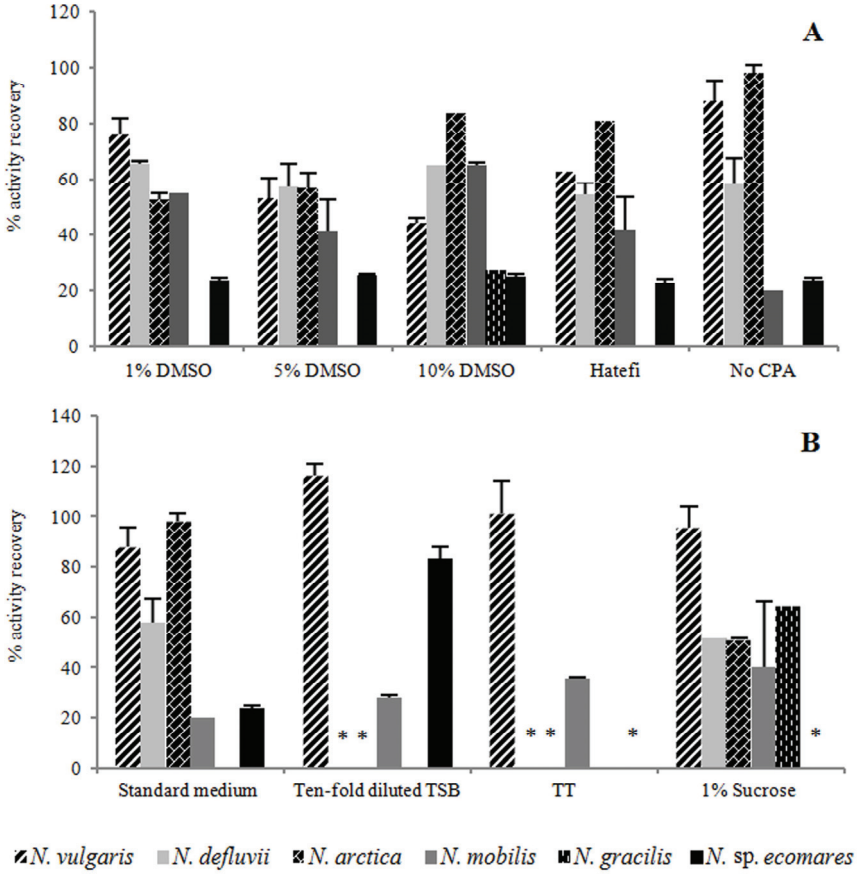

Figure 6.1. Cryoprotective effect of DMSO and hatefi (A) and organic carbon (B) as CPA. The average % activity recovery after cryopreservation compared to a control sample (n=2) is used to evaluate preservation success of each condition. For conditions with DMSO and hatefi, NOB strains were grown in standard mineral medium and subsequently preserved in mineral medium with the addition of the CPA. The preservation condition with hatefi was performed in 100% hatefi solution, and thus differs from the other preservation conditions. For conditions with organic carbon, NOB strains were grown and preserved in the carbon-rich media without the addition of an extra CPA. Standard deviations for each preservation condition are indicated by one-sided error bars. Absence of error bars refers to singular measurements (n=1) and is indicative for unstable preservation conditions. As indicated with an asterisk, *Nitrospira* sp. Ecomares 2.1 (TT and 1% sucrose), *Candidatus* N. defluvii A15 and *Candidatus* N. arctica 6680 (ten-fold diluted TSB and TT) were not tested in some carbon-rich media due to failure of pre-preservation growth.

Surprisingly, all strains besides *N. gracilis* 3/211 resuscitated well after cryopreservation without any cryoprotectant added to the culture (Fig. 6.1A) and without a significant increase of the lag phase (Table S6.3). *Candidatus* Nitrotoga arctica 6680 even reached a significantly higher recovery (97%) without use of a CPA. The fact that this is a cold adapted NOB isolated from permafrost soil from the Siberian arctic with seasonal freezing and thawing cycles, with an optimum growth temperature of 10°C might have contributed to this feature by the possible constant expression of cold induced proteins or other cold adapted survival mechanisms during growth [4]. Bacteria have already been shown to respond to low temperatures by the expression of cold induced proteins [16], which can function as important freeze protection mechanisms. Similar results were obtained previously with enrichment cultures [11] but were then mainly attributed to the additional protective power of exopolysaccharides in existing flocs. These observations suggest that the toxic effect of CPA addition, which can vary among compounds used but is indeed an issue when using DMSO, can impede successful preservation more than the freezing itself. Although DMSO is considered toxic, the threshold between protective versus toxic effect is strongly dependent of the organism of interest. Some bacteria can tolerate high DMSO concentrations and are even capable of growing in media containing 20-45% [17]. Nevertheless, care should be taken when using any potentially toxic CPA. The strain-dependent influence of concentration of DMSO as well as the success of cryopreservation without any protective additive demonstrates that simple tests or evaluation of small adaptations to established protocols are worthwhile to investigate.

Cryopreservation of NOB with the addition of organic carbon as CPA

All NOB strains known to date are obligate or facultative chemolithoautotrophs that are able to fix carbon dioxide as carbon source and oxidize nitrite to gain energy. Therefore, these cultures are always grown in a mineral medium containing no additional carbon or nutrient sources. We hypothesized that this standard practice, beneficial to avoid contamination, lie at the basis of the problematic preservation of specific NOB strains, as many carbon compounds can beneficially affect the (cryo)preservation of microorganisms [10, 11, 18, 19]. As is the case for other CPA, depending on the organism, these carbon compounds either are taken up by the cell to avoid intracellular ice formation, only penetrate the periplasmic space where they stabilize the cell membrane during ice formation by interaction with the polar head groups of the phospholipids (i.e. disaccharides such as trehalose and sucrose) [20], or remain extracellular and only protect the cells from external ice formation (i.e. polymers with high

molecular weight such as proteins or polysaccharides) [8, 18]. Therefore, in combination with hatefi or DMSO at various concentrations, improvements of preservation success through use of carbon-rich media, either as preservation medium or pre-preservation growth medium, were also assessed.

Growth with organic carbon. Firstly, growth of NOB strains in eleven carbon-rich media was verified because this would suggest no or very limited toxicity and potential carbon uptake (although this was not verified) and thus indicate its suitability for further testing as preservation medium. Carbon compounds were selected based on previous studies [10, 18, 21]. Specific activity rates of the strains in the carbon-rich media were monitored over a period of four weeks (Table S6.2) and six carbon-rich media supported growth of all strains. *Candidatus* Nitrotoga arctica 6680 was most sensitive to the addition of carbon-rich nutrients in the medium during growth, potentially because it's a highly enriched and not a pure culture. Available organic carbon most likely supported growth of the heterotrophs within the enrichment, which possibly negatively affected the activity and thus growth of *Candidatus* N. arctica 6680, either via depletion of essential nutrients or via production of inhibitory metabolites. Because of logistic reasons only three organic carbons - addition of 1% sucrose, ten-fold diluted TSB, and ten-fold diluted TSB with 1% trehalose - were selected for further testing as preservation and pre-preservation growth medium. This selection was supported by their previous successful use for cryopreservation of other oligotrophic bacteria [10, 11], their natural occurrence as CPA (e. g. sugars) [18], or compatible solutes (i.e. trehalose is used as a compatible solute by *Nitrobacter* (Spieck, unpublished data)).

Cryoprotective effect of preservation media containing organic carbon. After cultures were grown in their standard medium, selected carbon-rich media were used as preservation medium in combination with various concentrations of DMSO as CPA (hatefi buffer was only tested as a 100% solution, not in combination with these preservation media). For the non-marine strains, which were already efficiently preserved with or without DMSO, use of preservation media containing organic carbon did not significantly alter the activity recovery after cryostorage. *Nitrospira* sp. Ecomares 2.1. only achieved an activity recovery of around 20% using no CPA or only DMSO in mineral medium. But addition of either ten-fold diluted TSB without or with 1% trehalose did significantly increase activity recovery for all three DMSO conditions (Fig. 6.2A), up to around 80%. These results correlate well with previous observations of improved growth (i.e. higher cell yield and shorter generation time) of this

strain in the presence of low amount of organics [22]. For *N. gracilis* 3/211, obtained results are unfortunately less reliable because of unsuccessful resuscitation of replicates from several preservation conditions. However, the results confirmed the preference for 10% DMSO and also suggested a potential beneficial influence of sucrose and to a lesser extended of ten-fold diluted TSB with trehalose, which both allowed use of other DMSO concentrations (Fig. 6.3, Table S6.3). The latter conditions also drastically decreased the lag phase from about sixteen days to six days in 10% DMSO (Table S6.3). Due to their laborious nature and the confirmed suitability of 10% DMSO as CPA, additional preservation experiments for unequivocal confirmation were not performed.

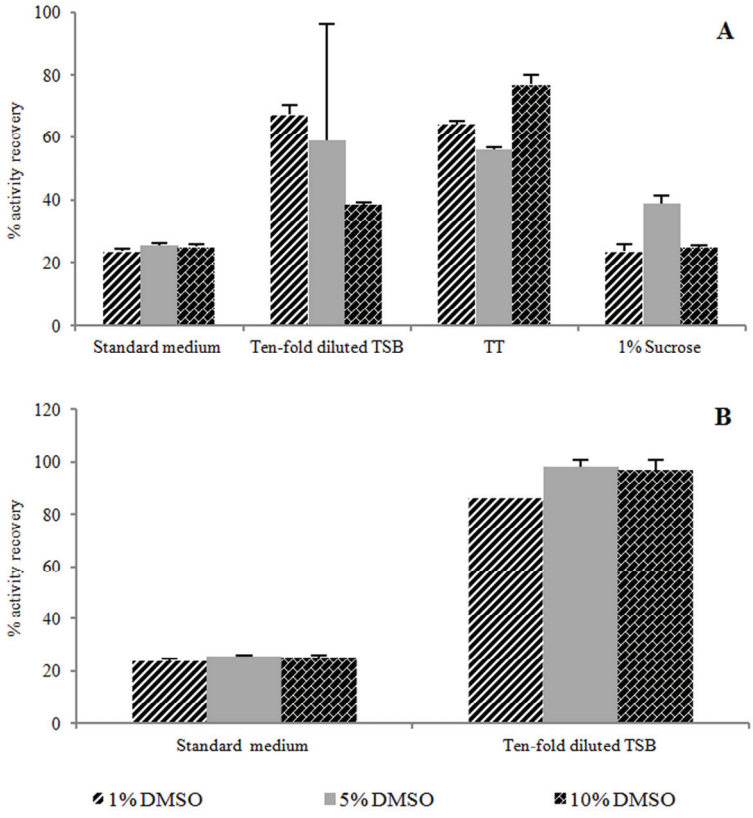

Figure 6.2. Combined cryoprotective effect of organic carbon and DMSO as CPA for *Nitrospira* sp. Ecomares 2.1 without (A) or with (B) pre-preservation growth in carbon-rich medium. The average % activity recovery after cryopreservation compared to a control sample (n=2) is used to evaluate preservation success of each condition. For (A), growth was performed in mineral medium, followed by the preservation in a

carbon-rich medium (x-axis) in combination with 1, 5 or 10% DMSO as CPA. Large error bars for the 5% DMSO in the ten-fold TSB medium are a result of large variations in the slopes of the replicates. For (B), pre-preservation growth was performed in ten-fold diluted TSB medium, followed by the preservation in the same medium in combination with 1, 5 or 10% DMSO as CPA. Other carbon-rich media (ten-fold diluted TSB supplemented with 1% trehalose, and standard medium with 1% sucrose) were not tested since pre-preservation growth failed. Standard deviations for each preservation condition are indicated by one-sided error bars. Absence of error bars refers to singular measurements (n=1) and is indicative for unstable preservation conditions.

Cryoprotective effect of pre-preservation growth and preservation in media containing organic carbon. The three selected carbon-rich media were used as pre-preservation growth medium as well as preservation medium, both with or without CPA. This was done to improve the intracellular uptake of the carbon during growth, which could allow a better cryoprotective effect than mere addition of organic carbon before preservation. However, despite the preliminary screening in carbon-rich media, some cultures showed unstable growth in some of the selected carbon-rich media, which sometimes resulted in a complete inhibition of activity (data not shown). Only for *Nitrospira* sp. Ecomares 2.1. this type of preservation conditions led to a significant improvement of preservation success, with activity recovery of around 100% (Fig. 6.2B) and a lag phase of less than one day (Table S6.3). Interestingly, all strains could also be successfully preserved with carbon-rich media as sole CPA during preservation (after a pre-preservation growth in these carbon-rich media) (Fig. 6.1B, Table S6.3).

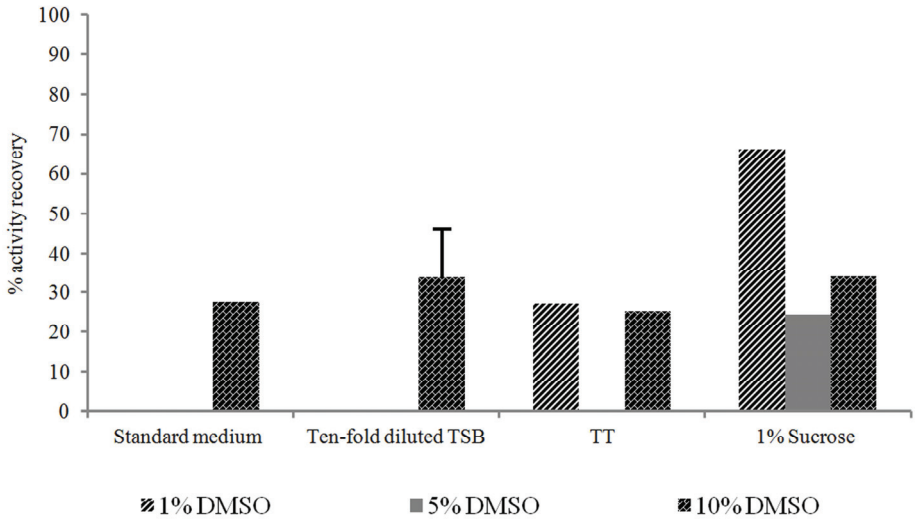

Figure 6.3. Combined cryoprotective effect of carbon-rich media in combination with a CPA for *Nitrospina gracilis* 3/211. The average % activity recovery after cryopreservation compared to a control sample (n=2) is used to evaluate preservation success of each condition. Standard deviations for each preservation condition are indicated by one-sided error bars. Absence of error bars refers to singular measurements (n=1) and is indicative for unstable preservation conditions.

Conclusions

We demonstrated that long-term cryopreservation of NOB is possible using a simple, cheap and rapid protocol. Based on obtained results we propose the use of 10% DMSO as CPA with optional ten-fold diluted trypticase soy broth as preservation medium and storage in liquid nitrogen as a standard procedure to store nitrite-oxidizing bacteria. The observations confirmed our hypothesis that preservation and/or pre-preservation growth in carbon-rich media can improve cryostorage of specifically difficult to maintain marine strains. They are also in agreement with earlier studies reporting a better preservation with organic carbon added during growth and/or preservation [10, 11, 23]. Nevertheless, the most optimal preservation condition as well as the suitability of organic carbon addition, is strain-dependent (Table S6.3) and preliminary tests should always be carried out before final application of a preservation condition. As a consequence of the presented work, the two included strains with the taxonomic status *Candidatus* can be deposited in public culture collections allowing their valid description as novel species.

6.4. ACKNOWLEDGEMENTS

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SUPPLEMENTARY INFORMATION

Table S6.1 Pearson correlation values between measurements for nitrite oxidation, growth (OD_{600nm}) and viability (ATP) monitored simultaneously for all cultures grown in different carbon-rich media.

Growth medium	*N. vulgaris* AB1			*N. mobilis* 231			*Candidatus* N. defluvii A17			*Nitrospira* sp. Ecomares 2.1.			*N. gracilis* 3/211			*Candidatus* N. arctica 6680		
	NO_2^- vs OD	NO_2^- vs ATP	OD vs ATP	NO_2^- vs OD	NO_2^- vs ATP	OD vs ATP	NO_2^- vs OD	NO_2^- vs ATP	OD vs ATP	NO_2^- vs OD	NO_2^- vs ATP	OD vs ATP	NO_2^- vs OD	NO_2^- vs ATP	OD vs ATP	NO_2^- vs OD	NO_2^- vs ATP	OD vs ATP
Standard medium	0.955	0.817	0.762	0.957	0.900	0.866	0.879	0.909	0.660	0.967	0.632	0.529	0.957	0.932	0.929	0.986	0.574	0.550
Ten-fold diluted TSB	0.965	0.813	0.794	0.966	0.937	0.747	-	-	-	0.980	0.900	0.811	0.986	0.990	0.930	-	-	-
TT	0.911	0.705	0.691	0.980	0.936	0.896	-	-	-	-	-	-	0.940	0.979	0.911	-	-	-
1% sucrose	0.946	0.832	0.742	0.983	0.975	0.940	0.851	0.921	0.847	-	-	-	0.917	0.834	0.699	0.810	0.762	0.911

Table S6.2 Overview of the relative growth success of different NOB strains in different carbon-rich media compared to standard medium. The average % activity recovery after cryopreservation compared to a control sample (n=2) is used to evaluate preservation success of each condition. Media in bold were selected for further testing of long-term preservation of NOB.

Carbon-rich media	***N. vulgaris*** AB1	***N. mobilis* 231**	***Cand.* N. defluvii A17**	***Nitrospira* sp. Ecomares 2.1**	***N. gracilis* 3/211**	***Cand.* N. arctica 6680**
TSB	100	29.93	14.84	43.11	94.07	0
Ten-fold diluted TSB	**100**	**69.45**	**100**	**65.76**	**57.69**	**34.66**
TT	**100**	**45.01**	**100**	**67.60**	**67.19**	**44.58**
Yeast extract 1%	100	30.04	23.29	64.65	86.34	0
Yeast extract 0,5%	100	29.12	24.75	57.68	83.17	0
Malt extract 1%	41.88	54.18	23.33	56.18	123.70	0
Malt extract 0,5%	64.44	37.06	32.87	71.18	129.34	17.28
Skimmed milk 1%	100	94.1	50	54.32	126.09	74.44
Trehalose 1%	100	100	100	60.38	94.09	8.38
Trehalose 5%	100	100	100	73.35	82.87	0
Sucrose 1%	**100**	**100**	**100**	**66.60**	**93.61**	**13.39**

Table S6.3 Overview of all the results of the different preservation conditions tested on each strain. The average % activity recovery after cryopreservation compared to a control sample (n=2) is used to evaluate preservation success of each condition. Lag phase is indicated as the average (n=2) increase in time (expressed in days) compared to a non-preserved control sample. For each strain the best preservation conditions are depicted in bold.

Strain	Pre-preservation growth medium	Preservation condition		% Activity recovery (± STDEV)	Lag phase (days) (± STDEV)
		Preservation medium	CPA		
***Nitrobacter vulgaris* AB1**	Standard medium	Standard medium	1% DMSO	75,87 (6,10)	0,15 (0,12)
	Standard medium	Standard medium	5% DMSO	52,93 (7,42)	0,27 (0,02)
	Standard medium	Standard medium	10% DMSO	44,31 (1,54)	0,01 (0,01)
	Standard medium	- [6]	Hatefi	62,83 [3]	1,44 [3]
	Standard medium	Standard medium	- [7]	87,95 (7,55)	0,32 (0,13)
	Standard medium	Ten-fold diluted TSB	1% DMSO	30,53 [3]	0,50 [3]
	Standard medium	Ten-fold diluted TSB	5% DMSO	28,19 (4,01)	0,41 (0,17)
	Standard medium	Ten-fold diluted TSB	10% DMSO	39,48 (4,60)	0,30 (0,03)
	Standard medium	TT	1% DMSO	100,68 (10,35)	0,22 (0,03)
	Standard medium	TT	5% DMSO	58,28 (2,23)	0,20 (0,03)
	Standard medium	TT	10% DMSO	82,64 (5,97)	0,09 (0,04)
	Standard medium	1% sucrose	1% DMSO	91,68 (8,88)	0,21 (0,21)
	Standard medium	1% sucrose	5% DMSO	39,56 (2,87)	0,49 (0,19)
	Standard medium	1% sucrose	10% DMSO	54,96 (13,99)	0,41 (0,27)
	Ten-fold diluted TSB	**Ten-fold diluted TSB**	**1% DMSO**	**120.91 (5.91)**	**0.00 (0.00)**
	Ten-fold diluted TSB	Ten-fold diluted TSB	5% DMSO	88,75 (0,58)	0,05 (0,03)
	Ten-fold diluted TSB	Ten-fold diluted TSB	10% DMSO	85,55 (0,67)	0,03 (0,01)
	Ten-fold diluted TSB	- [6]	Hatefi	65,74 (1,79)	0,05 (0,07)
	Ten-fold diluted TSB	**Ten-fold diluted TSB**	**- [7]**	**115.99 (5.06)**	**0.11 (0.09)**
	TT	**TT**	**1% DMSO**	**114.27 (0.81)**	**0.04 (0.01)**
	TT	TT	5% DMSO	87,05 (0,04)	0,02 (0,03)
	TT	TT	10% DMSO	87,40 (3,16)	0,04 (0,05)
	TT	- [6]	Hatefi	70,44 (3,84)	0,00 (0,00)
	TT	TT	- [7]	101,39 (12,85)	0,06 (0,08)
	1% sucrose	1% sucrose	1% DMSO	103,09 (15,95)	0,07 (0,10)
	1% sucrose	1% sucrose	5% DMSO	77,41 (18,58)	0,00 (0,00)
	1% sucrose	1% sucrose	10% DMSO	63,47 (1,14)	0,25 (0,13)
	1% sucrose	- [6]	Hatefi	88,60 (4,37)	0,10 (0,15)
	1% sucrose	1% sucrose	- [7]	95,50 (8,57)	0,20 (0,04)
***Nitrococcus mobilis* 231**	Standard medium	Standard medium	1% DMSO	54,61 [3]	1,33 [3]
	Standard medium	Standard medium	5% DMSO	41,55 (10,76)	0,32 (0,19)
	Standard medium	Standard medium	10% DMSO	64,82 (1,11)	0,11 (0,15)
	Standard medium	Standard medium	Hatefi	41,65 (11,72)	0,82 (0,90)
	Standard medium	Standard medium	- [7]	19,82 (0,31)	1,46 (0,11)
	Standard medium	Ten-fold diluted TSB	1% DMSO	31,82 (7,12)	1,68 (0,23)
	Standard medium	Ten-fold diluted TSB	5% DMSO	43,35 (3,06)	0,78 (0,36)
	Standard medium	Ten-fold diluted TSB	10% DMSO	46,96 (1,88)	1,21 (0,20)
	Standard medium	TT	1% DMSO	39,19 (4,72)	1,04 (0,28)
	Standard medium	TT	5% DMSO	39,34 (0,55)	0,79 (0,03)

Table S6.3. *continued*

Strain	Pre-preservation growth medium	Preservation condition		% Activity recovery (± STDEV)	Lag phase (days) (± STDEV)
		Preservation medium	CPA		
***Nitrococcus mobilis* 231**	Standard medium	TT	10% DMSO	37,21 (0,11)	1,18 (0,37)
	Standard medium	1% sucrose	1% DMSO	46,85 (7,62)	0,93 (0,00)
	Standard medium	1% sucrose	5% DMSO	49,73 (18,41)	0,93 (0,52)
	Standard medium	**1% sucrose**	**10% DMSO**	**76.49 (1.68)**	**0.33 (0.10)**
	Ten-fold diluted TSB	Ten-fold diluted TSB	1% DMSO	50,89 (5,51)	0,24 (0,34)
	Ten-fold diluted TSB	Ten-fold diluted TSB	5% DMSO	51,04 (0,30)	0,19 (0,15)
	Ten-fold diluted TSB	Ten-fold diluted TSB	10% DMSO	50,78 (1,64)	0,02 (0,02)
	Ten-fold diluted TSB	**- [6]**	**Hatefi**	**71.46 (3.88)**	**0.00 (0.00)**
	Ten-fold diluted TSB	Ten-fold diluted TSB	- [7]	27,85 (0,73)	0,96 (0,34)
	TT	TT	1% DMSO	54,70 (1,50)	0,20 (0,28)
	TT	TT	5% DMSO	43,20 (6,64)	0,20 (0,28)
	TT	TT	10% DMSO	39,03 (0,74)	0,00 (0,00)
	TT	- [6]	Hatefi	61,85 (4,15)	0,01 (0,02)
	TT	TT	- [7]	35,37 (0,71)	0,56 (0,11)
	1% sucrose	1% sucrose	1% DMSO	40,91 (5,65)	0,00 (0,00)
	1% sucrose	**1% sucrose**	**5% DMSO**	**69.81 (15.85)**	**0.00 (0.00)**
	1% sucrose	1% sucrose	10% DMSO	52,32 (12,63)	0,01 (0,02)
	1% sucrose	- [6]	Hatefi	52,13 (9,47)	0,00 (0,00)
	1% sucrose	1% sucrose	- [7]	39,99 (26,04)	0,89 (0,03)
***Candidatus* Nitrospira defluvii A17[1]**	**Standard medium**	**Standard medium**	**1% DMSO**	**65.39 (0.89)**	**1.90 (1.19)**
	Standard medium	Standard medium	5% DMSO	56,99 (8,38)	1,07 (0,16)
	Standard medium	Standard medium	10% DMSO	64,91 [3]	2,70 [3]
	Standard medium	Standard medium	Hatefi	54,20 (4,16)	0,35 (0,03)
	Standard medium	Standard medium	- [7]	57,93 (9,40)	3,23 (1,00)
	Standard medium	Ten-fold diluted TSB	1% DMSO	49,76 (0,37)	0,62 (0,88)
	Standard medium	Ten-fold diluted TSB	5% DMSO	48,88 (0,52)	0,42 (0,60)
	Standard medium	Ten-fold diluted TSB	10% DMSO	79,26 [3]	0,54 [3]
	Standard medium	TT	1% DMSO	39,55 (0,24)	0,19 (0,26)
	Standard medium	TT	5% DMSO	36,40 (0,35)	0,33 (0,46)
	Standard medium	**TT**	**10% DMSO**	**71.96 (0.13)**	**0.69 (0.49)**
	Standard medium	1% sucrose	1% DMSO	58,64 (2,17)	1,35 (0,21)
	Standard medium	1% sucrose	5% DMSO	47,11 (0,13)	0,55 (0,01)
	Standard medium	**1% sucrose**	**10% DMSO**	**68.89 (5.91)**	**1.30 (0.89)**
	1% sucrose	1% sucrose	1% DMSO	81,29 [3]	13,12 [3]
	1% sucrose	1% sucrose	5% DMSO	33,79 (0,99)	11,29 (1,99)
	1% sucrose	1% sucrose	10% DMSO	32,79 [3]	11,6 [3]
	1% sucrose	- [6]	Hatefi	88,34 [3]	16,66 [3]
	1% sucrose	1% sucrose	- [7]	51,71 [3]	6,72 [3]
***Nitrospira* sp. Ecomares 2.1.[2]**	Standard medium	Standard medium	1% DMSO	23,58 (0,99)	1,29 (1,00)
	Standard medium	Standard medium	5% DMSO	25,56 (0,55)	3,80 (1,09)
	Standard medium	Standard medium	10% DMSO	25,12 (0,77)	3,42 (2,65)
	Standard medium	Standard medium	Hatefi	22,66 (1,48)	0,83 (0,37)
	Standard medium	Standard medium	- [7]	23,60 (1,15)	4,23 (2,13)
	Standard medium	Ten-fold diluted TSB	1% DMSO	67,46 (2,86)	5,08 (2,95)

Table S6.3. *continued*

Strain	Pre-preservation growth medium	Preservation condition		% Activity recovery (± STDEV)	Lag phase (days) (± STDEV)
		Preservation medium	CPA		
***Nitrospira* sp. Ecomares 2.1.**[2]	Standard medium	Ten-fold diluted TSB	5% DMSO	58,73 (37,41)	1,67 (0,54)
	Standard medium	Ten-fold diluted TSB	10% DMSO	38,58 (0,62)	2,95 (2,97)
	Standard medium	TT	1% DMSO	64,47 (0,82)	0,51 (0,10)
	Standard medium	TT	5% DMSO	55,93 (0,79)	1,15 (0,49)
	Standard medium	TT	10% DMSO	77,11 (2,81)	0,38 (0,12)
	Standard medium	1% sucrose	1% DMSO	23,55 (2,45)	4,50 (1,86)
	Standard medium	1% sucrose	5% DMSO	38,95 (2,25)	2,64 (1,29)
	Standard medium	1% sucrose	10% DMSO	24,78 (0,54)	2,99 (0,23)
	Ten-fold diluted TSB	Ten-fold diluted TSB	1% DMSO	85,95 (0,13)	0,54 (0,01)
	Ten-fold diluted TSB	**Ten-fold diluted TSB**	**5% DMSO**	**98.00 (2.70)**	**0.14 (0.08)**
	Ten-fold diluted TSB	**Ten-fold diluted TSB**	**10% DMSO**	**96.00 (3.80)**	**0.10 (0.13)**
	Ten-fold diluted TSB	**-** [6]	**Hatefi**	**125.00 (7.57)**	**0.44 (0.62)**
	Ten-fold diluted TSB	Ten-fold diluted TSB	- [7]	83,37 (4,50)	1,52 (0,10)
***Nitrospina gracilis* 3/211**	Standard medium	Standard medium	1% DMSO	0 [4]	- [5]
	Standard medium	Standard medium	5% DMSO	0 [4]	- [5]
	Standard medium	Standard medium	10% DMSO	27,53 [3]	15,77 [3]
	Standard medium	Standard medium	Hatefi	0 [4]	- [5]
	Standard medium	Standard medium	- [7]	0 [4]	- [5]
	Standard medium	Ten-fold diluted TSB	1% DMSO	0 [4]	- [5]
	Standard medium	Ten-fold diluted TSB	5% DMSO	0 [4]	- [5]
	Standard medium	**Ten-fold diluted TSB**	**10% DMSO**	**33.77 (12.18)**	**13.16 (0.59)**
	Standard medium	TT	1% DMSO	27,24 [3]	8,75 [3]
	Standard medium	TT	5% DMSO	0 [4]	- [5]
	Standard medium	TT	10% DMSO	25,26 [3]	6,19 [3]
	Standard medium	1% sucrose	1% DMSO	66,23 [3]	11,83 [3]
	Standard medium	1% sucrose	5% DMSO	24,47 [3]	17,62 [3]
	Standard medium	1% sucrose	10% DMSO	34,02 [3]	3,11 [3]
	Ten-fold diluted TSB	Ten-fold diluted TSB	1% DMSO	0 [4]	- [5]
	Ten-fold diluted TSB	Ten-fold diluted TSB	5% DMSO	0 [4]	- [5]
	Ten-fold diluted TSB	Ten-fold diluted TSB	10% DMSO	0 [4]	- [5]
	Ten-fold diluted TSB	- [6]	Hatefi	31,03 [3]	12,98 [3]
	Ten-fold diluted TSB	Ten-fold diluted TSB	- [7]	0 [4]	- [5]
	TT	TT	1% DMSO	30,56 [3]	7,81 [3]
	TT	TT	5% DMSO	0 [4]	- [5]
	TT	TT	10% DMSO	0 [4]	- [5]
	TT	- [6]	Hatefi	0 [4]	- [5]
	TT	TT	- [7]	0 [4]	- [5]
	1% sucrose	1% sucrose	1% DMSO	36,73 [3]	2,00 [3]
	1% sucrose	1% sucrose	5% DMSO	0 [4]	- [5]
	1% sucrose	1% sucrose	10% DMSO	0 [4]	- [5]
	1% sucrose	- [6]	Hatefi	58,92 [3]	5,45 [3]
	1% sucrose	1% sucrose	- [7]	64,42 [3]	10,77 [3]
***Candidatus* Nitrotoga arctica 6680**[1]	Standard medium	Standard medium	1% DMSO	52,56 (2,48)	3,94 (2,30)
	Standard medium	Standard medium	5% DMSO	56,67 (5,66)	7,25 (1,72)

Table S6.3. *continued*

Strain	Pre-preservation growth medium	Preservation condition		% Activity recovery (± STDEV)	Lag phase (days) (± STDEV)
		Preservation medium	CPA		
***Candidatus* Nitrotoga arctica 6680**[1]	Standard medium	Standard medium	10% DMSO	83,91 [3]	0,55 [3]
	Standard medium	Standard medium	Hatefi	80,92 [3]	3,18 [3]
	Standard medium	**Standard medium**	**-** [7]	**97.94 (3.10)**	**5.14 (2.19)**
	Standard medium	**Ten-fold diluted TSB**	**1% DMSO**	**109.72 (7.95)**	**0.41 (0.58)**
	Standard medium	**Ten-fold diluted TSB**	**5% DMSO**	**104.31 (6.35)**	**0.09 (0.14)**
	Standard medium	Ten-fold diluted TSB	10% DMSO	79,10 [3]	3,46 [3]
	Standard medium	TT	1% DMSO	67,96 (6,99)	1,36 (1,92)
	Standard medium	TT	5% DMSO	62,32 (2,20)	0,91 (1,28)
	Standard medium	TT	10% DMSO	77,36 (1,24)	0,23 (0,33)
	Standard medium	1% sucrose	1% DMSO	62,17 (10,33)	4,30 (1,83)
	Standard medium	1% sucrose	5% DMSO	61,07 (0,78)	6,07 (0,50)
	Standard medium	1% sucrose	10% DMSO	65,30 (3,73)	4,49 (1,08)
	1% sucrose	1% sucrose	1% DMSO	40,42 (8,38)	0,00 (0,00)
	1% sucrose	1% sucrose	5% DMSO	48,01 (0,17)	0,00 (0,00)
	1% sucrose	1% sucrose	10% DMSO	48,96 (0,49)	0,00 (0,00)
	1% sucrose	- [6]	Hatefi	53,84 (3,01)	0,00 (0,00)
	1% sucrose	1% sucrose	- [7]	51,14 (0,57)	0,00 (0,00)

[1]: Pre-preservation growth in 10% TSB and TT failed

[2]: Pre-preservation growth failed in TT and 1% sucrose

[3]: Unreliable preservation conditions where only one out of two duplicates recovered. Only exact values of the recovered sample (so no average) are given.

[4]: No activity recovery observed

[5]: No lag phase available since no activity recovery was observed

[6]: Hatefi was used as a 100% solution, so without the addition of preservation medium.

[7]: No CPA was added

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Chapter 7

Preservation of marine sediment: effect of CPA and storage temperature

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Author's contributions:

Bram Vekeman (B.V.), Kim Heylen (K.H.) designed the experiment, B.V., Charles Dumolin (C.D.), students of the 3th bachelor in Biochemistry and Biotechnology 2012-2013 that participated in the Bachelorproef at LM-UGent performed the experiments, B.V., K.H. wrote the paper.

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7.1. INTRODUCTION

Environmental samples are a valuable microbial resource [1]. Despite the general consensus on its importance, research on storage of these kinds of samples is still in its infancies. Limited reports are currently available for bacterial co-cultures, enrichment cultures and consortia, and demonstrate that their stable cryopreservation is possible using simple protocols as for pure cultures [2, 3]. The use of dimethyl sulfoxide (DMSO) as cryoprotecting agent (CPA) is preferred since it succeeded in conserving both functionality and community structure of (i) a methanotrophic co-culture (MOB), (ii) an oxygen limited autotrophic nitrification/denitrification (OLAND) biofilm, and (iii) fecal material from a human donor [2]. The addition of a CPA allowed specific activity to be quicker resumed, while for the fecal sample it was only essential for community structure preservation. In addition to the protective action of the environmental matrix, this data suggests that the CPA provides an additional protection of the embedded Prokaryote cells, which seems logic as it does so for planktonic cells of axenic cultures. In contrast to axenic cultures, to date preservation of whole environmental samples is not frequently done. Nevertheless, stable storage of environmental samples is even more relevant as these are unique and finite samples, which can be important for comparative metagenomics, re-visiting research questions with novel techniques, and future biodiscovery [4]. As such, their preservation might boost the field of microbial ecology for further research.

This preliminary study aimed at validating the simple and rapid preservation protocol, which was recently found successful for fastidious axenic and mixed bacterial communities [2, 5–8], for marine sediments. The activity recovery of two functional guilds, aerobic methane oxidizers and anaerobic nitrous oxide producers was used as a proxy for their viability. The rationale behind this approach was that if we can protect representatives of two functional guilds broadly spread over Prokaryotic life, we can assume a general success of the storage strategy.

7.2. MATERIAL AND METHODS

Sediment sample was collected at the intertidal zone of the Koksijde beach (N. 51°07'56.04", O. 2°39'17.86") in March 2013. Four different cryopreservation conditions were tested: sediment was preserved at -80°C and -196°C with or without 10% DMSO as CPA. Prior to preservation, sediment was homogenized and aliquoted per 40g (=20mL volume) in eight 50 mL falcon tubes, each receiving 20mL media (composition see below) without or with CPA. Sediment samples were well mixed and two tubes per preservation condition were stored at -80°C and two at -196°C (liquid nitrogen; LN). Resuscitation of the sediment samples was performed after a preservation period of one month. Samples were thawed quickly in a warm water bath at 37°C. Subsequently, DMSO was removed by centrifugation (4°C, 6000g, 10 min) and repeated washing with medium without CPA. Details on the preservation procedure are described elsewhere [8, 9].

To evaluate the preservation success, recovery of specific activity was compared to activity of the sample before preservation. Aerobic methane oxidation and anaerobic nitrous oxide production were used as a proxy for viability recovery of the respective functional guilds. For this, ten gram of sediment was incubated at 28°C aerobically and anaerobically in a 1:1 (w/v) manner in dANMS medium prepared in natural seawater. Final media composition was 500 µM KNO_3, 500 µM NH_4Cl, 1 mM KH_2PO_4, 1 µM $CuSO_4.5H_2O$, 40 µM FeNaEDTA, 5 mM HEPES (pH7.8) and trace and salt elements according to Whittenbury [10]. Media was supplemented with 2 mM KNO_3 and 1 mM succinate for the anaerobic incubations. A final concentration of 25% methane and 10% acetylene was added to the aerobic and anaerobic cultures respectively at the start of the experiment. Methane and nitrous oxide concentrations were monitored over time with a Compact GC (Global Analyzer Solutions, Belgium), equipped with one channel connected to a flame ionization detector and two channels connected to a thermal conductivity detector. Values were converted to mM methane and nitrous oxide by compensating for change in gas pressure (measured with an Infield 7 pressure meter, UMS, Germany) and taking the solubility of the gases into account.

7.3. RESULTS & DISCUSSION

The activity of two different functional groups, aerobic methane oxidizers and anaerobic nitrous oxide producers, were monitored to evaluate the effect of temperature and 10% DMSO as CPA on the cryopreservation of marine sediment samples. Methane oxidation and nitrous oxide production activities comparable to activity prior to preservation were observed under at least one test condition (Fig. 7.1). However we observed a distinct difference in optimal cryopreservation condition between both functional groups. Based on functionality, preservation of methane oxidizers was successful in all tested conditions, without notable differences in activity. Still, addition of 10% DMSO resulted in a better activity recovery after preservation more resembling pre-preservation activity (Fig. 7.1B). This observation confirmed a previous report that 10% DMSO was the optimal concentration for the preservation of fastidious marine bacteria [8]. A protective effect of the surrounding matrix and biomass of bacterial aggregates or whole environmental samples has already been observed earlier [11]. For both investigated functional groups this protective effect was clearly observed (Fig. 7.1A & C). Especially for the nitrous oxide producers the sediment functioned as a very effective CPA with no measurable difference in activity before and after preservation (Fig. 7.1C). In contrast, the addition of DMSO seemed to have a detrimental effect on the specific cell activity of nitrous oxide producers, suggesting increased mortality due to the toxic effect of DMSO (Fig. 7.1D). However it should be noted that besides functionality also the community structure of the samples should be investigated to draw conclusions on the success of these preservation conditions. All members of the initial nitrous oxide producing community could have been preserved successfully. But, as DMSO can be used as a carbon source by many microorganisms, it might well be that sufficient DMSO remnants were present, despite multiple washing steps of the sample after preservation leading to less nitrous oxide produced by the same bacterial members [12] or alternatively by stimulating other, (non-)denitrifying microorganisms, resulting in a change in activity observed [13, 14].

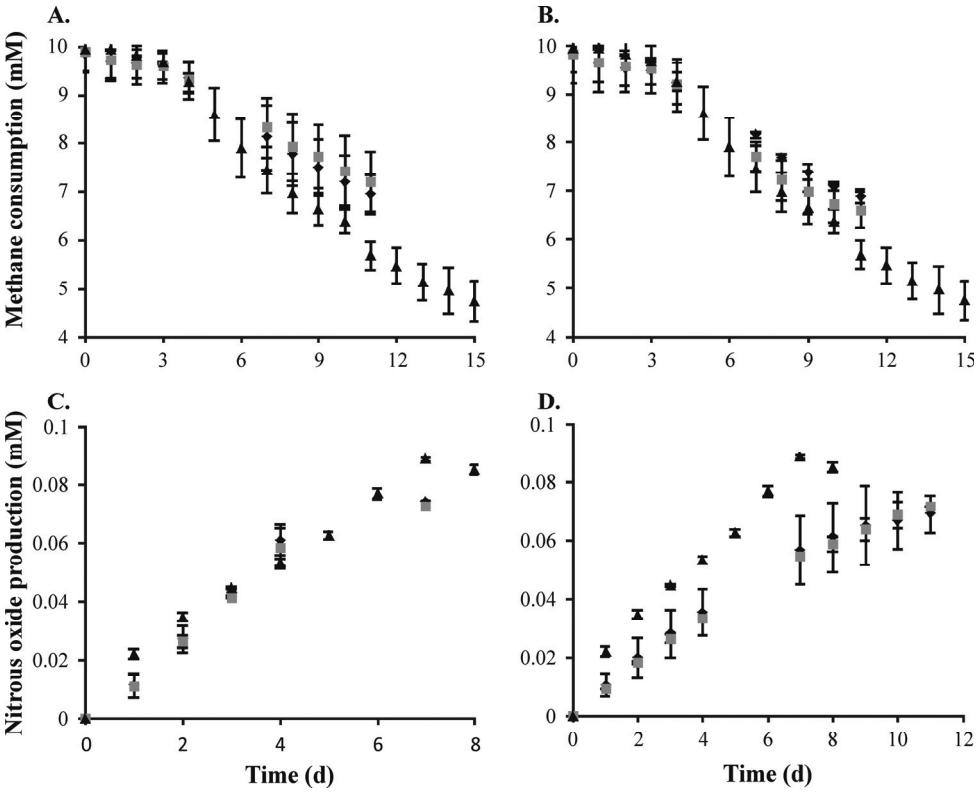

Figure 7.1. Average (n=2) activity recovery of methane oxidizers (A & B) and nitrous oxide producers (C & D) after one month preservation of sediment with (B & D) or without (A & C) 10% DMSO as CPA at -80°C (♦) and -196°C (■). Average (n=3) specific activity for each functional guild pre-preservation is indicated by (▲).

Next to the addition of a CPA for preservation, it remains important to decide whether to preserve the samples in widely available electronic freezers at -80°C or in well-monitored liquid nitrogen containers at -196°C, mostly limited to specialized labs. Preservation at a temperature below -135°C (water transition zone) is considered more stable over longer times as liquid water does not exist at these temperatures and cells that survive cooling to such temperatures remain intact indefinitely [15]. At -80°C, traces of unfrozen water can still exist, leading to recrystallization and resulting in a decreasing stability over time. In our sample no clear activity recovery difference was observed between both preservation temperatures (Fig. 7.1). This might be the result of a short preservation period of the samples (one month) in

combination with a controlled access to the freezers, resulting in fewer temperature fluctuations.

In conclusion, we demonstrate for the first time that cryopreservation of marine sediments can be simple and effective, with adequate activity recovery and without introduction of an extended lag phase. The choice of the preservation protocol might dependent on the objectives for further sample processing. This protocol is easily applicable by laboratories equipped with -80°C freezers and/or liquid nitrogen containers.

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Chapter 8

Discussion and Future perspectives

Deep-sequencing and meta-omics approaches provide an unprecedented window into microbial diversity and the active populations contributing to important ecosystem functions. It led to tremendous advances in microbial ecology over the past decades and completely overshadowed cultivation-based research. However, the last few years, microbial ecologists have become increasingly aware of the limitation of the exclusive focus on sequencing [1] and acknowledge the complementary need for functional genomics and physiology studies from axenic and enrichment cultures to provide understanding and context, mostly on functioning and regulation of newly discovered protein families, and ecophysiological preferences - such as pH or temperature optima, concentration ranges of nutrients or growth kinetics on various carbon sources - that cannot be deduced from genomic data. PCR-based techniques and downstream data analyses might also be biased towards already known diversity and can obscure novel or rare microbial diversity. Furthermore, (biotechnological) application of the metabolic potential of microorganisms requires the availability of cultures. As a result, there is a renewed interest in isolation of bacteria, especially those responsible for important ecosystem function and key players in biogeochemical cycles. The complexity and laborious nature of obtaining representatives of the broad microbial diversity into culture is already known for decades. Especially marine microorganisms are notoriously difficult to culture, with an estimated cultivable fraction between 0.001 – 0.25% [2]. Even culturing of highly abundant cyanobacteria appeared extremely laborious [3–5]. Also for marine MOB the discrepancy between the *in-situ* diversity and the limited number of axenic cultures, spanning only eight formally described species, is tremendous. Therefore the major focus of this dissertation was to gain insight in the determining factors of the cultivability and subsequent isolation and preservation of marine MOB, in addition to investigate the ecophysiology of retrieved strains. My main conclusions were:

(i) Sediment as adhesion material and headspace composition, especially the concentrations of carbon dioxide and methane, were identified as significant factors influencing methane oxidation rate.

(ii) Methanotrophs from offshore sediments resist *ex situ* growth more than those from coastal or estuary sediments and are less compatible with existing molecular detection tools. Still, it is relatively straightforward to cultivate representatives of as-yet uncultivated MOB clades with a low tech isolation approach when mimicking the *in situ* oxygen and nitrogen conditions.

(iii) The tight, probably metabolic, association with heterotrophs made the purification methanotrophs almost impossible. Knowledge of the identity of the methanotroph and use of methanol as carbon source was necessary to obtain axenic cultures.

(iv) A marine, pMMO-lacking, type II methanotroph was isolated. It belonged to the strict methylotrophic genus *Methyloceanibacter,* which could be distinguished in five ecotypes, coinciding with species barriers, using detailed growth experiments linked to genomic analyses for primary carbon and energy source, nitrogen sources, pH, temperature and salt. Four novel species of the genus were also described.

(v) Axenic key players of biogeochemical cycles as well as enrichment and environmental samples can be successfully cryopreserved enabling the stable and long-term storage of valuable microbial resources for future activity measurements, isolation campaigns, research or biotech purposes.

In this chapter, I will discuss my research strategy from a bird's eye perspective, touch upon data from preliminary experiments that were not incorporated in the previous chapters, and suggest interesting future experiments to continue research on this topic.

PART I. LESSONS LEARNT FROM A LARGE-SCALE STRATEGY TO ISOLATE METHANE-OXIDIZING BACTERIA FROM MARINE SEDIMENTS

Cultivation and isolation of marine MOB

Marine sediment comprises a large microniche space diversity resulting in a micro-scale biogeochemical heterogeneity [6]. As such sediment are more phylogenetically divers compared to other environment types [7]. To reflect this high microniche heterogeneity in marine sediment and subsequently maximize the retrieval of methanotrophic diversity, different realistic potential niches were mimicked by varying the concentration of inorganic nitrogen in the medium, in agreement with the in-situ range, and oxygen concentration in headspace. Starting from initial duplicate active methane-oxidizing enrichments per sediment sample, the combination of these different growth conditions resulted in a total of 288 enrichments. To my knowledge, tackling the isolation of MOB from marine ecosystems with such a large-scale approach is unprecedented. The choice of an isolation strategy solely relying on liquid culturing was motivated by a previous study reporting the inability of marine MOB to grow on solidified media [24], probably due to organic impurities. Furthermore, my own preliminary plating experiments demonstrated a general decrease in the ability of marine MOB to grow on gellan gum-solidified plates, quantified as a decrease in the number of methane-oxidizing colonies, along the investigated transect of the North Sea. Gellan gum is known to better support growth of a wide range of bacteria compared to agar, and thus allows a larger diversity to be cultured [12, 22, 23]. But despite the observed growth support of MOB from nearshore stations, albeit without rendering axenic cultures due to tight association with heterotrophs, it appeared inadequate for the isolation of more offshore MOB.

More than 70% of the enrichment cultures (i.e. 204 out of 288) were positive for methane oxidation **(Chapter 5)**. Next to known marine methanotrophs and *Methyloceanibacter methanicum*, representatives of deep-sea cluster 3 were brought into culture as well as two novel groups within deep-sea cluster 2. I therefore consider the applied large-scale strategy to retrieve marine methanotrophic cultures a success. The inclusion of several measures to increase cultivability might have contributed to this success. Aged seawater, originating from each sampling station at 3 m depth, was spiked with nutrients, including lanthanides, in accordance with the in-situ low nutrient concentrations (based on the MUMM database) for use as custom-made isolation medium. The use of low nutrient concentrations has become a widely adopted strategy to increase cultivated diversity for soil microbiota or bacterioplankton

[8–12] but has also proved successful in improving the diversity of MOB cultures obtained for lake sediments [13]. Natural seawater is also more suitable as low-nutrient growth environment for marine bacteria than synthetic seawater [14]. In addition, lower oxygen concentrations have already shown to select for a different MOB diversity [15] by avoiding self-intoxication [16, 17] or selective stimulation of low oxygen adapted species [18]. Growth on low oxygen concentrations might be the preferred condition for MOB representatives of the deep-sea cluster 2. Insights in their genomic landscape revealed the presence of different genes encoding enzymes involved in resistance to high atmospheric oxygen tensions **(Chapter 4)**. The interplay of the enzymes superoxide dismutase, catalase and the aerotolerant gene operon, all present in these genomes, have been proposed to inactivate toxic products of oxygen metabolism by eventually converting them to water in *Bacteroides fragilis* [19, 20]. To avoid the competition with heterotrophs, methanotrophs might prefer deeper layers of the sediment. Therefore the use of lower oxygen concentrations might further enhance the cultivation of marine methanotrophs and perhaps facilitate their isolation into pure cultures. Furthermore, I'm convinced that the addition of adhesion material (sediment) had a major impact on the cultivability of the MOB. Although differences in the bacterial community and abundance of MOB were not investigated between enrichments with and without the addition of sterilized natural sediment or acid-washed silicium dioxide, statistically significant differences in methane oxidation rates were observed **(Chapter 2)**. Although not experimentally verified, I assumed that increased methanotrophic activity related to increased MOB enrichment. Addition of adhesion material already previously revealed the potential to cultivate as-yet uncultivated bacteria [21].

Due to the scale of the isolation strategy, I was forced to work in a miniaturized format with 96-well plates for extinction culturing, which already previously proved successful for isolation and screening of MOB [22, 23]. Miniaturized extinction culturing allowed the use of various growth conditions closely mimicking different realistic niches in the native environment [24] in parallel and in duplicate, enabling the retrieval of a broader diversity of MOB into culture **(Chapter 5)**. It also enabled separation of slower growing but more abundant MOB from more easily cultured but less abundant heterotrophs. The inclusion of replicates from the same enrichment proved useful, as sometimes different MOB were retrieved, as exemplified by *M. methanicus* and *Methylocaldum marinum* found in duplicate extinction cultures of sample W04 under identical cultivation conditions (see further). However, at the start of my research I clearly overestimated the ease of obtaining axenic

marine methanotrophs via miniaturized extinction culturing. This led to an, in hindsight, overly ambitious experimental design with twelve sediment samples, i.e. six stations and different oxygenated depths, and resulted in an unmanageably large number of MOB enrichment cultures for a one man team to investigate in detail in the set timeframe of five years. Still, plating remained essential for obtaining axenic cultures after extinction culturing. With prior knowledge on the identity of the MOB, the use of methanol as carbon source, gellan gum as solidifying agent and a lot of dedication, I succeeded in the isolation of an axenic culture of the first marine type II *M. methanicus*. Additional attempts using alternative solidifying agents, such as Bacto-agar (Difco) and Agarose (Biozym) were also unsuccessful. The floating filter technique [25], already successful in the isolation of methanotrophic *Verrucomicrobia* unable to grow on solid media [3], was also tried but again without any success in obtaining an axenic culture. Lees and colleagues [24] found that Noble agar (Difco) at a concentration of 0.4% w/v allowed the growth of marine MOB when all other solidifying agents failed. I'm convinced obtaining axenic cultures for the other enriched MOB, giving priority to the unidentified cultures **(Chapter 5)**, would have been feasible via plating on methanol-based growth medium with sufficient time and manpower and when facilitated by prior analyses of the metagenomes of the enrichment cultures **(Chapter 3)**.

Five years ago, I consciously and maybe naively went for a "blind" and broad isolation strategy, without prior knowledge of the *in situ* methanotrophic diversity via *pmoA* sequencing, hoping to find novel MOB and convinced at the time that the available primers were biased towards already known diversity. Indeed, since then some drawbacks of the most common primer set (A189/mb661) have been reported. It fails to detect some of the clusters that have phylogenetic positions between *pmoA* and *amoA* sequences (e.g. the *pxma* cluster encoding a *pmoA* homologue, and RA21) [25, 26], it largely discriminates USCα and amplifies type IIb methanotrophs less efficiently [27, 28]. Also, our own results demonstrated the gross underestimation of marine diversity of methane oxidizers with PCR approaches and showed that even between closely related MOB mismatches in the primer target regions can occur **(Chapter 5)**. However, I'm convinced now that the laborious and challenging nature of isolating novel marine methanotrophs requires prior knowledge on the MOB diversity in natural samples or initial enrichments and is best performed using a targeted approach. In contrast to five years ago, shotgun sequencing has become affordable and mapping reads to a *pmoA* database can by-pass any primer bias. Alternatively, high time-resolved SIP using labeled methane followed by 16S rRNA amplicon sequencing would identify the active MOB

community which in turn could be screened for novel or as-yet uncultivated MOB diversity. Subsequent enrichment and isolation should follow a targeted approach, facilitated by custom-made primers/probes for qPCR or FISH to track the organism of interest. A more high-tech approach could bypass classic cultivation altogether through use of FISH-MAR or nanoSIMS to obtain insights in the ecophysiology of the MOB of interest, combined with its physically separation using single cell encapsulation combined with flow cytometry, micromanipulation and optical tweezers, filtration, cell sorting by flow cytometry or density gradient centrifugation. The end-result could also well be an axenic culture, avoiding the notorious separation of MOB from heterotrophic satellite cells.

Methane-driven food web

MOB can be seen as primary producers forming the basis of a methane-driven food web. Methane derived biomass of MOB can be grazed directly by zooplankton or transferred via other heterotrophic bacteria to zooplankton and further to macroinvertebrates up to fish [29]. As such methane oxidation by methanotrophs is a shaping force for the prokaryotic and microeukaryotic community [30, 31]. These interactions can be mutually beneficial, as the MOB provides carbon to the heterotroph, while the heterotroph removes toxic methane oxidation intermediates (e.g. methanol) and/or produces stimulating complex compounds (e.g. vitamins) for the MOB [32–34]. An unfortunate side effect of such interactions is the problematic purification of MOB during isolation, as heterotrophic bacteria, often non-methanotrophic methanol-utilizing methylotrophs, frequently co-purify and can be hard to get rid of [35]. Indeed, plating of the cultures on 1% gellan gum solidified medium under methane headspace always resulted in multiple colony morphologies, suggesting the persistence of heterotrophic alien cells in the enrichments. Picked colonies, subsequently exhibiting methane oxidation, unfortunately never represented a purified isolate. The inability to purify the MOB from enrichment cultures might indicate a high need for heterotrophic partners by marine MOB, probably because high intermediate concentrations of methanol, formaldehyde or formate that are formed during growth under high concentrations of oxygen or acetate, lactate, succinate and hydrogen gas under oxygen limiting conditions [34]. High inhibitory concentrations of intermediates have already been detected in high concentrations in methanotrophic pure cultures [36, 37]. Also in the methanotrophic strain *Methyloceanibacter methanicus*, the production of high concentrations of a waste/intermediate product was detected via chromatography (identification of the compound was unfortunately not possible).

Syntrophic associations, in which toxic or unwanted products of one partner are removed by the other partner and both are beneficial, might be exploited as a novel cultivation strategy for marine methanotrophs. This co-dependency between methanotrophs and heterotrophs (e.g. methylotrophs) has been observed several times in the past and the idea of co-cultivation is already being tested (Joerg Duetzmann & Rahalkar, unpublished data). However the heterotrophic strain selected for such an experiment is crucial as beneficial or harmful interactions can be strain-specific, thus can influence the MOB diversity that will be selected for [38]. Still, once essential growth-promoting factors produced by the heterotroph are identified, these can be synthetized and added to for example a sequence batch reactor or even a continuous membrane bioreactor, allowing the removal of excessive concentrations of toxic waste or intermediate compounds inhibiting MOB. Furthermore, the production and accumulation of toxic intermediate waste product is enhanced by growth under high oxygen tensions [36, 37]. Therefore growth under low oxygen tensions would result in a decreased need for "neighborly helpers" interfering with purification. Growth on low oxygen concentrations might be the preferred condition for MOB representatives of the deep-sea cluster 2. Insights in their genomic landscape revealed the presence of different genes encoding enzymes involved in resistance to high atmospheric oxygen tensions (see above) [19, 20]. In rice rhizospheres, it was already observed that methanotrophs can outcompete the heterotrophs under low oxygen concentrations (<5 μM) [39]. A similar competition between heterotrophs and methanotrophs for oxygen would take place in the top sediment layer, where methane and oxygen gradients overlap, and to avoid the competition with heterotrophs, methanotrophs might prefer deeper layers of the sediment. Therefore the use of lower oxygen concentrations might further enhance the cultivation of marine methanotrophs and perhaps facilitate their isolation into pure cultures.

Previous co-cultivation experiments pairwise combining methanotrophs with different heterotrophs showed a significant increase of MOB growth [38], furthermore addition of heterotrophic strains increased the methane oxidation rate [40]. However, these experiments were performed with "domesticated" MOB and heterotrophs, having a long *ex situ* cultivation history and retrieved from unrelated habitats. Therefore, re-visiting these experiments with microorganisms isolated from the same sample or same enrichment should result in even more pronounced positive effects of the addition of heterotrophs, which have a common origin and a possible history in cooperation *in situ* through coexistence and co-evolution in the same environment. I retrieved *M. methanicus* and *Methylocaldum marinum* from duplicate

extinction cultures of the same sample under identical cultivation conditions (20% O_2, 500 µM KNO_3, 500 µM NH_4Cl). Interestingly, both genera were also retrieved from the same enrichment obtained by Takeuchi and colleagues [41], which might reflect a similar niche preference/occupation. So both genera might have a preferential partnership in which *Methyloceanibacter methanicus*, preferentially growing on methanol **(Chapter 3)**, removes the excreted methanol produced by the methanotroph *Methylocaldum marinum*. In addition, I isolated a divers set of heterotrophs using a tenfold diluted tryptic soy agar medium and methylotrophs via an oligotrophic medium containing methanol as sole source of carbon and energy. A total of nine isolates belonging to the genera *Gramella*, *Roseovarius*, *Erythrobacter*, *Mesorhizobium*, *Arenibacter*, *Leisingera*, *Methylophaga*, *Methyloceanibacter* and *Labrenzia* were obtained. Due to time limitation, only a preliminary test with a simple design was performed, using randomly selected heterotrophs and without prior screening of their individual effect on MOB growth. The methane oxidation activity of the axenic culture of *M. methanicus* was compared with that of the axenic culture supplemented with all listed heterotrophic and methylotrophic isolates in equal amounts of biomass (based on OD_{600}), but surprisingly, no significant effect of the added bacteria on methane oxidation was observed. The effect of added heterotrophs might have cancelled each other out, as some heterotrophs stimulate methane oxidation while others are known to have an inhibitory effect [38]. Further pairwise co-cultivation testing after prior screening and gradually adding more heterotrophs is warranted to better understand methane-based food webs, competition and both positive and negative interactions involved. However, it is worth mentioning that, based on the shotgun sequencing data, not all microorganisms from this enrichment culture were retrieved and some, potentially specialized in MOB excretion products, resisted isolation.

The *Methyloceanibacter* species representing different ecotypes **(Chapter 3)** could surely be included in the pairwise co-cultivation experiments but also provide an ideal opportunity to conduct competition experiments gaining insight into the nature of their relationship *in situ*. Kin discrimination between sympatric conspecifics has already been observed for different microorganisms [42, 43]. Despite the growth on methane, *M. methanicus*, showed a preferential growth on methanol. However, as both the methane oxidizer and the methanol-oxidizing strains preferentially grew on methanol but with different maximal cell densities and growth rates, it would be interesting to see (i) how the different strains would compete for space and methanol in cocultivation under the low *in situ* concentrations of methanol and/or methane and (ii) potential differences in apparent methanol affinity. But if methane is the sole

source available, or more abundantly available than methanol, it seems logic that the methanotroph has a competitive advantage and a cooperation behavior will get established. The methanotroph will provide methanol and other intermediate compounds while the methylotrophs remove excess methanol concentrations preventing a self-intoxication of the methanotroph, in addition to other beneficial interactions. Similarly, long-term co-cultivation experiments under carbon source limitation might also allow the observation of HGT of sMMO operon from *M. methanicus* to one of the other *Methyloceanibacter* strains.

MOB are also found in close, often obligate, interactions with other organisms besides other Prokaryotes. Near hydrothermal vents and cold seeps, endo- and episymbiotic gammaproteobacterial MOB have been detected in and attached to the tissue of mussels, sponges, snails and tubeworms. These marine invertebrates rely on the methanotrophs to provide them with methane-derived carbon and in return the methanotrophs are provided with a stable environment with sufficient oxygen and methane to support their growth [44]. Furthermore, MOB were also shown to form stable consortia with algae, whereby the algae provide oxygen for methane oxidation and the MOB provides carbon dioxide that in return is fixed by the algae [45]. The perturbation caused by the vertical movement of bivalves and the interaction with both groups of organisms could be responsible for the retrieval of active MOB cultures found at deeper layers where no oxygen was detected. Representatives of the deep-sea clusters 1, 2 and 4 have already been detected as endosymbionts or epibionts of marine animals, but not consistently. Both genomes belonging to the MOB deep-sea cluster 2 enrichment cultures **(Chapter 4)** seemed to possess the *vapB*-*vapC* operon. The activity of the *vap* genes are involved in stress response and host–pathogen interactions [46]. Further, in the pathogenic strain *Rhodococcus equi* these genes have been suggested to play an important role in the activation of virulence genes upon acidification of the environment [47]. It has been proposed that the pH of actively hydrothermal vents is around 4.5, therefore this might be a mechanisms switching on symbiotic genes [48].

PART II. PRESERVATION OF MICROBIOMES: NECESSITY, FEASIBILITY AND STRATEGY FOR SAMPLE ARCHIVING IN MICROBIAL ECOLOGY

Beyond data archiving

Nobody disputes that high-throughput sequencing and the availability of enormous amounts of sequencing data has boosted microbial ecology research in all domains. In great contrast to this tedious data archiving and data sharing is the lack of care with which microbial ecologists handle and preserve their samples taking high risks of forever losing valuable and irreplaceable biological materials. Cary and Fierer [49] argued that sample archiving would stimulate world-wide comparative research in microbial ecology through revisiting of samples with other or novel technologies or another research focus while saving scarce public research funds by avoiding redundant collection efforts. The acquisition of environmental samples often costs a lot more time and money than subsequent sequencing and data analyses, especially for samples from secluded, inaccessible and extreme environments. I agree that, in analogy to the Svalbard Global Seed Vault, samples unique in both space and time, including from sites suffering major perturbation events like the Deepwater Horizon oil spill, are invaluable and need to be safeguarded for future descriptive, comparative or explorative research.

Storage of whole environmental DNA samples has become feasible as several robust technologies exist for high quality long-term preservation, even at room temperature. However I believe that a global, decentralized microbial biodiversity archive should contain actual environmental samples and microbiological life therein and not only the derived nucleic acids. Currently applied DNA extraction protocols might not be sufficient and future technical improvements will probably enable us to more efficiently retrieve the DNA from the whole microbial community. Even more importantly, in a sustainable bio-based economy, environmental microorganisms are essential resources. They are the foundation of major advances in a.o. drug discovery, industrial processes or waste management. For example, the discovery and subsequent enrichment of anaerobic ammonium oxidizing (anammox) bacteria [50, 51] less than twenty years ago has already led to more energy-effective full-scale wastewater treatment and may potentially make global water treatment energy-neutral or even energy-generating [52]. Investigation of Arctic/Antarctic environments of the last decade has given rise to industrial applications of cold-adapted enzymes, anti-freeze products and organisms capable to bioremediate cold soils. And just very recently, the enormous potential

of innovative strategies to grow and isolate 'uncultured' bacteria was demonstrated by the discovery of a new antibiotic, teixobactin, that inhibits cell wall synthesis and seems to avoid development of resistance [53]. If we want to tap into, manipulate or apply specific microbes' ecosystem services such as pollutant degradation, plant growth promotion and probiotic effects or harness degradable bio-molecules like pigments or polymers, then the physical microbe should be available for use to our advantage. Therefore, storage of whole environmental samples is preferred over a DNA archive to ensure future retrieval or isolation, study and use of the associated microbiota.

Technical feasibility

A suitable sample storage procedure for subsequent isolation of its associated microbiota should ensure viability of microbial cells. But the same holds true for DNA isolation. If not, cells lyse and DNA will be enzymatically degraded, especially at non-freezing temperatures, resulting in a changed microbial community of the stored sample. Therefore, regardless of the research domain or downstream use of the environmental sample, efforts for stable sample storage should be tackled with joined forces.

The matrix of an environmental sample - be it soil particles, sand particles, biofilms, flocs or aggregates - conveys an inherent protection to the microbial cells during storage, which significantly increases the viability and cell integrity of the embedded microorganisms during storage compared to pure cultures. Indeed, simple storage of the sample at different temperatures before DNA extraction had little influence on the microbial community structure of soil or human-related samples [54, 55]. However, contradicting results were found for the effect of storage at room temperature for example for human stool samples [56, 57], while others reported significant reductions of amount of extracted DNA (up to 90%) after storage of anaerobic digested sludges [58]. Thus far, subsamples simply stored under different temperatures, without additional precautions, have failed to render identical bacterial communities of fresh samples. However, small differences in the presence or relative abundance of taxa can become problematic for community comparison when advances in sequencing technology will allow for more resolved microbial community assessment [55].

To me, these reported changes in bacterial communities after storage are not surprising and the underlying reasons are evident. (i) Storage at room temperature and in cold rooms (4°C) is not stable and still allows residual cell activity, so the microbial community can shift during

storage. Rule of thumb is the lower the storage temperature and the frequency of temperature variation, the higher the stability and chance of successful preservation of a viable culture. Therefore, samples should always be cryopreserved, at -80°C for period up to five years or at -20°C for a few days to weeks but storage at non-freezing temperatures should always be avoided. (ii) Freezing of biological material is normally lethal (due to solution effects and intracellular freezing). The sample matrix alone does not sufficiently protect the microbial cells against freezing causing cell lyses and subsequent enzymatic DNA degradation. Therefore it makes sense to add cryoprotective agents to environmental samples before cryostorage, as done for pure cultures, to ensure a higher cell survival rate. Despite species- or strain-dependent variation, LM-UGent and collaborators have demonstrated the general applicability of dimethyl sulfoxide (DMSO) as suitable cryoprotectant for notoriously difficult-to-preserve bacteria such as aerobic and anaerobic methane or ammonium oxidizers or aerobic nitrite oxidizers [59–62]. When tested on complex bacterial microbiomes, DMSO addition succeeded in preserving both community structure and functionality of an oxygen limited autotrophic nitrification/denitrification (OLAND) biofilm and human stool samples after three months storage at -80°C [63]. This successful proof-of-principle demonstrated that high-quality, long-term sample archiving for subsequent DNA extraction, targeted isolation or metabolic activity assessment does not require different approaches and is technically feasible. The recent success of a very similar approach for cryopreservation of a photosynthetic picoeukaryote community [64] suggests that its general applicability is realistic. Furthermore our initial results indicate the potential use of this preservation protocol for the successful storage of whole sediment samples.

Strategy towards global sample archiving

I advocate a broad validation of the simple storage at -80°C with 5% DMSO as cryoprotective agent with attention to good practices in pre- and post-storage manipulations [65] by other research groups to ensure its reproducibility and reliability. We realize that cryopreservation at -80°C is less convenient than storage in cold rooms or -20°C but most research institutes have access to ultra-low temperature freezers and their use will be pivotal for sample storage over longer time periods. To assess the success of the cryostorage, guidelines for quality control and sample homogenization will be crucial. Quality control for pure culture preservation is based on viability, purity, identity and stability assessment after storage. For environmental samples, only 16S rRNA gene sequencing seems appropriate, despite inherent variability in DNA extraction and PCR amplification and limited interlaboratory

comparability. To be of value, stored subsamples should be representative of the original environmental bulk sample. So sample homogenization, which might be sample-type specific, prior to storage will be necessary to avoid intra-sample variability due to different microenvironments within the bulk sample harboring different microorganisms, as was recently demonstrated for human stool samples [66]. Results of these validation efforts and sample type-specific amendments should be made public, allowing convergence to a standard protocol.

Next, actual sample archives need to be constructed in research labs or institutes. In contrast to pure cultures, environmental samples, as well as DNA extracted from it, are finite biological materials, necessitating a tradeoff between sufficient sample size for repeated future use and storage space. In parallel, consensus on a Minimal Data Set for environmental samples should be developed. Archiving of environmental samples only makes sense when at least the associated metadata on origin, physicochemistry, microbial community analyses and other relevant sample type-specific information are available.

As a final step, I envisage the establishment of a global network of specialized Biological Resource Centers (BRC) that each become public, physical repositories for specific types of environmental samples such as soil, marine sediments, or human stool samples. Such a network could be a major enabler for the proposed Unified Microbiome Initiative (UMI) [67] and/or the International Microbiome Initiative (IMI) [68] to coordinate international microbiome research. With the support and expertise of the World Federation of Culture Collections (WFCC) and if well-embedded in existing renowned research facilities, these BRCs can bundle know-how and set standards for cryopreservation, DNA extraction and other sample-specific analyses. To overcome potential legal issues of sample sharing, the so-called "black box" regime could be invoked in analogy to the Svalbard Global Seed Vault, meaning that samples can only be retrieved by the institutions that deposited them.

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Summary

Methane is the most abundant organic greenhouse gas in our atmosphere, and has a strong infrared absorbance, being 25 to 30 times more effective than carbon dioxide on a 100 years scale. Methane therefore plays an important role in the climate warming regulation. Methanotrophs are microorganisms that can consume methane and utilize it as their sole source of carbon and energy. These organisms are the most important biological sink of methane. Their importance is especially demonstrated in marine ecosystems. While the oceans have the potential to produce enormous quantities of methane, a series of very effective microbiological oxidation processes results in the ocean being one of the smallest net global methane sources. Hence, it is of vital importance to understand who is there and what factors may positively or negatively impact the methane-oxidizing activity of these organisms. Despite the importance of the aerobic methane oxidizing bacteria (MOB) in the marine ecosystems currently only a limited amount of *ex situ* cultures is available. Nevertheless, they are indispensable to link physiology to genomic features and expand our knowledge about the specific habitat preferences of marine MOB. This study focused on aerobic marine MOBs and aimed at designing a large-scale enrichment and isolation strategy to retrieve a maximal MOB diversity from marine sediments.

First, the effect of adhesion material and headspace composition on the methane oxidation activity in marine sediment enrichments were investigated. The addition of sterilized natural sediment as well as acid-washed silicium dioxide significantly positively influenced methane oxidation. The exact mechanism of this positive effect needs further investigation but might be the facilitation of methane, carbon dioxide and oxygen gradients in addition to adhesion. Use of adhesion material might thus facilitate the cultivation and subsequent enrichment of members of this functional guild.

Next, using these insights, a large scale isolation strategy was performed from sediment collected at six different stations in the North Sea along a transect from estuary to open sea. An initial enrichment step with serial subcultivations was followed by miniaturized extinction culturing mimicking a range of nitrogen and oxygen microniches. A clear decreasing trend of

cultivability and detectability was observed along the investigated transect. Furthermore widely applied *pmoA* primers failed to amplify biomarkers in a large number of active methanotrophic cultures, suggesting enormous underestimation of methanotrophs *in situ* in PCR-based molecular surveys. Unfortunately, despite the numerous attempts we were not able to obtain axenic methanotrophic cultures, most likely due to tight mutualistic interactions with heterotrophic bacteria.

Shot gun sequencing of four methane-oxidizing enrichment cultures revealed the presence of a novel gammaproteobacterial MOB belonging to the deep-sea cluster 2 in two cultures and a novel alphaproteobacterial MOB belonging to the recently described methylotrophic genus *Methyloceanibacter* in the other two cultures. *Methyloceanibacter* methanicus represented the first MOB found in an exclusively methylotrophic genus, the first marine type II MOB and only the third taxon in which solely sMMO was resoponsible for methane oxidation. A targeted isolation using methanol as carbon source led to a axenic culture of the MOB, in addition to three closely related novel strict methylotrophic species, *M. superfactum*, *M. stevinii*, *M. marginalis*. Together with the previously described *M. caenitepidi*, these species exemplify an extreme niche differentiation, with a wide ecotypic variation related to growth kinetics on methanol, and preferences for nitrogen, pH, temperature and salt. Furthermore, the most striking difference of the deep-sea cluster 2 representatives demonstrated a striking difference with other gammaproteobacterial MOB in its lack of a calcium dependent methanol dehydrogenase encoded in the genome. The genome solely contained the genes for *xoxF5* for the lanthanide-containing methanol dehydrogenase.

Lastly, a preservation protocol was optimized for the long term storage of marine bacteria in order to successfully store the enrichments and axenic cultures obtained throughout this dissertation. Fastidious nitrite-oxidizing bacteria were used as model organisms. They demonstrated that optimal preservation conditions were strain-dependent whereby marine strains, appeared to be more sensitive to freezing than non-marine strains. Nevertheless, a general cryopreservation protocol using 10% dimethyl sulfoxide as cryoprotective agent with or without ten-fold diluted trypticase soy broth and trehalose as a preservation medium allowed successful preservation of all tested strains. Applying the same protocol on whole marine sediment samples allowed successful storage of different key players in the carbon and nitrogen cycle.

In conclusion marine MOBs are notoriously difficult to cultivate and isolate. Despite numerous attempts, I was only able obtain one axenic culture, in addition to over 200 enrichment cultures, from the oxygenated zones of North Sea sediments. The availability of a successful preservation protocol allows the storage of this axenic culture, ensuring that novel diversity does not get lost, but also further guaranties archiving of the obtained enrichment cultures and environmental samples. As such these are available for future isolation when novel insights in marine MOB and isolation strategies/techniques become available.

Samenvatting

Methaan is een belangrijk broeikasgas in onze atmosfeer, met een opwarmingspotentiaal dat 25 tot 30 keer zo sterk is als dat van koolstof dioxide op een schaal van 100 jaar. Methaan speelt daarom een belangrijke rol in de opwarming van het klimaat. Methannotrofen vormen de belangrijkste biologische bron van methaan verwijdering op onze planeet. Deze micro-organismen consumeren methaan als enige koolstof en energiebron. Het belang ervan wordt vooral aangetoond in mariene ecosystemen. Hoewel de oceanen enorme hoeveelheden methaan produceren, resulteert de zeer efficiënte en effectieve verwijdering van het methaan via microbiologische oxidatie erin dat de oceanen een van de kleinste netto mondiale methaan bronnen vormen. Daarvoor is het van vitaal belang om te begrijpen welke diversiteit aan methanotrofen aanwezig is en welke factoren een positief of negatief effect hebben op de methaan oxiderende activiteit van deze organismen. Ondanks het belang van de aërobe methaan oxiderende bacteriën (MOB) in mariene ecosystemen is er momenteel slechts een beperkte hoeveelheid aan culturen beschikbaar *ex situ*. De cultivatie is echter onmisbaar om fysiologische data en genomische functies te koppelen en verder onze kennis over de specifieke habitat-voorkeuren van mariene MOB uit te breiden. Om deze reden spitste dit onderzoek zich toe op marine MOB en het opzetten van een grootschalige aanrijkings en isolatie strategie die toelaat een maximale diversiteit aan MOB te bekomen.

Een eerste stap van deze studie focust zich op de optimalisatie van de aanrijkingstechniek via het onderzoeken van het effect van verschillende parameters op de methaan verwijderingsactiviteit in mariene sediment. Een onmisbaar effect van zand op de methaan verwijdering kon opgemerkt worden, waarbij geen activiteit werd waargenomen in culturen zonder zand. Aldus de toevoeging van zand stimuleert de activiteit en kan de groei van MOB *ex situ* vergemakkelijken. Het exacte mechanisme van dit positieve zand effect vereist verder onderzoek, maar het ontstaan van een methaan, koolstofdioxide en zuurstof gradiënt in het zand kan mogelijks een rol spelen. Op basis van deze inzichten werd vervolgens een grootschalige isolatie strategie opgezet met sediment verzameld van zes verschillende stations in de Noordzee. De aanwezige MOB werden aangerijkt door het combineren van een eerste

verrijking met seriële subcultivaties gevolgd door geminiaturiseerde verdunningen van de culturen in verschillende stikstof en zuurstof microniches. Een duidelijke dalende trend van cultiveerbaarheid en detecteerbaarheid van MOB werd waargenomen overheen de stations van de kust naar open zee. Bijkomend waren de algemeen toegepaste *pmoA* primers niet in staat om biomerker genen op te pikken in een groot aantal actief methaan verwijderende culturen, dit suggereert een enorme onderschatting van methanotrofen *in situ* bij PCR-gebaseerde moleculaire onderzoeksstrategieën. Helaas, ondanks de talrijke pogingen slaagden we er niet in om reinculturen van deze methanotrofen te bekomen in het laboratorium.

"Shot gun" sequenering werd gebruikt op vier methaan oxiderende culturen verkregen tijdens de studie. In twee van de onderzochte culturen, bleek de aanwezigheid van gammaproteobacteriële MOB. Deze behoren tot de diep-zee cluster 2 MOB. De overige culturen bleken sterk aangerijkt voor alphaproteobacteriële MOB, die behoren tot het genus *Methyloceanibacter*. Een selectieve isolatie met behulp van methanol als enige koolstofbron leidde tot de isolatie van *M. methanicus*, de eeste type II MOB van marine omgevingen, het derde genus met uitsluitend sMMO en bovendien de eerste MOB in een methylotroof genus. Aanvullend hierop werden leidde de isolatie tot de vondst van drie nieuwe nauw verwante en strict methylotrofe species, *M. superfactus*, *M. stevinii*, *M. marginalis*. Samen met de eerder beschreven M. caenitepidi, lichten deze species een extreem voorbeeld van niche differentiatie toe met een een brede ecotype variatie aan groei kinetiek van methanol, voorkeuren voor stikstof, pH, temperatuur en zout. Voor de vertegenwoordigers van de diep-zee cluster 2 leidde *i*nzichten in het genomische landschap, ten opzichte van andere beschikbare Gammaproteobacterial genomen, ertoe tot het concluderen van de afwezigheid van een calcium-afhankelijke methanol dehydrogenase

Ten slotte werd onderzoek gedaan naar het optimaliseren van een lange termijn bewaar-protocol voor de opslag van mariene bacteriën dat toelaat om de verkregen verrijking en zuivere culturen uit deze studie met succes te kunnen stockeren. Nitriet-oxiderende bacteriën werden hierbij als modelorganismen gebruikt. Uit het onderzoek bleek dat de voorwaarden voor optimaal behoud stam-afhankelijk zijn, waarbij mariene stammen, gevoeliger bleken voor bevriezing dan niet-mariene soorten. Echter, toevoeging van 10% dimethylsulfoxide met of zonder tienvoudig verdunde trypticase soja broth resulteerd tot een algemeen cryopreservatie protocol, die toeliet alle geteste stammen succesvol te bewaren. Verdere werd hetzelfde protocol getest voor behoud van volledige mariene sedimentmonsters. Deze

bewaring was succesvol en liet opslag van verschillende hoofdrolspelers in de koolstof- en stikstof cyclus toe.

Er kan worden geconcludeerd dat mariene MOBs moeilijk te cultiveren en te isoleren zijn. Ondanks de talrijke pogingen slaagden we er in slechts één reincultuur in het laboratorium te verkrijgen van de meer dan 200 verkregen aanrijkingen. De beschikbaarheid van een succesvol bewaar-protocol laat de opslag van deze reincultuur toe, wat ervoor zorgt dat nieuwe diversiteit niet verloren gaat, maar garandeert ook archivering van de verkregen aanrijkings culturen en omgevingsstalen. Als zodanig zijn deze beschikbaar voor toekomstige isolatie campagnes waarbij nieuwe inzichten in mariene MOB en isolatie strategieën/technieken bekomen worden.

Dankwoord

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Mijn studenten, Lien, Annelien, Serra, Gerleen, Eline, Charles, Fabian en Jasper, die ik begeleide gedurende de 5 jaar wil ik zeker bedanken voor hun motivatie, steun en inbreng. Ik wens jullie alle geluk toe bij de verdere ondernemingen die jullie aangaan. In het bijzonder Charles, Fabian en Jasper wens ik enorm veel succes toe met hun eigen PhD avontuur.

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Gent, 27 Mei 2016

Bram Vekeman

Curriculum vitae

Personalia

Bram Vekeman

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Educational Background

2010 – Present	PhD – LM-UGent, Faculty of Science, Ghent University. Dissertation: Methanotrophic microbiomes from North Sea sediment. Promotors: Prof. Paul De Vos, Prof. Peter Vandamme, Dr. ir. Nico Boon, Dr. Kim Heylen.
2007 – 2009	Ghent University, Ghent, Belgium Degree of Master of Science in Biology, achieved with great honour; Dissertation: Diversity of rhizobia bacteria in *Leguminosae* situated in the Brakel area: a molecular identification. Promotor: Prof. Dr. Anne willems
2004 – 2007	Ghent University, Ghent, Belgium Degree of Bachelor of Science in Biology, achieved with honour; Dissertation: wound reactions in multicellular green algae
2004	Koninklijk Technisch Atheneum Brakel Degree Secondary Education Science-Mathematics

Professional Experience

2014 **Visiting scientist,** department of Microbiology, Radboud University, NL

- Ion torrent Shotgun sequencing and analyzing genome data
- Validation of storage protocol for extremophile methanotrophs belonging to the phylum *Verrucomicrobia*
- Bacterial isolation using floating filter technique

2009 – 2010 UZ Brussel, Laboratorium of microbiology, lab technician

- Responsible for analyzing human diseases in blood serum samples in a ISO 15189 accredited lab

Specialist Courses

2014	Training in Intellectual property and valorisation (Sas, Benedikt, UGent)
	Global warming: the science of climate change (David Archer, University of Chicago)
	Creative thinking (Johan De Bruycker)
2013	Training in statistics: Introduction to R (ICES, Kristof De Beuf)
2012	Workshop Unisense , Aarhus, DK
	Safe handling of gasses by Air Liquide

Awarded Grants and Prices

2014 **FWO,** Fund for Scientific Reserch Flanders

- Grant for participation at a congress abroad, K1A6414N
- Travel Grant for a long stay abroad, V436514N

2013 Belspo-BCCM award for the best poster at the Annual Scientific Meeting "Microbial Diversity for Science and Industry for the poster "Exploring the diversity of methane oxidizing bacteria in marine ecosystems".

2012 **FEMS,** Federation of European microbiological society

- FEMS meeting attendance grant

2011 **IWT,** Agency for Innovation by Science and Technology

- Doctoral Scholarships Strategic Basic Research, Personal mandate (2 x 2years), IWT/111108

Scientific Output

A1 Publications

Vekeman B, Kerckhof F-M, Cremers G, De Vos P, Vandamme P, Boon N, Op den Camp H.J.M, Heylen K. (2016). Methane oxidation and differential growth responses suggest extreme niche specialization in marine methylotrophic Methyloceanibacter. Submitted.

Vekeman B, Speth D, Wille J, Cremers G, De Vos P, Op den Camp H.J.M, Heylen K. (2016). Genome characteristics of two novel Type I methanotrophs enriched from North Sea sediments containing exclusively a lanthanide-dependent XoxF5-type methanol dehydrogenase. Submitted.

Heylen K, **Vekeman B**. (2016) PERSPECTIVE: Necessity, feasibility and strategy for sample archiving in microbial ecology. Submission pending.

Vekeman B, Van Colen C, Dumolin C, Beeckman F, De Vos P, Boon N, Heylen K. (2016). Cultivability and detectability of marine methanotrophs in North Sea sediments decrease from estuarine to open sea areas. Submission pending.

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De Meyer SE, Van Hoorde K, **Vekeman B**, Braeckman T, Willems A. (2011) Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). Soil Biol. Biochem. 43 (12): 2384-2396.

B2 Publication

Vekeman B. & Heylen K., (in press). Preservation of microbial pure cultures and mixed communities, Hydrocarbon and Lipid Microbiology Protocols: isolation and cultivation, in press (McGenity TJ, Timmis KN & Nogales B, eds.). Springer-Verlag, Berlin Heidelberg. DOI: 10.1007/8623_2015_51.

Oral Presentation

Vekeman B, Wille J, Boon N, De Vos P, Heylen K. Exploring the diversity of methane-oxidizing bacteria in marine ecosystems. Oral Pitch: VLIZ Young Marine Scientists' Day, 2015, Brugge, Belgium.

Hoefman S, **Vekeman B**, Van Hoorde K, Vandamme P, Boon N, Heylen K, De Vos P. "Protecting the Fragile": long-term preservation of fastidious organisms. Oral Presentation: PhD Symposium Faculty of Science 2012, Gent, Belgium.

Vekeman B, Hoefman S, Spieck E, Boon N, De Vos P, Heylen K. Long-term preservation of nitrite-oxidizing bacteria: ICoN2-ENC Meeting 2011, Nijmegen, The Netherlands.

Invited talks

Vekeman B, Methanotrophic microbiomes from North Sea sediment. Max Planck Institute, 2016, Bremen, Germany.

Vekeman B, Preservation of microbial pure cultures and mixed communities. Tissue preservation and biobanking, 2015, Barcelona, Spain.

Poster Presentations

Vekeman B, Kerckhof FM, Vandamme P, Boon N, De Vos P, Op Den Camp H, Heylen K. Extreme example of niche differentiation: new methanotrophic species within methylotrophic genus Methyloceanibacter. BSM 2015, Brussels, Belgium.

Vekeman B, Beeckman F, Boon N, De Vos P, Heylen K. Niche specialization of aerobic methane oxidizers in marine sediments. Molecular Basis of Microbial One-Carbon Metabolism (GRC/GRS) 2014, South Hadley Maine, USA.

Vekeman B, Beeckman F, Boon N, De Vos P, Heylen K. Niche specialization of aerobic methane oxidizers in marine sediments. The 15th International symposium on microbial ecology (ISME), 2014, Seoul, South-Korea.

Vekeman B, Beeckman F, Boon N, De Vos P, Heylen K. Niche specialization of aerobic methane oxidizers in marine sediments. ENC Meeting 2014, Gent, Belgium.

Vekeman B, Beeckman F, Dumolin C, Boon N, De Vos P, Heylen K. Exploring the diversity of methane-oxidizing bacteria in marine ecosystems. BSM 2013, Brussels, Belgium.

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