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Optimizing growth conditions of new methane oxidizing and nitrifying bacteria

Bachelor thesis

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České Budějovice 2023

Eicher, D., 2023: Optimizing growth conditions of new methane oxidizing and nitrifying bacteria. Bc. Thesis, in English. – 50 p., Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic.

Annotation

By creating enrichment cultures within two different media, we attempted to isolate nitrifying bacteria from a peatland site. Furthermore, three different strains of methanotrophic bacteria were cultivated at different temperatures while their growth was observed. Additionally, the metabolic activity of one strain was examined when exposed to various carbon and nitrogen sources, to osmolytes, and to different pH levels in the presence of diverse amino acids.

Declaration:

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 13.12.2023

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Daniel Eicher

Acknowledgement

First and foremost, I would like to express my deepest gratitude to my supervisor Dr. Anne Daebeler for the warm welcome and for giving me the opportunity to be a part of her team. She helped me get familiar with the lab as well as all the equipment.

Secondly, I would like to thank my co-supervisors MSc. Magdalena Wutkowska, Ph.D. and Mgr. Vojtěch Tláskal, Ph.D. for providing valuable information on working with these bacteria. Both of them provided their assistance and were always available whenever I needed help.

Finally, I want to thank my family and friends, who unconditionally supported me throughout this journey.

Abstract

Nitrification as well as the oxidation of methane are key components of the global nitrogen and carbon cycle. Furthermore, levels of nitrate and methane are increasing due to anthropogenic activities. To counteract this trend, it is important to study the behaviours of nitrifying and methane oxidising organisms. Therefore, the isolation of nitrifying bacteria, detected within the peat soil of the Šumava National Park, was carried out in two different media. Thereby, growth was only detected within the mineral salt media mixed with autoclaved peat water. To further investigate nitrifying bacteria, additional studies should focus on replacing peat water with a defined, artificial medium. Moreover, the growth of three strains of methanotrophs, which had been isolated from the sediments of the hypertrophic fishpond Naděje, was tested under different temperatures. The gathered data shows that Wu1 (a *Methylobacter luteus*-like methanotroph) exhibited the fastest growth at temperatures ranging from 19–22°C, while Wu8 (a *Methylobacter tundripaludum*-like methanotroph) grew the fastest when incubated at 13–28°C. Furthermore, Wu6 (a *Methylomicrobium lacus*-like methanotroph) multiplied the quickest at a cultivation temperature of 28°C. Additionally, the growth behaviour of Wu6 was further assessed in the presence of various carbon and nitrogen sources, of osmolytes, and at different pH levels with the addition of various amino acids. The tests showed high metabolic activities of Wu6 at a pH of 5–8, as well as with the addition of sucrose. The obtained results are important for optimizing the cultivation conditions of these methanotrophs, leading to potential improvements in the process of their purification.

Table of Contents

1. Introduction 1
   1.1. Carbon and nitrogen cycle 1
   1.2. Nitrogen 1
   1.3. Nitrification and NOB 1
      1.3.1. *Candidatus* Nitrotoga arctica 4
   1.4. Methane 6
   1.5. Methanotrophs 7
      1.5.1. *Methylobacter* 8
      1.5.2. *Methylomicrobium* 9
2. Aims of the thesis 10
3. Material and Methods 11
   3.1. Origin and enrichment of the nitrifying bacteria 11
   3.2. Additional enrichment of NOB with MSM 13
   3.3. Monitoring activity of NOB 14
   3.4. Transfer of NOB cultures 15
   3.5. Strains of methanotrophs and cultivation 16
   3.6. Testing the growth of methanotrophs at different temperatures 17
   3.7. Growth measurments 18
   3.8. Statistical analyses 18
   3.9. Testing growth of a methanotroph on different osmolytes, under various pH levels, and with supplementation of different nitrogen and carbon sources 19
4. Results 20
   4.1. Enrichment of NOB 20
      4.1.1. Different media for NOB 20
      4.1.2. Enriching NOB in medium supplemented with peat water 20
   4.1. Characteristics of growth conditions of methanotrophs 22
      4.1.1. Optimal growth temperatures 22
      4.1.2. Capability of Wu6 to grow by using different carbon sources 26
      4.1.3. Growth of Wu6 using different nitrogen sources 27
      4.1.4. Growth of Wu6 in the presence of different osmolytes 28
      4.1.5. Growth of Wu6 at different pH levels in the presence of diverse amino acids 29
5. Discussion 30
   5.1. Enrichment of NOB 30
   5.2. Optimal growth conditions of three methanotroph strains 31
6. Conclusion 35

7. References/Literature 36
Appendix 45

List of Abbreviations:

AOA – Ammonia-oxidising archaea

AOB – Ammonia-oxidising bacteria

FNOM – Freshwater Nitrite Oxidiser Medium

MMO – Methane monooxygenase

MSM – Mineral salt medium

NMS– Nitrate mineral salt medium

NOB – Nitrite-oxidising bacteria

NXR – Nitrite oxidoreductase

OD600 – Optical density at 600 nm

SeW – Selenium-Wolfram solution

TES – Trace element solution

WWTP – Wastewater treatment plant

1. Introduction

1.1. Carbon and nitrogen cycle

The earth's ecosystems are closely interconnected by the carbon and nitrogen cycle, which has far-reaching impacts on biological diversity, soil fertility, climate regulation, and ecosystem health (Fowler et al., 2013; Schimel et al., 2015). As humans interfere with these cycles, it becomes more and more important to understand the mechanisms behind them in order to mitigate the anthropogenic impact ("Control Methane to Slow Global Warming — Fast," 2021; Diaz & Rosenberg, 2008). Therefore, it is crucial to study microorganisms that mitigate or produce molecules, that directly impact global biogeochemical cycling and climate on Earth.

1.2. Nitrogen

Nitrogen is an essential component of nucleic acids, amino acids, and adenosine triphosphate. Additionally, it is one of the most abundant elements on earth, as for example, the atmosphere consists of 78% dinitrogen gas, the most stable form of nitrogen. Nitrogen has a wide variety of redox states in different molecules, with a minimum of -III in ammonia (NH_3) and a maximum of +V in nitrate (NO_3^-). These molecules undergo transformation into one another through various oxidative and reductive reactions by different microorganisms. For instance, these reactions may occur through dissimilatory pathways, resulting in the formation of adenosine triphosphate (Gupta & Gupta, 2021).

1.3. Nitrification and NOB

During nitrification, ammonia is oxidised to nitrate. This process is carried out by ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB). Ammonia oxidising archaea (AOA) represent another group of microorganisms capable of ammonia oxidation (Sayavedra-Soto & Arp, 2011; Starkenburg et al., 2011). Furthermore, comammox bacteria can convert ammonia to nitrate within their own organism (Hu & He, 2017). The first step of the nitrification reaction is the oxidation of ammonia to nitrite (NO_2^-) by the AOB or AOA. Subsequently, the NOB oxidise the nitrite to nitrate. The crucial enzyme for the oxidation of nitrite is nitrite oxidoreductase (NXR), which is a molybdopterin-binding enzyme inside the dimethyl sulfoxide reductase family (Meincke et al., 1992; Yamanaka, 1988; Yamanaka et al., 1981). The functional NXR enzyme is built out of three subunits. The α subunit includes the catalytic site, while the β and γ subunit guide the electrons originating from nitrite to

downstream components of the respiratory chain. The γ subunit additionally acts as a membrane anchor of the enzyme (Daims et al., 2016).

AOB and NOB can benefit from each other via a mutualistic relationship (Koch et al., 2015). The AOB provide the NOB with nitrite, while these reduce nitrite, which is toxic when present in high amounts. This is the reason why NOB and AOB are often found in close proximity to each other (Koch et al., 2015). The process of nitrification is important for aquatic ecosystems and wastewater treatment plants (WWTP), as high concentrations of ammonia or nitrite can lead to health risks in humans and animals (Camargo & Alonso, 2006). Additionally, anthropogenically increased levels of nitrogen lead to large algal blooms in coastal areas and lakes (Diaz & Rosenberg, 2008). To reduce the amount of nitrogen released into the environment, sewage is processed in WWTPs. Thereby, microbial communities, consisting of nitrifying and heterotrophic denitrifying bacteria, turn ammonia into gaseous nitrous oxide (N_2O) and dinitrogen (N_2) gas (Siripong & Rittmann, 2007; Tian & Wang, 2020). Exploring and understanding these bacteria, including the discovery of new ones, is essential to enhance the efficiency of WWTPs. Without WWTPs, the amount of nitrogen input into the environment would be considerably higher.

Furthermore, nitrification acts as a key limiting step in the production of nitrous oxide emissions within peatlands (Siljanen et al., 2019). Thereby, the nitrification process contributes to the synthesis of nitrous oxide in two different ways (Hu et al., 2015; Kozlowski et al., 2016; Siljanen et al., 2019). It primarily influences the nitrous oxide production occurring through denitrification by regulating the amount of nitrite and nitrate in the environment as peatlands mainly receive their nutrients through atmospheric deposition (Bragazza et al., 2006; Siljanen et al., 2019). Additionally, nitrification can lead to the direct release of nitrous oxide, particularly in bare peat with minimal surface emissions during dry years (Gil et al., 2017).

In the year 1891 Sergei Winogradsky, as well as Grace and Percy Frankland, had already discovered the first nitrite-oxidising bacteria (reviewed in Sedlacek, 2020). However, due to the slow growth and dependence on syntrophic partners, it is rather difficult to cultivate these microbes and the purification of new strains of NOB can take years (Daims et al., 2016; Lebedeva et al., 2008). The importance of NOB is not only significant in terms of mitigation of anthropogenic nitrogen pollution. Besides the already described benefits of reducing nitrite in the environment, the produced nitrate is utilized as a valuable source of nitrogen by many plants and microorganisms (Gruber, 2008). However, excess amounts of nitrate can easily

leach into surface waters causing changes in biomass and productivity. The resulting eutrophication and acidification exert severe negative effects on the whole ecosystem (Hornung, 1999).

NOB are a diverse group of bacteria belonging to four different phyla: *Nitrospinae*, *Nitrospirae*, *Chloroflexi,* and *Proteobacteria.* (Spieck et al., 2021). However, new phyla containing NOB, such as the recently discovered *Ca*. Nitrosediminicolota, continue to be identified (Zhao et al., 2023). These phyla are divided into a total of seven genera, including the uncultured marine NOB *Candidatus* Nitromaritima, which is related to *Nitrospina* (Daims et al., 2016; Ngugi et al., 2016). All known NOB except *Nitrolancea hollandica*, which belongs to the phylum of *Chloroflexi*, contain a gram-negative cell envelope (Daims et al., 2016; Sorokin et al., 2012). Within the phylum *Proteobacteria*, nitrite oxidisers are found in the classes of *Alpha-*, *Beta-* and *Gammaproteobacteria* belonging to the respective genera of *Nitrobacter*, *Nitrotoga*, and *Nitrococcus* (Lücker et al., 2013; Spieck et al., 2021). The genus *Nitrospira* was first included in the phyla of *Deltaproteobacteria* (Teske et al., 1994), and later reclassified to the phylum *Nitrospirae* (Lücker et al., 2013; Schloss & Handelsman, 2004).

NOB exist in many different habitats. 16S rRNA sequences of nitrite oxidisers have already been observed in engineered systems like WWTPs, as well as in natural habitats like soil, freshwater, marine, and hypersaline waters (Daims et al., 2016). Additionally, some NOB have been found in geothermal areas and subsurfaces, for example in groundwater, caves, or living endolithicaly (Daims et al., 2016). The occurrence of different genera strongly varies across different ecosystems (Daims et al., 2016). Members of the *Nitrospinae* prevail in marine environments (Beman et al., 2013; Daims et al., 2016; Füssel et al., 2012; Jorgensen et al., 2012; Nunoura et al., 2015). These bacteria can reach abundances of 10% within microbial communities in deep-sea waters, mesophilic zones, oxygen minimum zones, and sediments (Beman et al., 2013; Daims et al., 2016; Füssel et al., 2012; Jorgensen et al., 2012; Nunoura et al., 2015). The phylum *Nitrospirae* contains the genus *Nitrospira*, the most diverse genus of all the NOB (Daims et al., 2001, 2016; Lebedeva et al., 2008, 2011). *Nitrospira* are divided into at least six phylogenetic sublineages and among them are the only known thermophilic NOB. These bacteria exist in geothermal areas with temperatures up to 65°C (Edwards et al., 2013; Lebedeva et al., 2011; Marks et al., 2012).

Furthermore, NOB can be distinguished by the orientation type of their NXR in the cell. In species of the genera *Nitrobacter*, *Nitrococcus*, and *Nitrolancea*, the α subunit of the enzyme is located in the cytoplasm (Daims et al., 2016; Lücker et al., 2010; Sorokin et al., 2012; Spieck

et al., 1996; Starkenburg et al., 2006; Sundermeyer-Klinger et al., 1984). In *Nitrospira*, *Nitrospina,* and *Candidatus* Nitromaritma it was found in the periplasm (Daims et al., 2016; Koch et al., 2015; Lücker et al., 2010, 2013; Ngugi et al., 2016; Spieck et al., 1998; Sundermeyer-Klinger et al., 1984). Additionally, a new type of periplasmic NXR was discovered in the *Candidatus* Nitrotoga, which is a phylogenetically distinct genus (Kitzinger et al., 2018; Spieck et al., 2021).

1.3.1. *Candidatus* Nitrotoga arctica

The NOB *Candidatus* Nitrotoga arctica (Figure 1) was originally isolated from permafrost soil of the Siberian Arctic in 2007 (Alawi et al., 2007). Since then, more *Ca.* Nitrotoga have been discovered in other natural and artificially created ecosystems. Successful enrichments have been created from freshwater habitats, marine water bodies, coastal sediments, and groundwater cave systems (Daims et al., 2016). Additionally, *Nitrotoga*-like bacteria have been detected in WWTPs. Thereby, after the incubation of the activated sludge at different temperatures, three distinct groups of NOB were discovered (Lantz et al., 2021; Spieck et al., 2021).

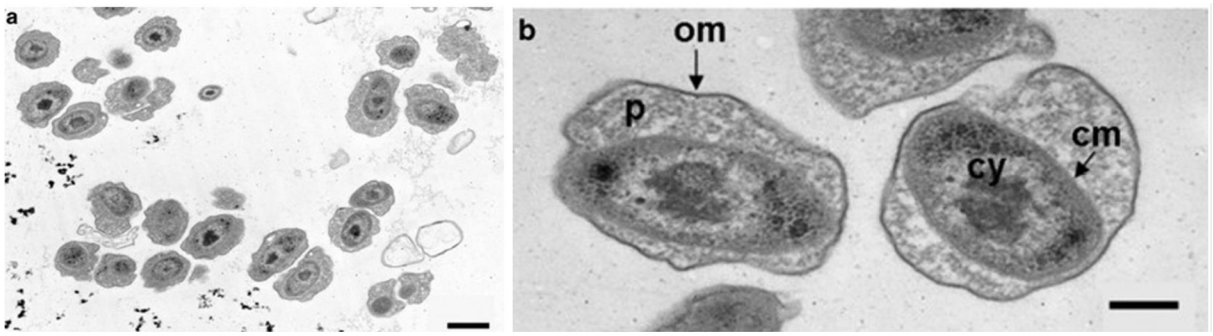

Figure 1: Electron micrographs of an enrichment containing *Candidatus* Nitrotoga arctica, (a) bar = 200 nm, **(b)** bar = 500 nm, Abbreviations: Cytoplasm (cy), cytoplasmic membrane (cm), outer membrane (om), periplasm (p) (Alawi et al., 2007)

The genus *Nitrotoga* belongs to the order of *Nitrosomanadales*, which is within the class of *Betaproteobacteria*. The taxonomically closest related and isolated organism to *Ca.* Nitrotoga arctica, which has been isolated into pure culture, is *Gallionella ferruginea* (Alawi et al., 2007). These are iron oxidising bacteria, which were already discovered by Ehrenberg in 1836 (Hallbeck et al., 1993). *Gallionella* have a characteristic twisted extracellular stalk, which consists of precipitated iron oxide (Hallbeck et al., 1993).

The genus *Nitrotoga arctica* was the first psychrophilic NOB that could be enriched. Its growth temperatures range between 4 and 22°C with an optimum at 10°C (Alawi et al., 2007). No other *Nitrospira* or *Nitrobacter* has been grown at such low temperatures (Spieck et al., 2021). This microorganism is additionally adapted to a low concentration of nitrite of 0.3 mM with a tolerance limit of 1.2 mM (Alawi et al., 2007). The cells of *Ca.* Nitrotoga arctica are irregularly shaped encompassing coccoid or rod-shaped forms, ranging from 0.4 to 0.7 × 1.0 µm and form flocs via cell aggregates (Alawi et al., 2007). While this organism exhibits an extraordinarily wide periplasmic space, it does not possess carboxysomes or intracytoplasmic membranes (Alawi et al., 2007). Additionally, these aerobic chemolithotrophs are non-motile, replicate via binary fission, and utilize carbon dioxide (CO_2) for the assimilation of carbon (Alawi et al., 2007).

It is exceedingly difficult to isolate these bacteria, which is the reason why until 2021 only two cultures of *Ca.* Nitrotoga could be purified (Ishii et al., 2020; Kitzinger et al., 2018). There are several reasons leading to complications. First, the growth rate of *Ca.* Nitrotoga is very slow, with a doubling time of up to 54 hours (Ishii et al., 2017; Nowka et al., 2015). Furthermore, several combined methods are required to eliminate the adhering heterotrophic microbes and to reduce organic material, which is excreted by the NOB (Spieck et al., 2021). Finally, some special cofactors and metabolites like ammonium chloride (NH_4Cl) or pyruvate are most likely required to increase the growth rate of *Ca.* Nitrotoga (Ishii et al., 2020; Watson et al., 1986; Wegen et al., 2019). Additionally, the conservation of these bacteria is possible via a special cryopreservation method (Vekeman et al., 2013). However, it was reported that the success rate of reactivation of cryopreserved cultures of *Ca.* Nitrotoga is worse than for other NOB (Spieck et al., 2021). Consequently, no strain of *Nitrotoga* has been validly published and described in the International Journal of Systematic and Evolutionary Microbiology, and therefore it is still named *Candidatus* (Oren et al., 2020).

1.4. Methane

Methane (CH_4) plays an important role in the global carbon cycle, influences to the global economy as an important fuel source and contributes to climate change as a greenhouse gas. It is a 34 times more active greenhouse gas than carbon dioxide and since the beginning of 1800, the atmospheric levels of methane nearly tripled reaching a concentration of 1.9 ppm in 2022 (Monaco et al., 2021; Nisbet, 2022). An average methane molecule lasts approximately 12 years in the atmosphere, a time, which is much shorter than that of other greenhouse gases. Once released into the surrounding mainly by methane producing microorganisms, it is either biologically converted into carbon dioxide via microorganisms or chemically converted into water as well as a methyl radical by reacting with hydroxyl radicals, especially in the stratosphere (Cicerone & Oremland, 1988; Stevenson et al., 2020). Current international recommendations emphasize the need to reduce the amount of methane released into the atmosphere, which would lead to a reduction in the temperature rise ("Control Methane to Slow Global Warming — Fast," 2021). Some studies even estimate, that with the help of new technologies, the amount of methane released into the atmosphere could be halved, which could prevent a temperature increase of up to 0.3°C until 2050. ("Control Methane to Slow Global Warming — Fast," 2021; Monaco et al., 2021).

There are three major sources of methane: thermogenic, abiogenic, and biogenic sources. 60% of the total emissions are caused by anthropogenic activity (Nisbet, 2022). The primary sources are livestock, rice fields, waste treatment, and wastewater treatment, which altogether make up more than half of these man-made emissions (Conrad, 2009). The methane released via these sources originates from methanogenic archaea, which thereby contribute immensely to the worldwide methane output into the atmosphere. These microbes can also be found in peatlands, wetlands, and oceans (Guerrero-Cruz et al., 2021).

1.5. Methanotrophs

Methanotrophs are microorganisms that oxidise methane, which serve as an important biological sink of methane, as they have developed a way to use this molecule to their advantage. (King, 1992). The oxidation of methane can occur in anaerobic as well as aerobic microorganisms (Guerrero-Cruz et al., 2021). As for this thesis the experiments were only conducted with aerobic methanotrophs, all further text will not focus on the anaerobic organisms.

Aerobic methanotrophs utilize four enzymes to completely oxidise methane to carbon dioxide: methane monooxygenase (MMO), methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase (Anthony & Ghosh, 1998; Lee et al., 2016; Ophem et al., 1993). MMO utilizes methane for energy generation, ultimately leading to the growth of these methanotrophs (Khider et al., 2021). There are two types of MMO, the soluble form, which is found in the cytoplasm and the particulate form, which is fused with the system of intracellular membranes (Khider et al., 2021). The soluble form has iron atoms at the active centre while the particulate type contains primarily copper atoms (Murrell et al., 2000). Methanotrophs can genomically encode and synthesise either both or only one of these two enzyme types (Khider et al., 2021). However, it should be noted that most species produce the particulate type and only some the soluble type (reviewed in Dedysh & Knief, 2018). In those species where both enzymes are produced the MMO expression depends on the so-called ‘copper switch’(Khider et al., 2021; Stanley et al., 1983). When the copper-to-biomass ratio is high, the particulate type is expressed, as the active centre requires a high concentration of copper. Otherwise, the soluble form is produced (Khider et al., 2021; Morton et al., 2000).

In general, these methanotrophs were discovered in the phyla of *Verrucomicrobia*, *Actinobacteria*, and *Proteobacteria*, including the classes of *Alphaproteobacteria* and *Gammaproteobacteria,* which contain the order of *Methylococcales* (Dedysh & Knief, 2018). Within the order of *Methylococcales* there are the families *Crenotrichaceae*, *Methylococcaceae*, and *Methylothermaceae* (J. P. Bowman, 2018; Orata et al., 2018). Furthermore, the genera *Methylomicrobium* and *Methylobacter* are part of the family *Methylococcaceae* (Dedysh & Knief, 2018).

1.5.1. *Methylobacter*

Methylobacter luteus (Figure 2a) and *Methylobacter tundripaludum* (Figure 2b) are both non-motile methanotrophs that can use methane as their only carbon and energy source (reviewed in Collins et al., 2017). They are usually cultured in mineral medium such as nitrate mineral salts (NMS) medium without organic supplements (J. Bowman, 2006; Whittenbury, Davies, et al., 1970). The medium was originally developed in 1970 and has been modified over the years to improve its effectiveness (J. Bowman, 2006; Whittenbury, Davies, et al., 1970).

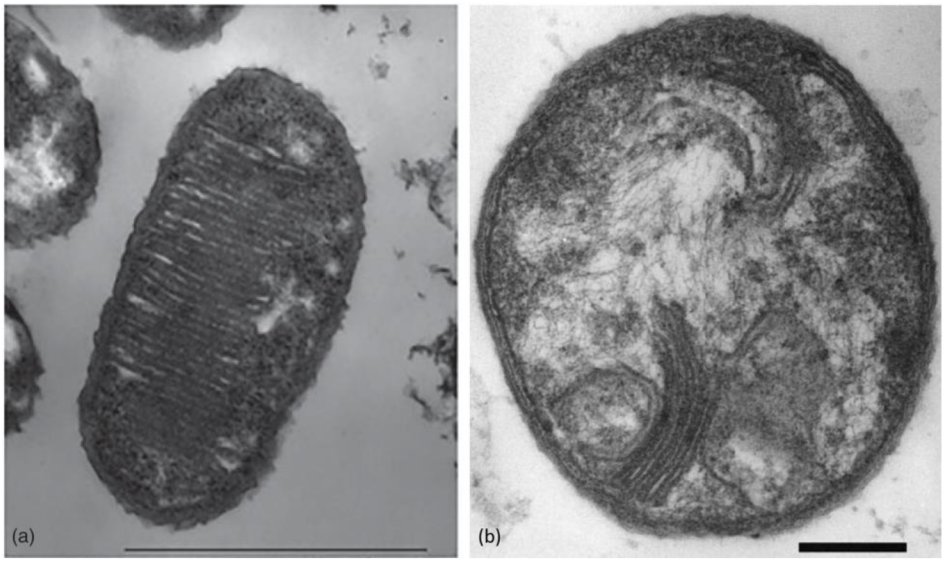

Figure 2: Electron micrograph of one cell of *Methylobacter luteus* sp. VKM-53B (a) and *Methylobacter tundripaludum* SV96 (b), bar=1 µm (a)**,** bar=200 nm (b) (Collins et al., 2017; Wartiainen et al., 2006)

M. luteus was isolated from sediments of freshwater rivers and lakes (Collins et al., 2017; Romanovskaia et al., 1978). It grows between 15–40°C with an optimal temperature of 30°C and at a pH between 5–9 (Collins et al., 2017; Romanovskaia et al., 1978). The cells of *M. luteus* are cocci or elliptical rods with a size of 1.2–1.5 x 1.5–2.0 µm (Collins et al., 2017; Romanovskaia et al., 1978). These bacteria synthesise a yellow-green pigment (Collins et al., 2017; Romanovskaia et al., 1978), whereas the cells of *M. tundripaludum* SV96 are initially pale pink, which fade in older cultures during methane starvation (Wartiainen et al., 2006). *M. tundripaludum* was first discovered and isolated from Arctic wetlands (Wartiainen et al., 2006). The cells of this methanotroph have a straight, fat rod-shaped form with a diameter of 0.8–1.3 µm and a length of 1.9–2.5 µm (Wartiainen et al., 2006). *M. tundripaludum* mostly appears in pairs or long chains but can also exist as single cells. These bacteria can multiply at temperatures ranging from 10–30°C with the optimum at 23°C and at a pH of 5.5–7.9 (Wartiainen et al., 2006).

1.5.2. *Methylomicrobium*

Methylomicrobium lacus (Figure 3) (previously *Methylosarcina lacus*) belongs to the order of *Methylococcales* and was first isolated from lake sediments (Kalyuzhnaya et al., 2005). These microbes are not motile, reproduce via binary fission and form coccoid cells, which comprise a length of 1.0–1.2 µm and a respective width of 0.7–1.0 µm (Kalyuzhnaya, 2016). *M. lacus* develops extracellular fibrils as well as holdfast-like structures. Their colonies, which can be grown under methane/air atmosphere on mineral salt medium, consist of 1–2 mm white, smooth, and convex circles (Kalyuzhnaya et al., 2005). The necessary nitrogen can be taken up via aspartate, glutamate, nitrate, or serine. *M. lacus* grows at a wide temperature range of 4–35°C, with the optimum at 28–30°C and at a pH of 4.0–7.0. (Kalyuzhnaya et al., 2005).

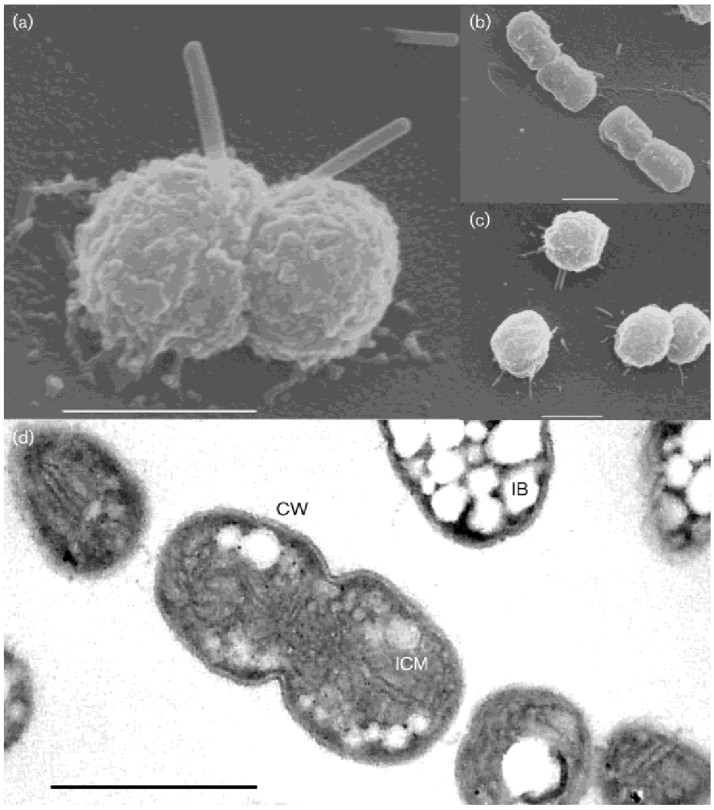

Figure 3: Electron micrographs of *Methylomicrobium lacus*, bars = 1 µm, Abbreviations: cell-wall (CW), internal cytoplasmic membranes (ICM), inclusion bodies (IB) (Kalyuzhnaya et al., 2005)

2. Aims of the thesis

- Enriching NOB out of peat soil
- Describing the physiology of three available isolates of methanotrophs
    - Characterising optimal temperatures for growth
    - Characterising optimal pH and osmolarity for growth
    - Characterising range of sources of carbon and nitrogen supporting growth

3. Material and Methods

3.1. Origin and enrichment of the nitrifying bacteria

Peat soil samples were collected by members of the hosting laboratory on the 16th of October 2021 in the first zone of the Šumava National Park, Czech Republic (49.0166411N, 13.5685650E). Three soil cores B, C, and D (10×10 cm) were taken within an area of 60 m² from a fen-type peat site called fen. The soil from each core was separated into an upper layer (0-10 cm depth) and a lower layer (10-30 cm depth). The soil was then stored in separate bags at 4°C.

In order to enrich NOB, a nutrient-limited medium (Table 1) was prepared as follows: 500 ml of a so-called Freshwater Nitrite Oxidiser Medium (FNOM) (Table 2) (Boddicker & Mosier, 2018; Lantz et al., 2021) with its pH adjusted to 4.6 was autoclaved and 5 ml of potassium dihydrogen phosphate (KH_2PO_4), as well as 0.5 ml of trace element solution (TES) (Table 3), were added. Sodium nitrite ($NaNO_2$) was added to reach a final nitrite concentration of 0.3 mM. Both the pH and nitrite concentration were chosen according to measurements of the field conditions. Then 50 ml of this medium were distributed to 6 sterile 100 ml bottles. Next, soil (< 1 g) from one of the six bags was added into each bottle. All the work was done within the laminar flow hood to reduce the possibility of contamination. The bottles were then incubated in the dark at room temperature for static cultivation.

Table 1: Nutrient-limited medium

Component	For approximately 500 ml
FNOM	500 ml
KH_2PO_4	5 ml
TES	0.5 ml
$NaNO_2$	Conc. of 0.3 mM (approximately 0.0105 g)

Table 2: Composition of the FNOM

Component	For 1 l
Milli-Q water	1 l
NaCl	1 g
$MgCl_2 \times 6H_2O$	0.4 g
$CaCl_2 \times 2H_2O$	0.1 g
KCl	0.5 g
10× vitamin solution	0.1 ml
$NaHCO_3$ (1 M)	1 ml
$NaNO_2$ (1 M)	0.3 ml
HCl	Until pH of 4.6

Table 3: Trace elements stock solution

Component	For 1 l
Milli-Q water	1 l
$FeSO_4$ x $7H_2O$	2 g
$ZnSO_4$ x $7H_2O$	0.1 g
$MnCl_2$ x $4H_2O$	0.03 g
$CoCl_2$ x $6H_2O$	0.02 g
$CuCl_2$ x $2H_2O$	0.079 g
$NiCl_2$ x 6H2O	0.02 g
Na_2MoO_4 x $2H_2O$	0.0255 g
Na_2EDTA	5 g

3.2. Additional enrichment of NOB with MSM

On the 25th of November 2021, additional enrichments targeting NOB were set up according to Table 4. This time sterile peat water was mixed with mineral salt medium (MSM), which should represent a more similar environment to peat. The composition of MSM can be seen in Table 5. To reduce the amount of the required material, the cultures were set up in a sterile 24-well plate. First 2.0 ml of sterile peat water, 0.4 ml of MSM, and 0.4 ml of potassium dihydrogen phosphate (KH_2PO_4) were pipetted into each of the twelve wells. Afterwards, TES, SeW (Selenium-tungstate solution), and sodium nitrite ($NaNO_2$) were added to each well at concentrations as described in Table 4. Finally, chunks of peat material were added into twelve wells. The other twelve empty wells served as borders between peat soil samples collected from the cores B, C, and D. The plate was then sealed with parafilm so that no contamination could occur and water would be retained. Afterwards, it was incubated in the dark at room temperature.

Table 4: Final cultivation medium for second enrichment of NOB

Component	Concentration	For one well
Sterile peat water	-	2.0 ml
MSM	-	0.4 ml
KH_2PO_4	0.054 g/l	0.4 ml
TES	-	0.001 ml
SeW	-	0.001 ml
$NaNO_2$	1 M	Conc. of 1 mM

Table 5: Composition of the MSM

Component	Mass concentration
Milli-Q water	-
NaCl	0.585 g/l
KCl	0.075 g/l
$CaCl \times 2H_2O$	0.054 g/l
$MgSO_4 \times 7H_2O$	0.49 g/l

3.3. Monitoring activity of NOB

Every other week the cultures were tested in the laminar flow hood with Quantofix NO_2^-/NO_3^- strips (Macherey-Nagel) for activity of nitrite oxidation. These strips qualitatively detect nitrite and nitrate (upper field) or nitrite (lower field) by turning purple, as seen in Figure 4. To ensure that the colour change in the upper field did not result from the nitrite, culture sample was mixed 1:1 with 100 mM sulfamic acid. The nitrite reacts with the sulfamic acid and turns into dinitrogen gas, while any present nitrate is not reacting. Subsequently, the mixture was pipetted onto another test stripe and if a colour change occurred, it indicated the presence of nitrate. Such a change displays ongoing nitrite oxidation by NOB in the medium.

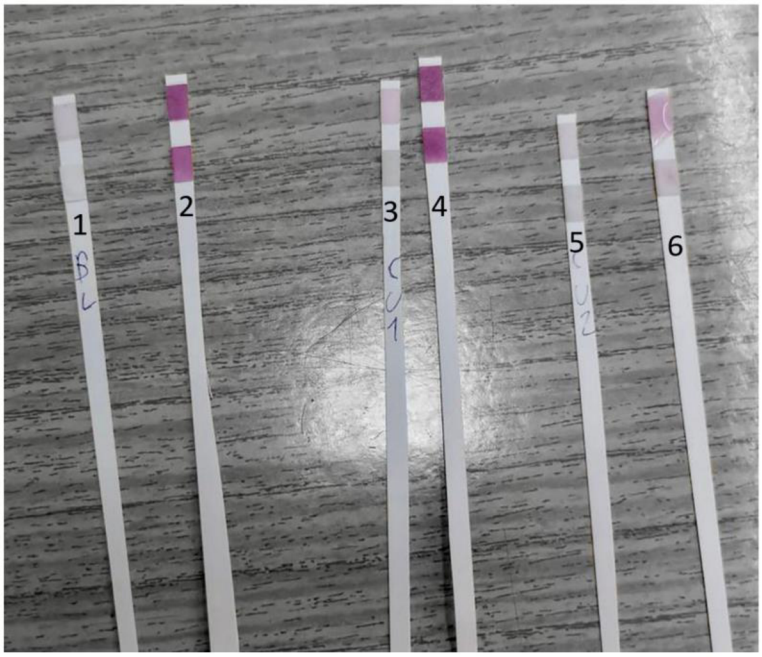

Figure 4: Quantofix NO_2^-/NO_3^- strips; The left test strip of each pair was used to test for nitrate after nitrite was reduced, while the right one shows the colour change before the reaction with 100 mM sulfamic acid. Both fields turning purple of test stripes 2 and 4 indicate that nitrite is still present, while in test stripes 3, 5, and 6 only the upper field is slightly purple implying the presence of nitrate. Due to no colouration on test stripe 1, it indicates the absence of nitrate.

3.4. Transfer of NOB cultures

On the 22nd of March 2022 (after ~17 weeks), the NOB enrichment cultures from the second attempt were transferred into a new plate for further isolation. First, well number six and seven were refilled with 1 ml of sterile peat water, as most of the fluid had evaporated. Afterwards, 2.5 ml of sterile peat water and 0.01 ml, of enrichment culture from the first cultivation plate were pipetted into a new well on a second plate, except for well six and seven from which 0.02 ml of liquid were transferred from the older plate (Table 6). Then, 0.001 ml of 1M sodium nitrite ($NaNO_2$), 0.001 ml TES (Table 3), and 0.001 ml SeW were added into each new well. Both 24-well plates were closed, sealed with parafilm, and incubated in the dark at room temperature. The new plate was then regularly checked via the colour change on the test strips.

Table 6: Set up for the transfer of NOB enrichments from the second cultivation attempt

Component	For one well
Culture from the old well	0.01 ml or 0.02 ml
$NaNO_2$ (1 M)	0.001 ml
Sterile peat water	2.5 ml
TES	0.001 ml
SeW	0.001 ml

3.5. Strains of methanotrophs and cultivation

Three methanotrophs (tentatively named Wu1, Wu6, and Wu8) were isolated by my co-supervisor Magdalena Wutkowska in 2021 from the sediments from the hypertrophic fishpond Naděje, located in the southwestern part of the Czech Republic (49.0369167N, 14.4345556E). By sequencing the genomes and subsequent comparative genomics, based on a consensus single-copy gene taxonomy of the 16S rRNA, it was determined that Wu1 is mostly related to *Methylobacter luteus*, Wu6 to *Methylomicrobium lacus*, and Wu8 to *Methylobacter tundripaludum*.

The methanotrophs were grown in an NMS medium, which was prepared according to Table 7 (J. Bowman, 2006; Whittenbury, Davies, et al., 1970). Specifically, 1 g of potassium nitrate (KNO_3) and 0.54 g of potassium dihydrogen phosphate (KH_2PO_4) were added into a 1 l flask containing 800 ml of Milli-Q water. After the chemicals had been dissolved via a magnetic stirrer the pH was raised to 6.8 with a 1 M sodium hydroxide (NaOH) solution. The flask was then filled up to 1 l with Milli-Q water. Before the other mineral salts were added, the medium was autoclaved at 121°C for 20 minutes. After cooling 1 ml of 1.62 M magnesium sulfate ($MgSO_4 \times 7H_2O$) solution, 1 ml of 0.1 M calcium chloride ($CaCl_2 \times 2H_2O$) solution, and 1 ml TES were added under aseptic conditions. For additional information on the TES see Table 3.

The cultures were maintained in 120 ml bottles sealed with butyl rubber stoppers and amended with ~15% methane in the headspace. During weekly transfers, 0.5 ml of well-established culture was added into 19.5 ml of NMS medium (Table 7). To reduce the possibility of contamination, the cultures were transferred to the fresh medium within the laminar flow hood.

Table 7: Composition of the NMS medium

Component	Molarity	For 1 l
Milli-Q water	-	1 l
KNO_3	-	1 g
KH_2PO_4	-	0.54 g
NaOH	1 M	Until pH of 6.8
$MgSO_4 \times 7H_2O$	1.62 M	1 ml
$CaCl_2 \times 2H_2O$	0.10 M	1 ml
TES	-	1 ml

3.6. Testing the growth of methanotrophs at different temperatures

Before larger, full-size experiments were conducted, smaller version tests were performed in advance. The three distinct cultures of methanotrophs were grown in duplicates at 3–5°C and 13°C. The methanotrophs were incubated as described above. On day 0, 3, and 8 these incubations were well-mixed. Additionally, 0.2 ml of culture was added to a 96-well plate in triplicates and measured for growth (see next section for details).

For the final experiment, the same three methanotroph cultures were incubated in duplicates at 5 different temperatures: 4.5, 13, 19, 22, and 28°C. Additionally, to the beforehand used procedure for the cultivation, all cultures were kept on shaking platforms at 90 rpm and shielded from light. The cultures were subsampled on day 0, 3, 5, 7, and 10 of the experiment. Each time, the cultures were taken from their shaking platform and mixed well. The subsamples were taken with a sterile needle and syringe after disinfection of the stopper surface with 70% ethanol. Approximately 0.8 ml of culture was collected at each time point and then distributed to three wells of a 96-well plate by pipetting 0.2 ml of culture into each well.

3.7. Growth measurments

The optical density of a culture was measured at 600 nm (OD600) as a proxy for the cell density of a culture ("Monitoring Growth of Suspension Cultures in Microplates," 2020). Growth was calculated as the difference in OD600 over time. This method builds on the fact that cultures become more turbid during bacterial growth and light passing through is increasingly scattered. Therefore, the fraction of light reaching the detector decreases. The amount of light, which is lost depends on cellular characteristics like cell size, how the cell membrane is built, and the cell anatomy, as well as on instrumental optics (Latimer, 1982). The OD600 was measured in triplicates within 96-well plates using a plate reader (Agilent BioTek Synergy 2). The average of each technical triplicate was calculated and the bacterial absorbance of day 0 was subtracted from the values of the later days to determine the increase of the OD600 values since the start of the incubation. Additionally, the standard deviation was calculated from each biological triplicate. The results were plotted in Excel (Version 2311).

3.8. Statistical analyses

To validate the results statistical analyses were performed. Therefore, a script was written in R (R Core Team, 2022), while RStudio (RStudio Team, 2022) was utilized to process the output. The first null hypothesis stated that no changes in the growth for a specific strain at different temperatures occurred, while the second null hypothesis postulated that there was no difference in the growth between different strains at given temperature. To test the hypotheses one-way ANOVA tests were conducted with subsequent Tukey's honestly significant difference (Tukey's HSD) tests to account for multiple comparisons. All tests were conducted at a significance level of $p < 0.05$.

3.9. Testing growth of a methanotroph on different osmolytes, under various pH levels, and with supplementation of different nitrogen and carbon sources

The growth of one methanotroph isolate — Wu6, was tested on 96-well Biolog Plates (Biolog, Inc.). In total four different plates were used: PM1, PM3B, PM9, and PM10. PM1 is designed to test the growth of the cultures on different carbon sources, PM3B – for nitrogen sources, PM9 – for growth in the presence of different osmolytes, and PM10 – for growth at different pH values in the presence of diverse amino acids. Further information on the plates can be found in the Appendix. As per manufacturers instructions, 0.05 ml of a tenfold diluted culture and 0.05 ml of 100× diluted Biolog Redox Dye Mix G were added to each well of the respective Biolog plate (Table 8). If the cells are metabolically active, the dye is reduced and changes its colorimetric properties, which was measured on a plate reader (see below for details).

Table 8: Components necessary for one 'Biolog' plate

Component	For one plate
100× diluted dye	5 ml
Milli-Q water	4.95 ml
Bacterial culture	0.5 ml
NMS	4.5 ml

The culture was handled in the laminar flow. All plates were incubated in the presence of 15% methane and an extra PM1 plate was used for comparison of substrate use in the absence of methane. The absorbance was measured on a plate reader (Agilent BioTek Synergy 2) at 590 nm for the PM1 plates on day 0, 2, 5, and 7, while the other plates were only measured on day 0 and 7 to reduce the risk of contamination. These measurements were not replicated. The values of the inoculation day were then subtracted from the measured absorbance of day 7. Additionally, the obtained results for the PM1 plate, which was incubated in absence of methane, were subtracted again from the values of the PM1 plate incubated in the presence of methane. Subsequently, key findings were graphically represented in plots processed in Excel (Version 2311).

4. Results

4.1. Enrichment of NOB

4.1.1. Different media for NOB

Through amplicon sequencing the presence of *Candidatus* Nitrotoga-like nitrite oxidizers had already been detected in the peat soil. Two different media were employed to isolate these NOB from the peat soil samples collected in the Šumava National Park. First, a nutrient-limited medium was prepared. To create an environment more closely resembling the fen site, a second medium was prepared by mixing autoclaved peat water with a mineral salt medium. As NOB tend to grow slowly, no colour change on the nitrite/nitrate test stripes, which would have indicated the formation of nitrate, was visible for several weeks. On the 3rd of March, after more than 3 months, the potential presence of nitrate was detected within the 24-well plate containing the enrichments of the second medium. In the other enrichment, no sign of NOB activity was detected during the entire project.

4.1.2. Enriching NOB in medium supplemented with peat water

Despite precautions, the evaporation of medium could not be avoided, leading to five wells of the culturing plate drying out. As shown in Figure 5 all except one of the enrichments from the experimental site D had dried out after 17 weeks. The same occurred in one of the lower layer samples of B and C.

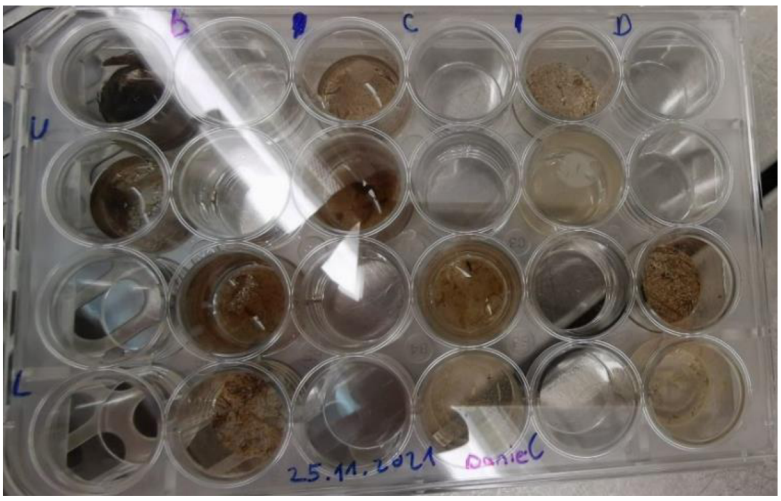

Figure 5: 24 well-plate containing NOB enrichments; every two columns contain material from the same soil core, while the first two rows contain peat samples from the upper layers (0-10 cm) and the lower rows contain peat from the lower layers (10-30 cm).

After 17 weeks of incubation, the most significant nitrate production was observed in enrichments from the upper layer of fen core B. Furthermore, no nitrite was detected anymore in this enrichment. The enrichments of the deeper layer from core B also showed nitrate formation, though less intense than the upper layer. The not completely dried enrichments of the cores C and D also provided evidence for NOB with a faint colour change indicating nitrate production. Transfer of these cultures into a new 24-well plate, using the same medium, did not provide positive results in the following two months. Nevertheless, the original cultures continued to transform nitrite into nitrate.

4.1. Characteristics of growth conditions of methanotrophs

4.1.1. Optimal growth temperatures

After ten days of incubation, the most significant increase in cell density by the culture Wu1 was detected at temperatures of 19 and 22°C, as seen in Figure 6. Significantly lower OD600 values were detected within bottles incubated at lower (4.5 and 13°C) and higher (28°C) temperatures, which was confirmed via Tukey's HSD post-hoc test (ANOVA, $p_{adj} < 0.001$). Thereby, significant differences in OD600 between the bottles incubated at 19 and 22°C to the bottles incubated at 4.5, 13, and 28°C were revealed. At the lowest temperature tested (4.5°C), there was no observable increase in OD600 at all during the ten days of incubation.

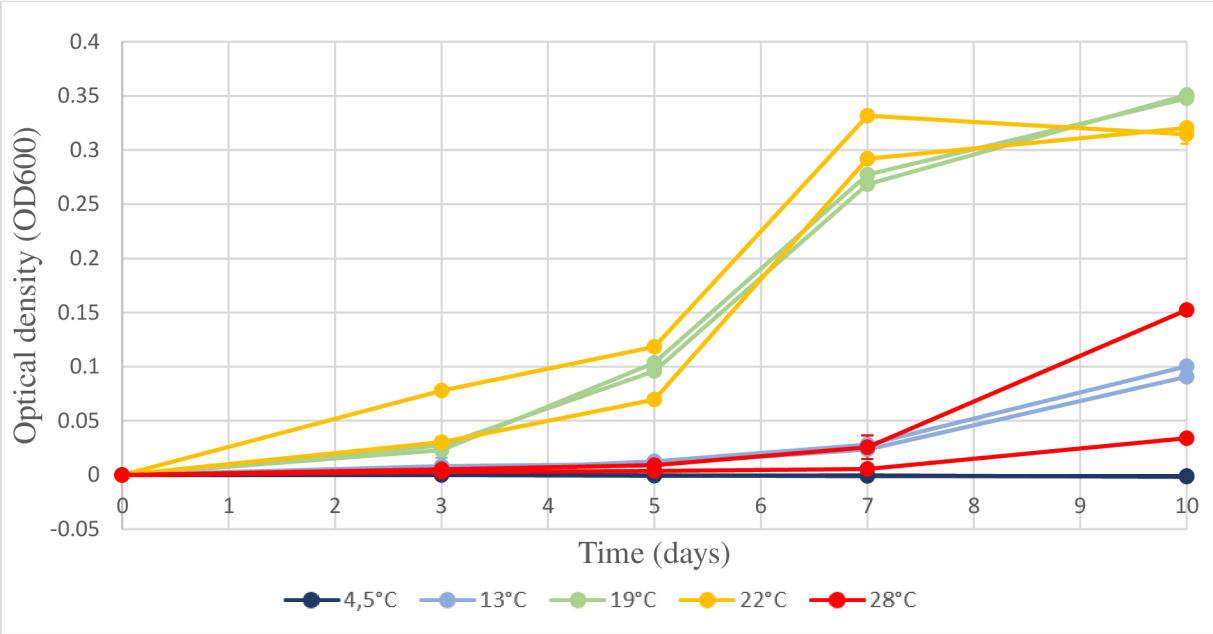

Figure 6: Optical density measurements of isolate Wu1 (a *Methylobacter luteus*-like methanotroph) incubated at different temperatures. Each dot represents the average optical density of the three measurements of one bottle. Error bars represent standard deviation. In certain cases, due to small values, the dots on the graph might cover the error bars.

During the ten-day incubation, the most notable growth enhancement in isolate Wu6 was observed at temperatures of 19 and 28°C (Figure 7). The comparison between the two bottles maintained at 22°C revealed remarkable differences in OD600 values. One of the two treatment duplicates reached values similar to the isolates incubated at 19 and 28°C whereas the second bottle showed a relatively low increase of OD600 and remained stagnant after the third day. This suggests that an error occurred during the incubation process of the second bottle. For this reason, we conducted a one-way ANOVA for the two bottles at 22°C independently (ANOVA, $p_{adj} < 0.001$). A Tukey's HSD post-hoc test showed that the bottles maintained at 19, 28, and one at 22°C differed not significantly in OD600, indicating similar OD600. In addition, by comparing the OD600 values after already three days of incubation the highest one was detected at 28°C (ANOVA, $p_{adj.} < 0.001$), nearly reaching similar amounts as after ten days of incubation.

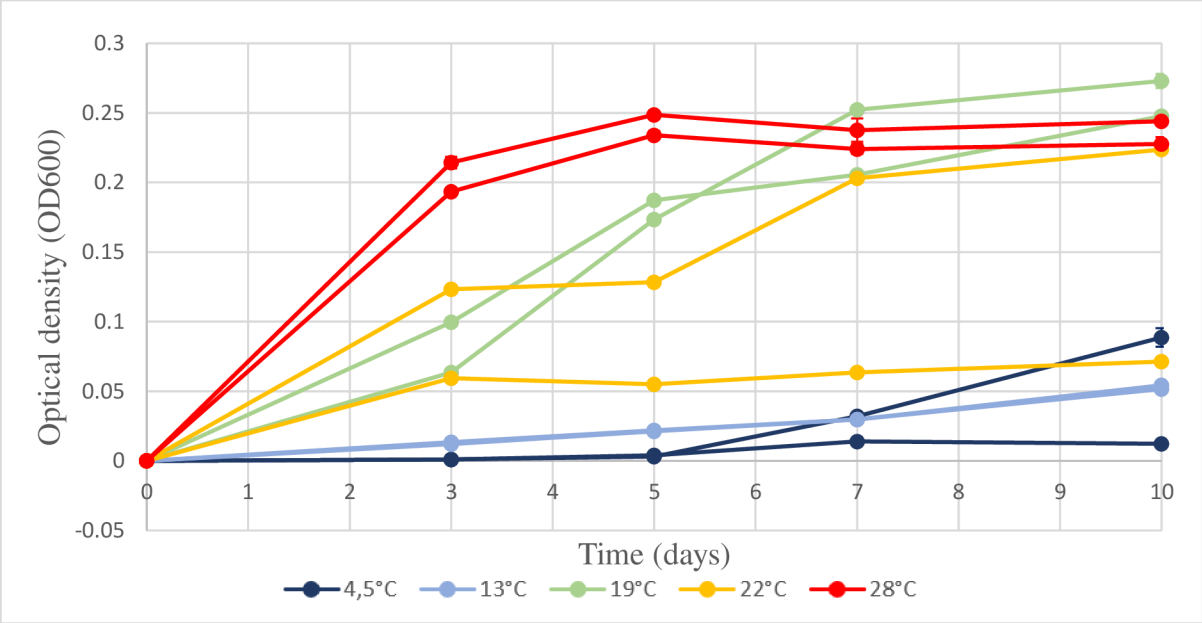

Figure 7: Optical density measurements of isolate Wu6 (a *Methylomicrobium lacus*-like methanotroph) incubated at different temperatures. Each dot represents the average optical density of the three measurements of one bottle. Error bars represent standard deviation. In certain cases, due to small values, the dots on the graph might cover the error bars.

Throughout the ten-day experiment, Wu8 reached similar cell densities at 13, 19, 22°C and in one of the treatment duplicates at 28°C (Figure 8). By comparing the two bottles incubated at 28°C, significant differences in OD600 values became observable, indicating a potential error in the incubation process in one of these bottles. Therefore, a one-way ANOVA was conducted separately for each of the two bottles at 28°C (ANOVA, $p_{adj} < 0.001$). A followed Tukey's HSD test for multiple comparisons detected no significant difference in OD600 at 19, 22°C, and in one of the bottles at 28°C. Furthermore, this test resulted in no significant difference in OD600 between the other treatment duplicate incubated at 28°C and the two bottles maintained at 4.5°C. Thereby, generally no increase was shown.

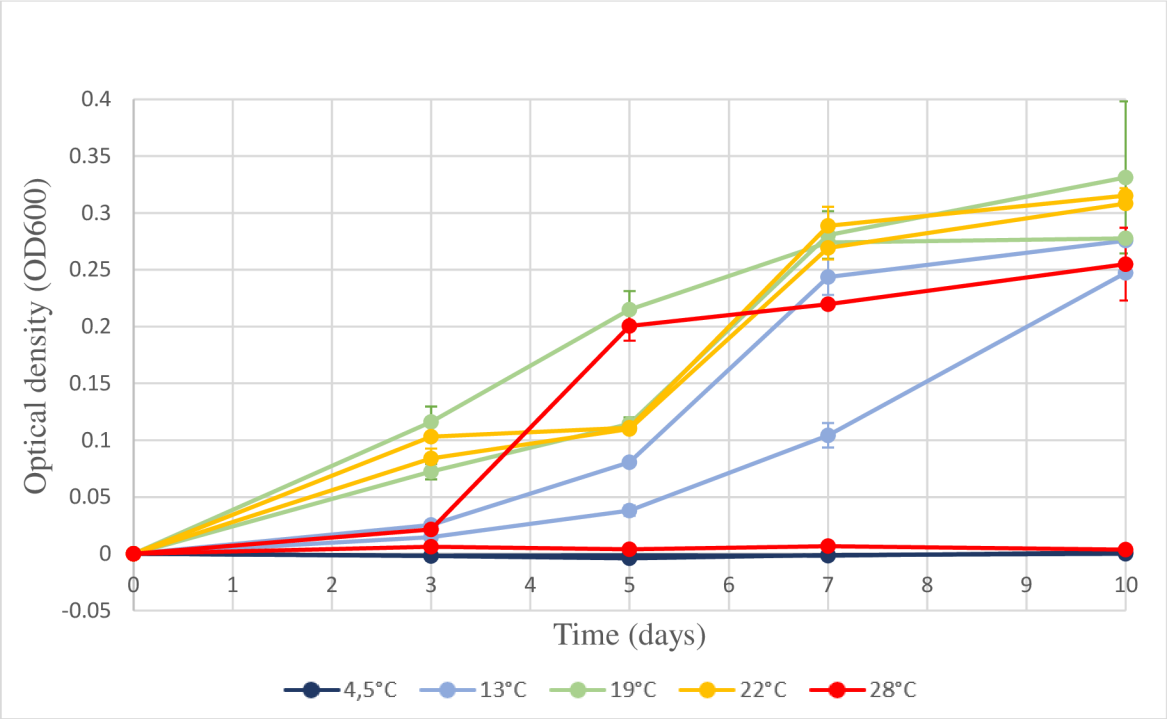

Figure 8: Optical density measurements of isolate Wu8 (a *Methylobacter tundripaludum*-like methanotroph) incubated at different temperatures. Each dot represents the average optical density of the three measurements of one bottle. Error bars represent standard deviation. In certain cases, due to small values, the dots on the graph might cover the error bars.

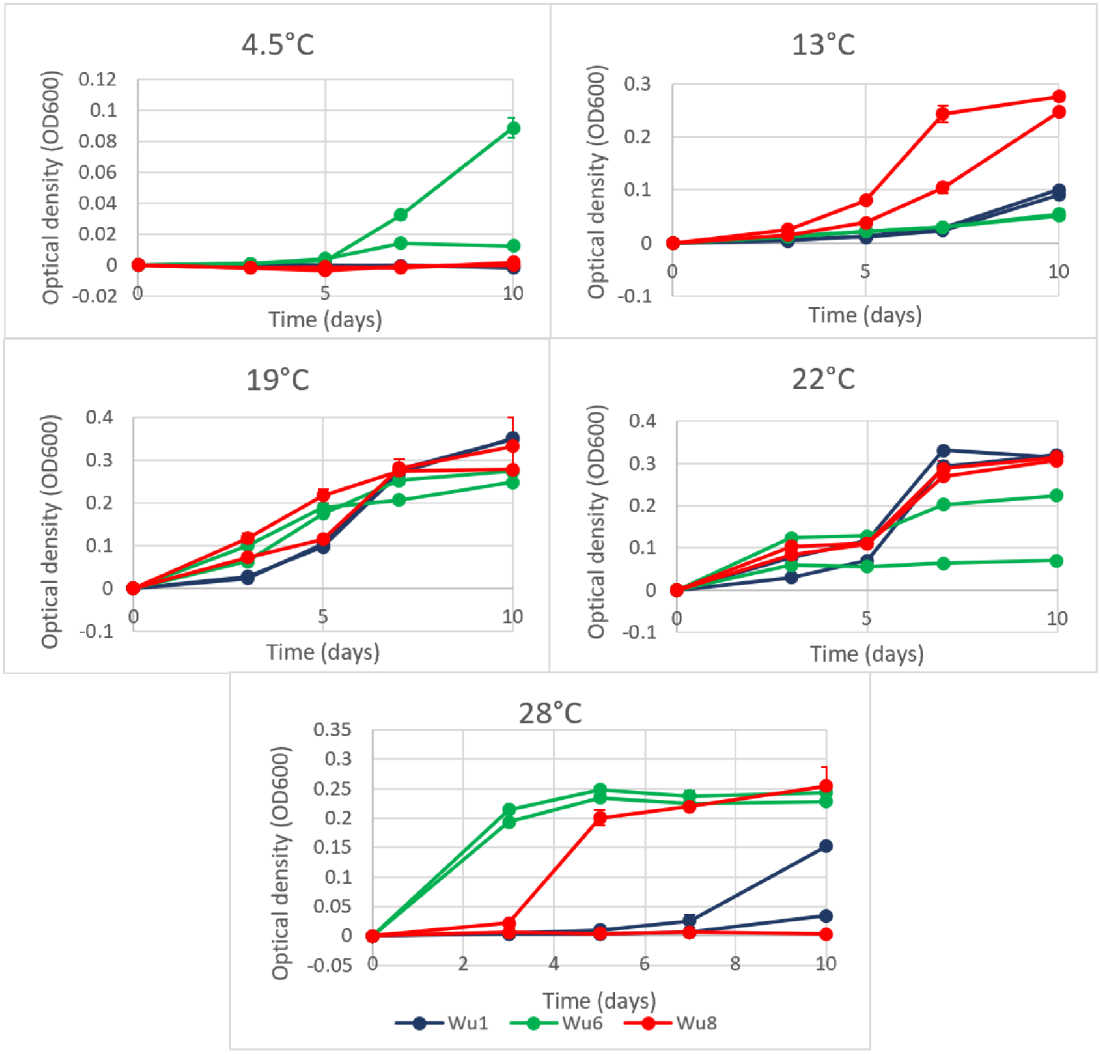

Figure 9: Optical density measurements of methanotroph isolates Wu1, Wu6, and Wu8 incubated at the same temperatures. Each point is a mean of the three measurements of on bottle. Error bars represent standard deviations. In certain cases, due to small values, the dots on the graph might cover the error bars.

We observed significant differences in growth for Wu1, Wu6, and Wu8 at many of the tested temperatures (Table 9). At 4.5°C only Wu6 showed a significant increase in OD600 (Figure 9). When the incubation temperature was elevated to 13°C, Wu8 accelerated its growth, attaining significantly higher OD600 levels than the other two strains after ten days. At 19°C, there was no significant difference between the isolates, while at 22°C, Wu6 grew significantly less than the two other strains. At 28°C, the Wu6 strain reached a plateau after only three days, much earlier than the other two strains. Notably, Wu1 grew significantly less than Wu6 at this temperature.

Table 9: The statistical significance (Tukey's HSD test) of the different optical densities from methanotroph isolates Wu1, Wu6, and Wu8 attained after ten days of incubation at the same temperatures.

	4.5°C	13°C	19°C	22°C	28°C
Wu1-Wu6	0.006	< 0.001	0.002	< 0.001	0.04
Wu1-Wu8	**0.99**	< 0.001	**0.11**	**0.98**	**0.77**
Wu6-Wu8	0.008	< 0.001	**0.11**	< 0.001	**0.13**

4.1.2. Capability of Wu6 to grow by using different carbon sources

From all the carbon sources on the Biolog Plate PM1, the isolate Wu6 grew best in the presence of sucrose, reaching an absorbance of 0.327 (Figure 10). The second best growth was observed for the amino acid — L-glutamic acid (absorbance value > 0.300). Growth in the presence of other carbon sources was considerably less, for example with D-cellobiose reaching only half the absorbance of L-glutamic acid. Moreover, the wells containing monosaccharides L-arabinose and D-galactose only showed very little growth with an absorbance around 0.100. On the other hand, the presence of 2-amino-ethanol yielded the lowest growth, which resulted from the additional presence of methane, lowering the observed growth.

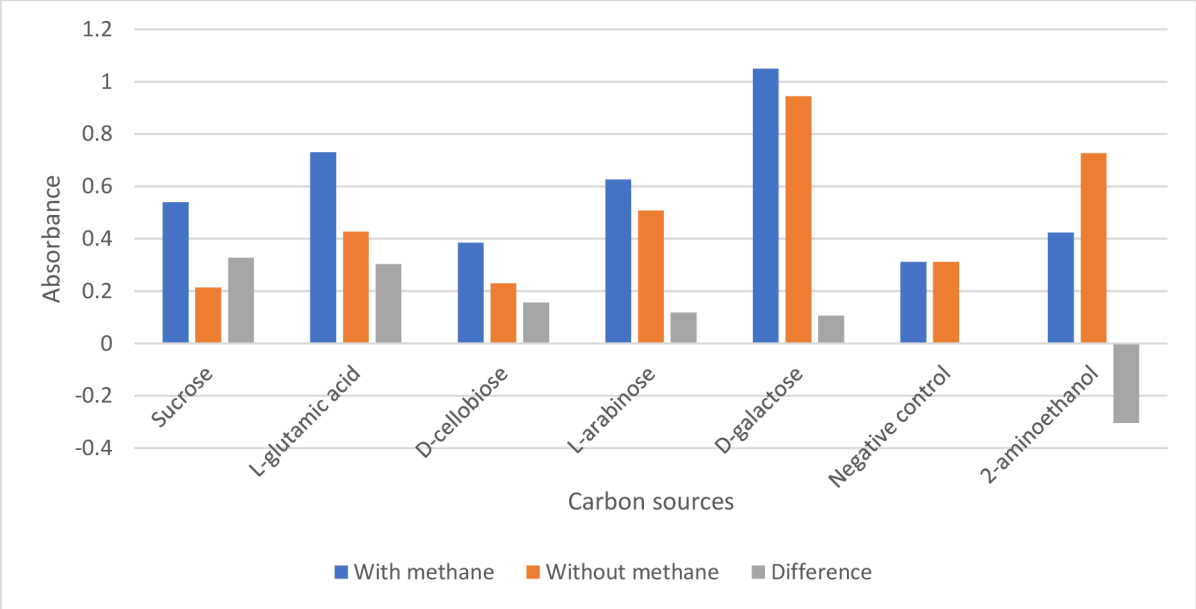

Figure 10: Absorbance values from incubations with the tested carbon substrates leading to the highest growth response of Wu6 incubated in the presence and absence of methane. For all substrates see Appendix Table 10. The measurements were not replicated.

4.1.3. Growth of Wu6 using different nitrogen sources

The nitrogen source, which led to the best growth over the seven-day incubation was the dipeptide Ala-Glu (alanine-glutamate). Additionally, as depicted in Figure 11, the presence of the peptides Ala-Gln (alanine-glutamine), Gly-Gln (glycine-glutamine), L-isoleucine, Ala-Leu (alanine-leucine), and Ala-Asp (alanine-aspartic acid) resulted in similar growth with absorbance values of over 0.300. This was notably higher compared to the negative control, where the increase in absorbance was only a tenth. The incubation with L-serine resulted in an increase of absorbance of about 0.102. However, with the addition of D-serine and nitrite, a lower absorbance was measured than in the negative control. No increase or even a decrease in absorbance was detected in the presence of D,L-α-amino-N-butyric acid, hydroxylamine, and D,L-α-amino-caprylic acid.

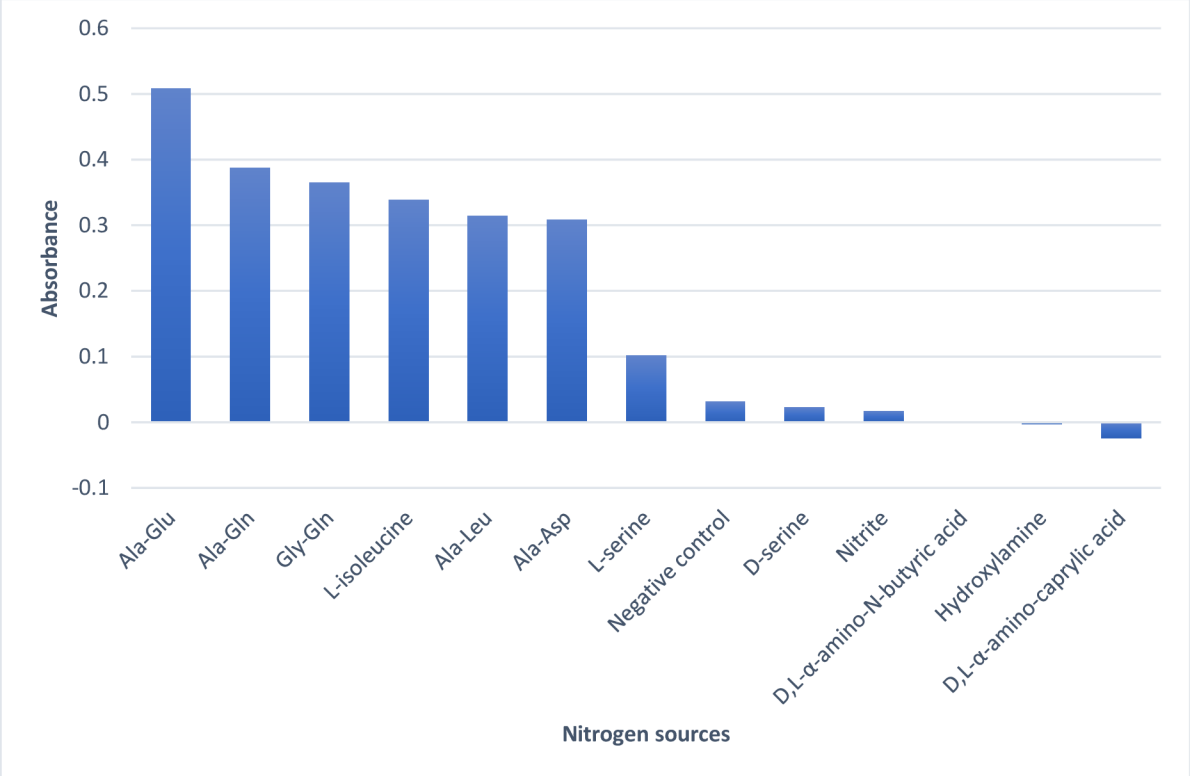

Figure 11: Absorbance values from incubation with nitrogen sources that resulted in changes compared to a control after seven days of incubation. For all substrates see Appendix Table 11. The measurements were not replicated. Abbreviations: Ala-Glu (alanine-glutamate), Ala-Gln (alanine-glutamine), Gly-Gln (glycine-glutamine), Ala-Leu (alanine-leucine), Ala-Asp (alanine-aspartic acid)

4.1.4. Growth of Wu6 in the presence of different osmolytes

The strongest increase in absorbance of Wu6, measured on Biolog Plate PM9, was observed within the well containing 1% sodium lactate (Figure 12). However, when the concentration of sodium lactate was raised to 2%, no increase in absorbance was detected after seven days of incubation. Moreover, by increasing the sodium chloride (NaCl) concentration from 2% to 6%, the absorbance dropped from 0.587 to 0.019. However, when choline was added to 6% NaCl, the absorbance was raised to 0.154. By increasing the amount of potassium chloride (KCl) or urea by a few percent the absorbance was reduced to 50%. A similar effect was detected by increasing the amount of sodium nitrate ($NaNO_3$) or sodium nitrite ($NaNO_2$). In contrast, by raising the concentration of sodium sulfate (Na_2SO_4) from 2% to 3%, an increase in the absorbance was observed. Nevertheless, further addition decreased the absorbance.

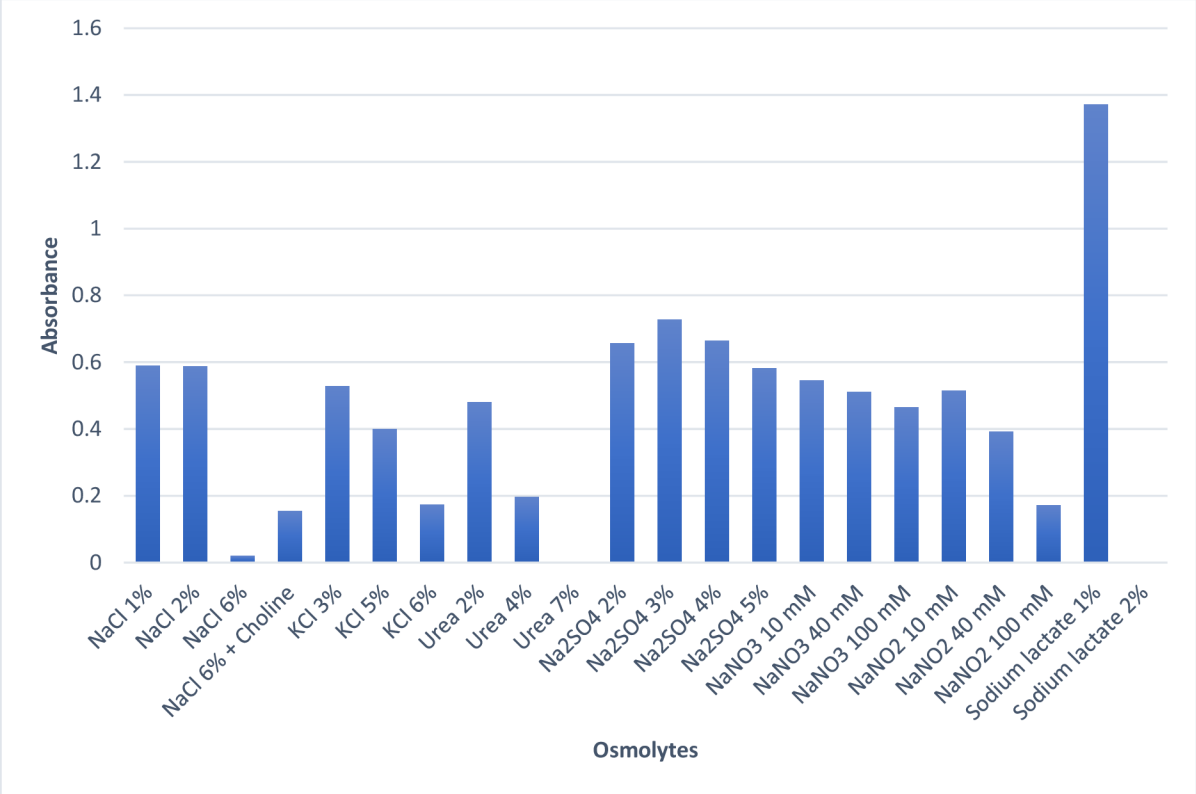

Figure 12: Absorbance values of the Wu6 culture after seven days of incubation with different osmolytes. For all substrates see Appendix Table 12. The measurements were not replicated. Abbreviations: NaCl (sodium chloride), KCl (potassium chloride), Na_2SO_4 (sodium sulfate), $NaNO_3$ (sodium nitrate), $NaNO_2$ (sodium nitrite)

4.1.5. Growth of Wu6 at different pH levels in the presence of diverse amino acids

As presented in Figure 13, the methanotroph Wu6 culture showed an increase in absorbance only in the pH range of 5–10, with the highest values at 5-8. The very strong inhibiting effect of pH 4.5 disappeared with the addition of D-lysine or L-norleucine, which caused an increase in absorbance not observed for any other pH without additions. Parallel, to the acidic conditions, the inhibition of pH 9.5 was alleviated by adding the amino acids hydroxy-L-proline, L-proline, or anthranilic acid. Here the absorbance reached the highest values of 1.450 to 1.525.

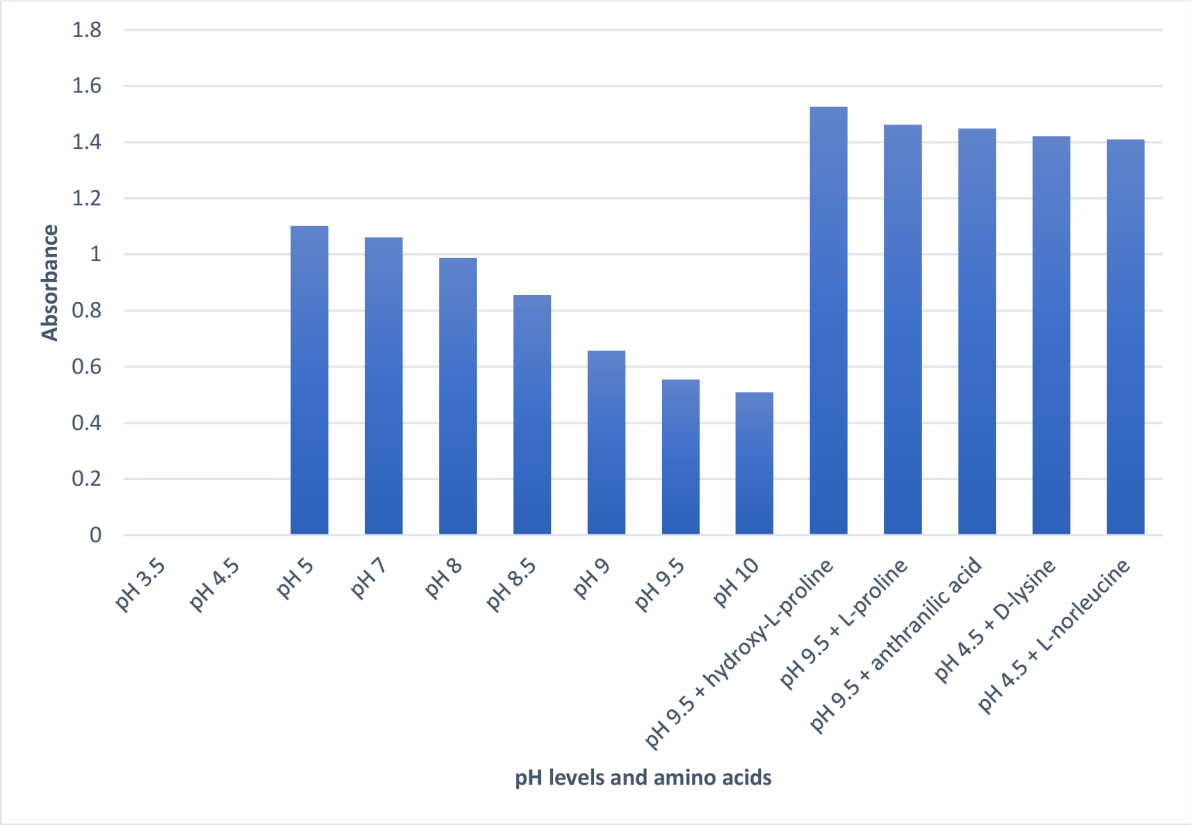

Figure 13: Absorbance values of the Wu6 culture after seven days of incubation at different pH levels in the presence of diverse amino. For all substrates see Appendix Table 13. The measurements were not replicated.

5. Discussion

5.1. Enrichment of NOB

Through the increased use of fertilizer, more nitrogen in the form of ammonium or urea is released into the environment which in turn enhances the level of nitrite in freshwater habitats and peatlands. NOB living in those environments are able to oxidise the present nitrite into nitrate, thereby playing an important role in the cycling of nitrogen. The isolation of NOB adapted to low temperatures or low pH prevailing in peatlands can further enhance the knowledge about the diversity of this microbial guild. Subsequent study of their physiology and metabolic pathways could lead to discoveries of adaptive mechanisms. (Camargo & Alonso, 2006; Ishii et al., 2020; Spieck et al., 2021). Therefore, one purpose of this project was to enrich NOB from acidic peat soil, where they had been detected previously by sequencing of the 16S rRNA gene.

For the enrichment of *Nitrotoga*-like bacteria from peat two different media were employed. However, after several months of incubation, nitrite-oxidising activity was only detected within the medium that contained autoclaved peat water, not in the artificial medium. This might be explained by the abundance of trace elements and vitamins present in peat water, which depend on the composition of nearby rocks and sediments (Shotyk, 1988). To potentially increase the already slow growth rate of *Ca.* Nitrotoga certain cofactors and metabolites such as ammonium chloride (NH_4Cl) or pyruvate could be essential (Ishii et al., 2020; Watson et al., 1986; Wegen et al., 2019). Consequently, the addition of peat water might contribute to the enhanced growth of the naturally occurring NOB. However, further studies need to be conducted to discover the exact composition of the peat water.

Furthermore, the absence of growth in the artificial medium contrasts with the results reported by Lantz et al. (2021), who enriched other *Ca.* Nitrotoga bacteria by using the same fully artificial medium. Thereby, growth was observed at a pH of 5.0-8.0. Additional studies on the physiology of *Ca.* Nitrotoga bacteria further demonstrate that these bacteria exhibit optimal culturing conditions at near-neutral pH (Alawi et al., 2007; Ishii et al., 2017; Lantz et al., 2021). This suggests that the pH level of 4.6 used in our enrichment was too low for cultivation in an artificial medium, despite their original habitat being acid.

Moreover, the upper layer of core B contained more nitrate than other layers or sites (Vojtech Tlaskal, personal communication). This fits well to the observed nitrite-oxidising activity in enrichments that used peat soil from this layer and site. The responsible NOB may have been members of the genera *Nitrobacter* and *Nitrotoga*, as their presence has been confirmed at the site by an earlier 16S rRNA survey (Daňková, 2022). However, further cultivation of the enrichments showed no sign of activity for months. As mentioned above, the growth of NOB is rather slow. To achieve detectable results, a longer incubation time may be needed, but this would have exceeded the time frame for this bachelor thesis (Ishii et al., 2017). Therefore, it might be possible for further studies to continue isolating these NOB by multiple enrichment steps.

5.2. Optimal growth conditions of three methanotroph strains

Methanotrophs have been isolated from a plethora of different habitats that encompass combinations of diverse physicochemical conditions (Dedysh & Dunfield, 2014; Meruvu et al., 2020; Whittenbury, Phillips, et al., 1970). As a group they span over several phyla, and each species is adapted to a unique set of conditions, such as temperature. For instance, some *Methylobacter* species were found thriving in the polar or subpolar environments, such as *Methylobacter* sp. S3L5C showing optimal growth temperatures in polar ponds at 3–12°C (Khanongnuch et al., 2022). On the other side of the temperature spectrum are species that thrive in hot springs, with some growing at temperatures as high as 72°C, like the *Methylothermus* strain HB (Bodrossy et al., 1999).

The three methanotrophic species examined in this study, although coming from the same temperate pond in central Europe, exhibited different thermal preferences. By comparing the isolates at each cultivation temperature, distinct growth behaviours among them became evident. The strain Wu1 always showed a lower value in the optical density than other isolates except when grown at 19 and 22°C. On the other hand, the methanotroph of Wu6 already increased its turbidity at 4.5°C, while its optimal growth condition was observed at 28°C.

These differences suggest a better adaption of the strain Wu6 towards various temperature changes. Moreover, the growth at higher temperatures implies that Wu6 could be more heat resistant than the other cultures. However, the methane concentration additionally influences the growth speed at certain temperatures (Tveit et al., 2023). High methane concentration can either increase growth at higher temperatures observed in *Methylobacter tundripaludum* SV96 or facilitate growth at lower temperatures like in *Methylobacter sp. G7* (Tveit et al., 2023).

This indicates that the comparison of growth between different species also depends on the methane concentration. Additionally, as the optical density values depend on cellular characteristics, final conclusions about the comparison between the three different species cannot be made.

The isolate Wu1 grew optimally at 19–22°C, which is less than the optimal temperature of 30°C of the closely related methanotroph *Methylobacter luteus* (Collins et al., 2017; Romanovskaia et al., 1978). *Methylobacter luteus* can be classified as a mesophilic organism, whereas the *Methylobacter luteus*-like isolate tested here may rather be a psychrotolerant bacterium. These differences in temperature preference between two so closely related organisms could indicate that they are different ecotypes occupying distinct ecological niches (Madigan et al., 2021).

The comparison of different growth temperatures for the isolate Wu6, which is closely related to *Methylomicrobium lacus*, showed an increase in growth up to the highest tested temperature of 28°C. Therefore, it may be that the optimal temperature for growth of this strain lies beyond 28°C, and additional tests at even higher temperatures need to be conducted. These findings align exactly with the study of *Methylomicrobium lacus* (28–30°C) conducted by Kalyuzhnaya (2005). Furthermore, after ten days of incubation, there was no significant difference in growth between 19, 28°C and one of the 22°C treatments duplicates. The other one of the 22°C treatments exhibited significantly slower growth, which could be due to unsuccessful inoculation.

The third isolate Wu8, a *Methylobacter tundripaludum*-like strain, grew fastest at 13°C to 28°C. Due to the limited number of replicates and some large variation among them, the optimal growth temperature could not be identified within this range. These results agree with the findings of Wartiainen (2006), who identified the optimal growth temperature to be 23°C, even though those methanotrophs were isolated from an Arctic wetland soil. One of the bottles incubated at 28°C, exhibited no growth at all, which most probably was a result of unsuccessful inoculation.

In addition to the optimal growth temperature, the isolate Wu6 was examined for the influence of various carbon and nitrogen substrates, of pH, and of osmolytes on its growth to improve the knowledge of this strain.

When comparing different pH levels, it became apparent that this strain exhibits a preference for growth within a pH range of 5–10, with the highest growth in absence of amino acids at a pH of 5–8. This finding supports previous research conducted by Kalyuhnaya (2016; 2005), which evidenced that slightly acidic environments favour the growth of the species *Methylomicrobium lacus*. The medium, that was used for isolating and growing Wu6, had a pH of 6.8 (J. Bowman, 2006; Whittenbury, Davies, et al., 1970). However, the pH of porewater from the Naděje sediments oscillated around 7.5 (Wutkowska et al., unpublished), and it most likely undergoes diurnal and seasonal fluctuations (Gobler et al., 2017; Søndergaard, 1990). Work in this thesis shows that the medium with much higher pH and addition of hydroxy-L-proline, L-proline and anthranilic acid, would significantly benefit the growth, therefore some of the next steps in designing an optimal medium should test the addition of these compounds.

Additionally, the experiments with osmolytes showed that high salinity prevents the growth of this methanotroph, which indicates that this strain would not grow in marine environments. The porewater in hypertrophic environments most likely contains a mixture of osmolytes that could possibly allow for better growth of Wu6 in their native environment. However, further studies would need to be conducted to identify the concentration of the specific osmolytes within the porewater that could further promote the growth of Wu6.

The highest growth within diverse nitrogen sources was detected in the presence of certain dipeptides such as alanine-glutamate, alanine-glutamine, or glycine-glutamine. By comparing these results to the negative control, in which the methanotroph got its nitrogen from nitrate within the medium, a significant increase could be observed. This can be explained by the utilization of dipeptides via special carriers or extracellular hydrolysis in certain bacteria, which promotes their growth (Fernández et al., 2004; Law, 1977). Consequently, for the optimization of the growth rate, the addition of such dipeptides would be a viable approach in the development of a new medium. Furthermore, nitrite was shown to inhibit the growth of this strain, which was anticipated given that nitrite is toxic to various lifeforms at already low concentrations (Camargo & Alonso, 2006).

Furthermore, the results of this study indicate that sucrose and L-glutamic acid enabled the highest metabolic activity of Wu6 when compared with other carbon sources. These findings are contrary to the expectations that methanotrophs solely use methane as their main energy source. Hence, the culture was tested for other bacteria after the entire project (Magdalena Wutkowska, personal communication), revealing a contamination by *Pseudomonas sp*. As these bacteria are capable of using 2-aminoethanol as a carbon and nitrogen source, their

presence could account for observed metabolic activity in the presence of organics (Krysenko & Wohlleben, 2022). This contamination might also be the reason for the increased growth observed upon the addition of sodium lactate within the plate containing different osmolytes, as these bacteria have the ability to utilize lactate (Gao et al., 2012).

There are strong concerns towards rapid changes to the habitats of methanotrophs caused by climate change or other man-facilitated phenomena, such as eutrophication. These factors can cause substantial physicochemical alterations in multiple parallel ways, especially in and in proximity to agricultural soils, which in turn leach excess nitrogen to surface water and freshwater bodies making them hostile for methanotrophs. In this work, it has been clearly shown that some of the isolated methanotrophs are very sensitive to rising temperatures, and probably to other environmental parameters such as an increase in salinity.

The primary objective of this part of the thesis was to find the optimal growth conditions for the three strains of methanotrophs isolated from the hypertrophic fishpond Naděje. Through their capability to utilize methane as their energy source, methanotrophs play a crucial role in combating climate change. Identification of the optimal conditions for the growth of these bacteria benefits further cultivation and research on these methanotrophs.

6. Conclusion

One of the main objectives of the current study was to enrich *Nitrotoga*-like bacteria from acidic peat soil. After approximately 17 weeks, activity of NOB was detected within the enrichment containing autoclaved peat water mixed with a mineral salt medium. The major limitation of the study was that NOB tend to grow very slowly, which was probably the reason why additional isolation provided no results. Ultimately, it would be advantageous for further research to create a medium with known composition in which these bacteria multiply, instead of an undefined medium containing peat water. The cultivation of NOB could then be easily repeated, enabling further experiments focused on their physiology.

Additionally, an experiment was conducted on the three strains of methanotrophs obtained from the hypertrophic fishpond Naděje. The goal was to determine their optimal growth condition. This study found that the optimum incubation temperature for isolate Wu1 (a *Methylobacter luteus*-like methanotroph) lies between 19–22°C, while Wu8 (a *Methylobacter tundripaludum*-like methanotroph) exhibited the highest increase in growth at temperatures ranging from 13–28°C. The isolate Wu6 (a *Methylomicrobium lacus*-like methanotroph) grew fastest when incubated at 28°C. Further studies could explore whether this methanotroph could also grow at even higher temperatures. Additionally, the growth of Wu6 was further tested in the presence of different carbon sources, nitrogen sources, osmolytes, and at different pH levels in the presence of diverse amino acids. The isolate showed high metabolic activity at a pH from 5–8. Contrary to the expectations, one of the highest metabolic activities was observed in the presence of sucrose. Additional tests need to be carried out to confirm these results, as there is the possibility that the plates were contaminated.

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Appendix

Table 10: Carbon substrates of PM1, contains substrates all substrates after 7 days of incubation.

Location	Substrate	Absorbance	Location	Substrate	Absorbance
D11	Sucrose	0.327	E4	D-Fructose-6-Phosphate	0.002
B12	L-Glutamic Acid	0.303	G9	Mono Methyl Succinate	0.001
F11	D-Cellobiose	0.156	A1	Negative Control	0
A2	L-Arabinose	0.118	B2	D-Sorbitol	0
A6	D-Galactose	0.106	D10	Lactulose	0
F10	Glyoxylic Acid	0.072	D12	Uridine	0
A9	D-Alanine	0.071	E8	ß-Methyl-DGlucosid	-0.001
H7	Glucuronamide	0.07	G10	Methyl Pyruvate	-0.001
G6	L-Alanyl-Glycine	0.069	B10	Formic Acid	-0.002
G11	D-Malic Acid	0.068	D2	D-Aspartic Acid	-0.003
G1	Glycyl-LGlutamic Acid	0.063	D9	α-D-Lactose	-0.003
H11	Phenylethylamin	0.062	H8	Pyruvic Acid	-0.005
A4	D-Saccharic Acid	0.06	A10	D-Trehalose	-0.008
A8	L-Proline	0.058	F12	Inosine	-0.008
C12	Thymidine	0.054	A11	D-Mannose	-0.01
B1	D-Serine	0.052	H9	L-Galactonic Acid-γ-Lactone	-0.01
D1	L-Asparagine	0.05	C10	Maltose	-0.011
H3	m-Hydroxy Phenyl Acetic Acid	0.039	F9	Glycolic Acid	-0.011
E9	Adonitol	0.038	C3	D,L-Malic Acid	-0.012
H10	D-Galacturonic Acid	0.034	D3	D-Glucosaminic Acid	-0.013
C8	Acetic Acid	0.033	F5	Fumaric Acid	-0.014
H6	L-Lyxose	0.033	C4	D-Ribose	-0.015
F4	D-Threonine	0.028	E10	Maltotriose	-0.016
H2	p-Hydroxy Phenyl Acetic Acid	0.024	E2	m-Tartaric Acid	-0.018
F2	Citric Acid	0.022	D8	α-Methyl-DGalactoside	-0.018
H1	Glycyl-L-Proline	0.022	F3	myo-Inositol	-0.022
C5	Tween 20	0.021	B11	D-Mannitol	-0.023
D5	Tween 40	0.021	G2	Tricarballylic Acid	-0.024
B6	D-Gluconic Acid	0.02	B8	D-Xylose	-0.028
E11	2-Deoxy Adenosine	0.02	D4	1,2-Propanediol	-0.029
G12	L-Malic Acid	0.02	F6	Bromo Succinic Acid	-0.031
D6	α-Keto-Glutaric Acid	0.018	C11	D-Melibiose	-0.032
A3	N-Acetyl-DGlucosamine	0.014	G4	L-Threonine	-0.032
F8	Mucic Acid	0.014	H5	D-Psicose	-0.032
A12	Dulcitol	0.013	E7	α-Hydroxy Butyric Acid	-0.034

C1	D-Glucose-6-Phosphate	0.013	G3	L-Serine	-0.037
E1	L-Glutamine	0.011	B3	Glycerol	-0.04
G8	N-Acetyl-ß-DMannosamine	0.01	E5	Tween 80	-0.041
C9	α-D-Glucose	0.01	E12	Adenosine	-0.041
F1	Glycyl-L-Aspartic Acid	0.009	D7	α-Keto-Butyric Acid	-0.044
H4	Tyramine	0.009	A7	L-Aspartic Acid	-0.063
B5	D-Glucuronic Acid	0.008	F7	Propionic Acid	-0.063
E3	D-Glucose-1-Phosphate	0.008	E6	α-Hydroxy Glutaric Acid-γLactone	-0.072
A5	Succinic Acid	0.007	G7	Acetoacetic Acid	-0.077
B4	L-Fucose	0.007	C6	L-Rhamnose	-0.15
B9	L-Lactic Acid	0.004	B7	D,L-α-GlycerolPhosphate	-0.155
C2	C2 D-Galactonic Acid-γ-Lactone	0.003	C7	D-Fructose	-0.242
G5	L-Alanine	0.003	H12	2-Aminoethanol	-0.304

Table 11: Nitrogen sources of PM3B, contains all substrates of the plate after 7 days of incubation with their absorbance.

Location	Substrate	Absorbance	Location	Substrate	Absorb-ance
H3	Ala-Glu	0.508	G5	Allantoin	0.053
H2	Ala-Gln	0.387	C10	L-Citrulline	0.05
H9	Gly-Gln	0.365	E10	D-Mannosamine	0.05
B4	L-Isoleucine	0.339	F2	Adenine	0.048
H6	Ala-Leu	0.314	B12	L-Tryptophan	0.04
H1	Ala-Asp	0.308	E6	Glucuronamide	0.037
B5	L-Leucine	0.287	B11	L-Threonine	0.035
B9	L-Proline	0.27	F7	Guanosine	0.033
G11	δ-Amino-N-Va-leric Acid	0.267	A11	L-Cysteine	0.032
D6	N-Amylamine	0.265	E9	D-Galactosamine	0.032
G8	γ-Amino-N-Bu-tyric Acid	0.26	D2	N-Phthaloyl-L-Glutamic Acid	0.031
D12	Agmatine	0.25	A1	Negative Control	0.031
E8	D-Glucosamine	0.246	E12	N-Acetyl-D-Galactosa-mine	0.03
C7	D-Lysine	0.233	A5	Urea	0.029
E2	ß-Phenylethyl-amine	0.232	A6	Biuret	0.029
A12	L-Glutamic Acid	0.231	H11	Gly-Met	0.025
B1	L-Glutamine	0.231	C8	D-Serine	0.023
A8	L-Arginine	0.225	F12	Inosine	0.022
B8	L-Phenylalanine	0.221	A4	Nitrate	0.022
C2	L-Valine	0.217	G1	Xanthine	0.021

D7	N-Butylamine	0.217	F11	Uridine	0.02
D3	L-Pyroglutamic Acid	0.216	F5	Cytosine	0.018
D11	Putrescine	0.215	A2	Ammonia	0.017
C6	D-Glutamic Acid	0.199	A3	Nitrite	0.017
H10	Gly-Glu	0.199	E11	N-Acetyl-D-Glucosamine	0.017
H4	Ala-Gly	0.185	F3	Adenosine	0.017
F8	Thymine	0.181	C5	D-Aspartic Acid	0.016
H5	Ala-His	0.174	D1	N-Acetyl-L-Glutamic Acid	0.016
H8	Gly-Asn	0.166	F1	N-Acetyl-D-Mannosamine	0.016
A7	L-Alanine	0.165	E7	D,L-Lactamide	0.015
C3	D-Alanine	0.161	B7	L-Methionine	0.015
A9	L-Asparagine	0.158	D5	Methylamine	0.014
G12	α-Amino-N-Va-leric Acid	0.151	F4	Cytidine	0.014
A10	L-Aspartic Acid	0.15	G2	Xanthosine	0.013
C1	L-Tyrosine	0.149	G9	ε-Amino-N-Caproic Acid	0.013
C12	L-Ornithine	0.148	D8	Ethylamine	0.012
H7	Ala-Thr	0.145	E3	Tyramine	0.012
E1	Histamine	0.138	E4	Acetamide	0.012
G6	Parabanic Acid	0.136	E5	Formamide	0.012
B6	L-Lysine	0.135	C4	D-Asparagine	0.011
D9	Ethanolamine	0.123	C11	L-Homoserine	0.011
B3	L-Histidine	0.103	G4	Alloxan	0.011
B10	L-Serine	0.102	F9	Thymidine	0.009
F10	Uracil	0.071	C9	D-Valine	0.007
H12	Met-Ala	0.064	D10	Ethylenediamine	0.004
F6	Guanine	0.062	G7	D,L-α-Amino-N-Butyric Acid	0.001
G3	Uric Acid	0.056	D4	Hydroxylamine	-0.003
B2	Glycine	0.055	G10	D,L-α-Amino-Caprylic Acid	-0.024

Table 12: Osmolytes of PM9, contains all osmolytes used and their absorbance reached after 7 days of incubation.

Loca-tion	Substrate	Absorb-ance	Location	Substrate	Absorbance
F1	Sodium Lactate 1%	1.372	E10	Urea 5%	0.06
G5	Sodium Phosphate pH 5.2 20 mM	1.301	C11	NaCl 6% + Octopine	0.04
D6	Sodium sulfate 3%	0.728	B10	NaCl 6% + Creatine	0.029
D7	Sodium sulfate 4%	0.664	B6	NaCl 6% + MOPS	0.027
D5	Sodium sulfate 2%	0.657	B2	NaCl 6% + Betaine	0.026
E1	Sodium formate 1%	0.627	B11	NaCl 6% + Creatinine	0.026
E2	Sodium formate 2%	0.601	C12	NaCl 6% + Trigonelline	0.022

G3	Sodium Phosphate pH 7 100 mM	0.6	B3	NaCl 6% + N-N Dimethyl glycine	0.021
A1	NaCl 1%	0.589	C6	NaCl 6% + Glutathione	0.021
A2	NaCl 2%	0.587	B7	NaCl 6% + Ectoine	0.02
D8	Sodium sulfate 5%	0.581	C8	NaCl 6% + Trehalose	0.02
A3	NaCl 3%	0.572	A7	NaCl 6%	0.019
G1	Sodium Phosphate pH 7 20 mM	0.546	B1	NaCl 6%	0.019
H1	Sodium Nitrate 10 mM	0.546	C9	NaCl 6% + Trimethylamine-N-oxide	0.018
G2	Sodium Phosphate pH 7 50 mM	0.543	C1	NaCl 6% + KCl	0.017
D1	Potassium chloride 3%	0.528	C10	NaCl 6% + Trimethylamine	0.017
G4	Sodium Phosphate pH 7 200 mM	0.528	C2	NaCl 6% + L-proline	0.016
H7	Sodium Nitrite 10 mM	0.514	C7	NaCl 6% + Glycerol	0.014
H4	Sodium Nitrate 60 mM	0.512	A8	NaCl 6.5%	0.012
H3	Sodium Nitrate 40 mM	0.51	C4	NaCl 6% + ß-Glutamic acid	0.01
H2	Sodium Nitrate 20 mM	0.502	C5	NaCl 6% + γ-Amino-n-butyric acid	0.008
H8	Sodium Nitrite 20 mM	0.5	C3	NaCl 6% + N-Acethyl L-glutamine	0.004
E7	Urea 2%	0.48	G6	Sodium Phosphate pH 5.2 50 mM	0.003
H5	Sodium Nitrate 80 mM	0.477	B5	NaCl 6% + Dimethyl sulphonyl propionate	0.002
A4	NaCl 4%	0.467	G7	Sodium Phosphate pH 5.2 100 mM	0.002
H6	Sodium Nitrate 100 mM	0.464	E3	Sodium formate 3%	0.001
G11	Ammonium sulfate pH 8 50 mM	0.452	E11	Urea 6%	0.001
G9	Ammonium sulfate pH 8 10 mM	0.445	G8	Sodium Phosphate pH 5.2 200 mM	0.001
D9	Ethylene glycol 5%	0.443	A10	NaCl 8%	0
E8	Urea 3%	0.423	A12	NaCl 10%	0
D10	Ethylene glycol 10%	0.422	E4	Sodium formate 4%	0
D3	Potassium chloride 5%	0.399	E5	Sodium formate 5%	0
H9	Sodium Nitrite 40 mM	0.391	E12	Urea 7%	0
D11	Ethylene glycol 15%	0.38	F2	Sodium Lactate 2%	0
D12	Ethylene glycol 20%	0.356	F3	Sodium Lactate 3%	0
D2	Potassium chloride 4%	0.356	F4	Sodium Lactate 4%	-0.001
G10	Ammonium sulfate pH 8 20 mM	0.353	F6	Sodium Lactate 6%	-0.001
H10	Sodium Nitrite 60 mM	0.313	F7	Sodium Lactate 7%	-0.001
A6	NaCl 5.5%	0.26	F8	Sodium Lactate 8%	-0.001
H11	Sodium Nitrite 80 mM	0.248	F10	Sodium Lactate 10%	-0.001
G12	Ammonium sulfate pH 8 100 mM	0.229	F12	Sodium Lactate 12%	-0.001
A5	NaCl 5%	0.202	E6	Sodium formate 6%	-0.001
E9	Urea 4%	0.196	F5	Sodium Lactate 5%	-0.001
D4	Potassium chloride 6%	0.173	F11	Sodium Lactate 11%	-0.001

H12	Sodium Nitrite 100 mM	0.172	F9	Sodium Lactate 9%	-0.002
B8	NaCl 6% + Choline	0.154	A11	NaCl 9%	-0.002
B9	NaCl 6% + Phosphoryl choline	0.138	A9	NaCl 7%	-0.017
B12	NaCl 6% + L-Carnitine	0.098	B4	NaCl 6% + Sarcosine	-0.024

Table 13: Different pH in the presence of diverse amino acids of PM10, displays the increase of absorbance from all wells of the plate reached after 7 days.

Location	Substrate	Absorb-ance	Location	Substrate	Absorb-ance
F9	pH 9.5 + Hydroxy-L-Pro-line	1.525	F12	pH 9.5 + L-Homoserine	0.698
F3	pH 9.5 + L-Proline	1.461	A10	pH 9	0.656
G1	pH 9.5 + Anthranilic Acid	1.447	G2	pH 9.5 + L-Norleucine	0.652
D7	pH 4.5 + D-Lysine	1.419	G3	pH 9.5 + L-Norvaline	0.614
D3	pH 4.5 + L-Norleucine	1.408	D8	pH 4.5 + 5-Hydroxy Ly-sine	0.562
E6	pH 9.5 + L-Glutamic Acid	1.384	A11	pH 9.5	0.554
D6	pH 4.5 + L-Cysteic Acid	1.348	A12	pH 10	0.509
F10	pH 9.5 + L-Ornithine	1.345	E8	pH 9.5 + Glycine	0.43
E7	pH 9.5 + L-Glutamine	1.308	F6	pH 9.5 + L-Tryptophan	0.36
E12	pH 9.5 + L-Lysine	1.29	F7	pH 9.5 + L-Tyrosine	0.331
F8	pH 9.5 + L-Valine	1.211	G5	pH 9.5 + Cadaverine	0.108
E10	pH 9.5 + L-Isoleucine	1.207	G6	pH 9.5 + Putrescine	0.095
E4	pH 9.5 + L-Asparagine	1.181	G9	pH 9.5 + Tyramine	0.061
H9	X-β-D-Galactosaminide	1.153	D1	pH 4.5 + Anthranilic Acid	0.038
H11	X-PO4	1.127	D9	pH 4.5 + 5-Hydroxy Tryptophan	0.031
E5	pH 9.5 + L-Aspartic Acid	1.119	E9	pH 9.5 + L-Histidine	0.027
A4	pH 5	1.101	B7	pH 4.5 + L-Glutamine	0.002
B6	pH 4.5 + L-Glutamic Acid	1.092	A3	pH 4.5	0.002
G7	pH 9.5 + Histamine	1.091	D11	pH 4.5 + Trimethyla-mine-N-oxide	0.002
F2	pH 9.5 + L-Phenylalanine	1.082	A1	pH 3.5	0.002
H10	X-α-D-Mannoside	1.082	B1	pH 4.5	0.001
H8	X-β-D-Glucosaminide	1.069	B3	pH 4.5 + L-Arginine	0.001
A7	pH 7	1.059	B8	pH 4.5 + Glycine	0.001
H12	X-SO4	1.051	B12	pH 4.5 + L-Lysine	0.001
A5	pH 5.5	1.046	C3	pH 4.5 + L-Proline	0.001
E3	pH 9.5 + L-Arginine	1.043	C7	pH 4.5 + L-Citrulline	0.001
E11	pH 9.5 + L-Leucine	1.04	C8	pH 4.5 + L-Valine	0.001
A6	pH 6	1.035	C11	pH 4.5 + L-Homoargi-nine	0.001
H4	X-α-D-Galactoside	1.019	A2	pH 4	0
H7	X-β-D-Glucuronide	1.019	B2	pH 4.5 + L-Alanine	0
F4	pH 9.5 + L-Serine	1.01	B4	pH 4.5 + L-Asparagine	0

H5	X-β-D-Galactoside	1.004	B5	pH 4.5 + L-Aspartic Acid	0
H6	X-α-D-Glucuronide	0.99	B9	pH 4.5 + L-Histidine	0
A8	pH 8	0.986	B10	pH 4.5 + L-Isoleucine	0
H2	X–α-D-Glucoside	0.972	B11	pH 4.5 + L-Leucine	0
H3	X-β-D-Glucoside	0.969	C1	pH 4.5 + L-Methionine	0
E1	pH 9.5	0.968	C4	pH 4.5 + L-Serine	0
G12	pH 9.5 + Urea	0.938	C5	pH 4.5 + L-Threonine	0
F5	pH 9.5 + L-Threonine	0.916	C6	pH 4.5 + L-Tryptophan	0
D4	pH 4.5 + α-Amino-N-Butyric Acid	0.895	C9	pH 4.5 + Hydroxy-L-Proline	0
G10	pH 9.5 + Creatine	0.886	C10	pH 4.5 + L-Ornithine	0
H1	X-Caprylate	0.857	C12	pH 4.5 + L-Homoserine	0
A9	pH 8.5	0.854	D10	pH 4.5 + D,L-Diamino-Pimelic Acid	0
F1	pH 9.5 + L-Methionine	0.832	D12	pH 4.5 + Urea	0
E2	pH 9.5 + L-Alanine	0.822	D2	pH 4.5 + L-Norleucine	-0.013
G11	pH 9.5 + Trimethylamine-N-oxide	0.79	C2	pH 4.5 + L-Phenylalanine	-0.022
G4	pH 9.5 + Agmatine	0.77	D5	pH 4.5 + p-Amino-Benzoic Acid	-0.121
F11	pH 9.5 + L-Homoarginine	0.735	G8	pH 9.5 + Phenylethylamine	-0.495