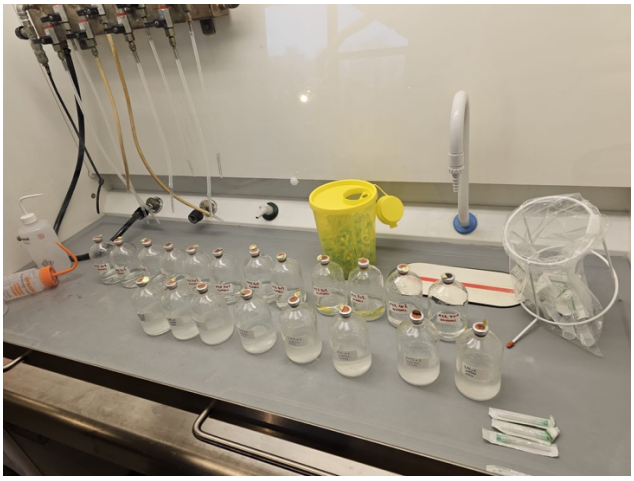
Biokinetic Characterization of Methane Oxidizing Microbiomes Co-metabolizing Trace Organic Chemicals

Hugo Möller



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Abstract

The presence of organic micropollutants (OMPs) in wastewater effluents is concerning, as they pose a threat to human and aquatic health. Biodegradation via methane-oxidizing bacteria (MOB) is an attractive option, as the method is cheap and effective. MOB can co-metabolize a variety of substrates, there among organic micropollutants. Methane-oxidizing microbiomes (MOMs) were cultivated with different SRT, methane loading rate, O_2:CH_4 ratio and nitrogen sources. The MOMs were spiked with OMPs for adaptation. Most of the MOMs generated similar results with specific growth rates of 0,40-0,66 d^{-1} and biomass yields on methane of 0,32-0,64 g CDW·g CH_4^{-1}. The MOMs with higher methane loading had the highest methane uptake rates, as well as the highest yields on nitrogen. The MOMs grown on nitrate instead of ammonia had higher methane and nitrogen uptake rates. The MOMs with 15 days SRT, high methane loading, 3:2 O_2:CH_4 ratio and nitrate as the nitrogen source generated the highest biokinetic values overall.

The MOMs degraded sulfamethoxazole (SMX) between 81-85%, reaching removal rates of 4,5-5,3 $L \cdot g^{-1} \cdot d^{-1}$. Metoprolol, diclofenac and dimethyl sulfide were moderately degraded between 22-50%. The degradation of SMX generated higher removal rates than some previous studies, there among degradation via activated sludge and membrane bioreactors. The MOMs that had not been adapted to OMPs had poor removal efficiencies in general, highlighting the importance of adaptation. The MOMs with low methane loading generated the highest removal efficiencies. A microbial community analysis is needed to further conclude how the communities differ. Further research is needed to grasp why the high methane loading bacteria had lower removal efficiencies while simultaneously oxidizing methane to a higher extent.

Preface

This thesis was written at Lund University with the experiments being conducted at the Technical University of Denmark (DTU). The project was performed and written from September 1st, 2023, until January 15th, 2024.

Firstly, I would like to thank my main supervisor Carolina Suarez Rodriguez for being kind, helpful and offering detailed and encouraging feedback.

Secondly, I would like to thank all the people at DTU for welcoming me with open arms. I would like to extend a warm thank you to my on-site supervisor Borja Valverde Perez, for treating me well, offering extensive feedback and being patient. I would like to thank all the members of the research group that I took part in at DTU Sustain. Especially my co-supervisors, Francesco Savio and Alen Simonic, for helping me out with my experiments and for being flexible and kind in general. I would also like to thank Emma Egholm Graveshorst for helping me in general, especially with GC and performing my VFA analyses, Mikael Olsson for running my OMP samples, Isabella Christiansen for running my (many) nutrient samples and Hanne Bøggild for helping me with VFA.

Populärvetenskaplig sammanfattning

Uppreningsförsök av organiska mikroföroreningar i avloppsvatten

Dagens extensiva användning av olika organiska kemikalier såsom läkemedel och smink, lämnar restprodukter i avloppsvatten som till viss del hamnar i dricksvatten och i naturen. Dessa restprodukter antas kunna skada både mänsklig och vattenlevande hälsa, till exempel genom att förhindra tillväxt och endokrina funktioner. Därför är det viktigt att rena vattnet ordentligt. Dagens befintliga tekniker är kostsamma och tidskrävande och behovet av ett billigare och effektivare alternativ efterfrågas.

Detta projekt har undersökt nedbrytningen av organiska mikroföreningar i avloppsvatten med hjälp av mikroorganismer. Mikroorganismer som konsumerar metan, så kallade metanoxiderande bakterier, kan bryta ner vissa mikroföreningar via en process som kallas co-metabolism. Co-metabolism innebär att mikroorganismer bryter ner och omvandlar ett ämne, utan att ta del av deras näringsinnehåll. De behöver med andra ord näring i annan form, i detta fall metan.

Under en fyramånadersperiod, odlades mikroorganismer i förslutna bägare. Mikroorganismerna odlades med variationer i deras tillväxtförutsättningar, med b.la. tillförsel av olika mängder metan samt olika kvävekällor. Till bakterierna tillsattes även en uppsättning mikroföreningar för att undersöka eventuell påverkan på tillväxt. Nedbrytningen mättes sedan under 48 timmar för att undersöka hur mycket av varje ämne som bröts ner samt för att generera hastighetskonstanten k_{bio} för varje ämne, för att kunna jämföra med litteraturvärden för tidigare försök.

Resultaten visade bland annat att bakterierna gynnades av högre metantillförsel och nitrat istället för ammoniak som kvävekälla. Bland de olika mikroföroreningarna var det endast ett, sulfamethoxazole, som bröts ner signifikant (81–85%). Bland resten av mikroföroreningarna, bröts tre ner mellan 22–50%, metoprolol, diclofenac och dimethylsulfid.

Resultaten påvisar att vissa mikroföreningar kan brytas ner till viss del av mikroorganismer. Framtida forskning kommer behövas för att utförligare undersöka vilka parametrar som gynnar ytterligare nedbrytning.

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List of abbreviations

OMP	Organic micropollutant
WWTP	Wastewater treatment plant
MOM	Methane oxidizing microbiome
MOB	Methane oxidizing bacteria
MMO	Methane monooxygenase
pMMO	Particulate methane monooxygenase
sMMO	Soluble methane monooxygenase
MABR	Membrane-aerated bioreactor
MBR	Membrane bioreactor
SRT	Solid retention time
dNMS	Dilute nitrate mineral salt
dAMS	Dilute ammonia mineral salt
TSS	Total suspended solids
SS	Short SRT
LS	Long SRT
LM	Low methane
HM	High methane
ALA-OA	Alachlor oxanilic acid
ALA-ESA	Alachlor ethane sulfonic acid
ATZ	Atrazine
BAM	2,6-dichlorobenzamide
BTZ	Bentazon
BTA	Benzotriazole
CBZ	Carbazole
CDZ	Chloridazon
DFC	Diclofenac
DMT-OA	Dimetachlor oxanilic acid
DMT-ESA	Dimetachlor ethane sulfonic acid
DMS	Dimethyl sulfide

GPS	Glyphosate
ISP	Isoproturon
MCP	Mecoprop
MTP	Metoprolol
SMX	Sulfamethoxazole

1. Introduction

1.1 Motivation

1.1.1 Background

The lack of clean drinking water is an issue expected to intensify because of climate change and a growing population. Access to clean and readily available domestic water is essential for public health (WHO, 2023). Today, over 96 % of urban wastewater in Sweden undergoes both chemical and biological treatment. According to Swedish EPA and Statistics Sweden (2020), 97 % of the biochemically degradable organic matter and phosphorus are purified for wastewater treatment plants (WWTPs) connected to populations above 2000 people. As for nitrogen, the degree of purification for the larger WWTPs amounts to about 74 % (Åkerblom, 2020). However, many pharmaceuticals and other contaminants remain largely undegraded in water. Up to 90 % of e.g. oral compounds can excrete human bodies as active substances. The urgency towards upgraded removal technologies is essential as the micropollutants reside in water and maintain their pollution towards terrestrial and aquatic environments (Euronews Digital, 2023; Water Science School, 2018).

Organic micropollutants (OMPs) include e.g. pharmaceuticals, personal care products and pesticides. OMPs can be potentially harmful to human as well as aquatic health as numerous reports suggest their potential threats. Bioaccumulation, growth disruption as well as endocrine disruption in aquatic organisms are among the primarily reported potential hazards (EEA, 2011; Rozas et al., 2016; Verlicchi et al., 2012). Bioaccumulation in crops irrigated with wastewater containing OMPs has been reported by Christou et al. (2017) and Goldstein et al. (2014) posing a potential risk to humans and other organisms if consumed. As the usage of pharmaceuticals and urbanization is increasing, the presence of OMPs is expected to expand. (Golovko et al., 2021)

Presently, OMPs are only partially biodegraded in conventional WWTPs using biological treatment (Falås et al., 2016) (Gros et al., 2010). More advanced technologies to remove OMPs from wastewater are available but are unsustainable and/or expensive. Alternatives that exist are various advanced oxidation processes such as UV/hydrogen peroxide treatment, ozonation and photo-Fenton which have all been proven successful in the past (Giannakis et al., 2015; Kudlek, 2018; Wols & Hofman-Caris, 2012). There is, however, a need for a low-cost and efficient alternative to the highly costly options that are currently available.

OMPs are degraded based on their physico-chemical properties and the range of degradation varies greatly. Some conventional WWTPs can degrade over 90 % of certain compounds e.g. ibuprofen using only secondary treatment. Other compounds e.g. antibiotics require further treatment for degradation (Peake et al., 2016). For pharmaceuticals, the rate of absorption in humans ranges between around 20-80 % before excretion (Corcoran et al., 2010). When treated in WWTPs, the OMPs and their metabolites are either degraded, bound to sludge or biosolids, or remain dissolved in the water phase (Trudeau et al., 2005).

1.1.2 Methane oxidizing microbiomes (MOM)

Methane oxidizing microbiomes (MOM) are typically complex and diverse communities, consisting of multiple species of bacteria, archaea, and fungi. A key component in MOMs are the methane-oxidizing bacteria (MOB) which are a group of bacteria that have the capability of growing on methane as their sole carbon and energy source. They play an important role in the global carbon cycle by reducing methane emitted to the atmosphere, making them relevant for global warming studies. Methanotrophs are present in a variety of environments, including soils, sediments, wetlands, and aquatic environments. They can also be present in extreme environments such as hot springs, hypothermal vents, and polar regions (Kalyuzhnaya et al., 2018).

Methanotrophs can be divided into four subgroups: *Gammaproteobacteria* (type I), *Alphaproteobacteria* (type II), *Verrucomicrobia* as well as members of NC10 phylum. Aerobic methanotrophic bacteria use oxygen to activate methane and oxidize methane into methanol (Kalyuzhnaya et al., 2018).

1.1.3 MMO and its applications

The ability to degrade OMPs has made MOBs valuable, particularly in the field of environmental remediation. Methane monooxygenase (MMO) is the main enzyme responsible for the oxidation of methane into methanol (Kalyuzhnaya et al., 2018). Figure 1 illustrates the main pathway for methane oxidation.

MMO exists in two forms that are found in MOBs – soluble methane monooxygenase (sMMO), located in the cytoplasm and particulate methane monooxygenase (pMMO), which is bound to the membrane (Gęsicka et al., 2021). sMMO has a broader substrate range than pMMO, however, both are capable of degrading various OMPs. Both forms of the enzyme are capable of selectively hydroxylating methane, which involves the addition of a hydroxyl group to the methane molecule, converting it into methanol. Some

methanotrophs can express either form of the enzyme. The availability of copper is the factor that governs the expression of the two enzymes. A metabolic switch mediated by copper ions regulates the expression of sMMO and pMMO. When grown under high concentrations of copper (>5 µM), pMMO is produced, whereas sMMO is expressed under low availability of copper (<0,1 µM) (Stanley et al., 1983; Kalyuzhnaya et al., 2018).

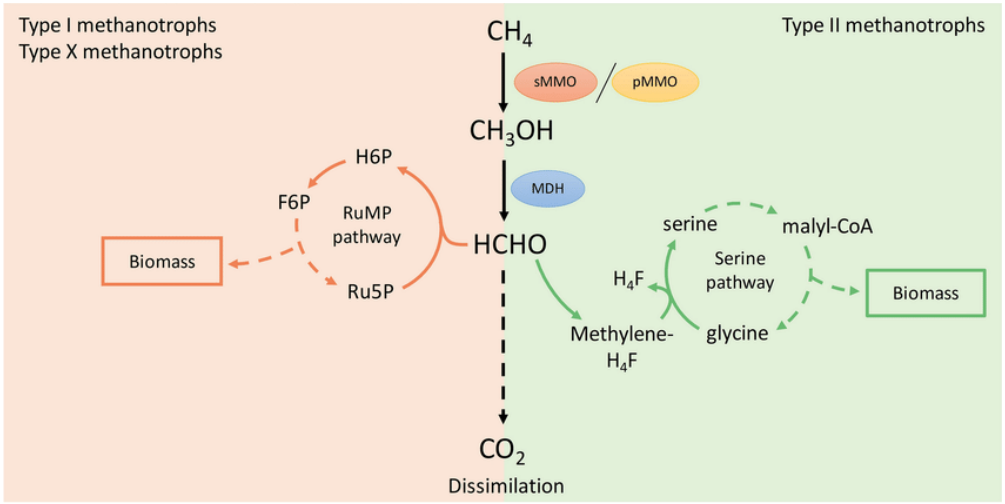


Figure 1: Metabolic pathways of methanotrophs type I, II and X (Khider et al., 2021). CC by 4.0 DEED

1.1.4 Metabolism of OMPs

The ability of MMO to act on a wide range of substrates allows it to degrade various OMPs. This includes hydrocarbons (like alkanes, cycloalkanes, alkenes), haloalkenes, ethers, and even aromatic and heterocyclic hydrocarbons. By transforming these pollutants into less harmful or more biodegradable forms, MMO aids in cleaning up contaminated environments (Mortensen et al., 2023). OMPs can be degraded through primary metabolism as well as co-metabolism. Co-metabolism refers to the simultaneous degradation of two compounds, where the degradation of the secondary compound (in this case, the pollutant) depends on the presence of the primary compound (methane). It can also be described as the transformation of a non-growth substrate (Fenner et al., 2021).

1.1.5 Operational Parameters

Solid retention time (SRT) has been recognized as one of the major process parameters of importance for biological systems. SRT is defined as the time the solid fraction of the wastewater spends in a treatment unit. Achermann et al (2018) found that longer SRT is mainly associated with higher oxidative

biotransformation when comparing the first-order rate constants between SRTs of 3 and 15 days.

Methane loading rate has also been found to be a significant factor in biodegradation performance. Mortensen et al (2022) discovered that biomass productivity and removal rate of sulfamethoxazole and 1H-benzotriazole increased linearly with a higher methane loading rate in a membrane-aerated bioreactor (MABR).

Different nitrogen sources have also been found to have an impact on biodegradation and productivity. Using ammonia instead of nitrate has been reported to have a varying response in different strains of methanotrophs with growth inhibition/stimulation as well as the production of toxic intermediates e.g. hydroxylamine and nitrite. Ammonia can function as a competitive inhibitor to MMO but has also been reported to facilitate biomass growth and degradation of compounds like trichloroethylene. (Hoefman et al., 2014; Chu & Alvarez-Cohen, 1998)

Finally, the O_2:CH_4 ratio has been proven to have a significant impact on methane oxidation. Ren et al (1997) found that the rate of CH_4 oxidation by strains of pure cultures of Alphaproteobacteria and Gammaproteobacteria were at maximum with O_2 concentrations of 0,45-20 % (v/v) in the presence of non-limiting dissolved CH_4 and inorganic nitrogen. Concentrations ranging from 20-63 % of O_2 on the other hand, depressed the activity with up to 23 %.

1. 2 Project aims

The thesis aims to evaluate the performance of the different batch operations with respect to OMP removal. The different parameters used in the different batches will be correlated with the removal rate of OMPs.

The project aims are as follows:

- Perform batch experiments with MOMs with variations of four operational parameters (SRT, CH_4 loading rate, O_2:CH_4 ratio and nitrogen source). A biokinetic characterization will be carried out to compare and deduce which parameters implicate the highest biomass productivity. Half of the batches will be adapted to OMPs to examine the impact of bacterial growth.
- Perform 48-hour biodegradation tests to generate and assess the biodegradation constant k_{bio} and to measure the removal efficiencies.

2. Materials and Methods

2.1 Cultivation media and inoculum

Two different cultivation media were used, dilute nitrate mineral salts (dNMS) and dilute ammonia mineral salts (dAMS) (Whittenbury, et al., 1970). A detailed recipe can be found in table A1 and A2. The initial inoculum consisted of a mixed community derived from the groundwater treatment plant Gilleleje Vandværk. Since methane was reported to be present in the influent (GEUS. 2018), methanotrophs were believed to inhabit the tanks. Two L of inoculum were retrieved from the effluent of the aeration tank, placed in a Pyrex bottle, and stored at 4°C for one week. The inoculum was replaced after four weeks without considerable growth. The new bacteria were provided by Xu et al., (2021) and consisted of a mixed methanotrophic seed derived from a lab-scale fermenter, dominated by *Methylomonas* and *Methylophilus.* The inoculum was adapted to dNMS for one week before the batch experiments. The methane content in the gas phase was maintained at 10-30 %. Oxygen and methane were provided in a 2:1 ratio. The MOB culture was kept in a shaker at 25°C. The media was autoclaved before the experiments.

2.2 Experimental setup

250 mL serum bottles were used with 100 mL of working volume with 3 mL of MOB culture. The serum bottles were sealed with rubber stoppers and aluminum lids. Half the bottles were spiked with 1 mL of OMP stock solution (See section 2.3). After the addition of media, the bottles were flushed with N_2 for 5-10 minutes each. For the bottles with a high methane loading rate, 50 mL of headspace was removed. The same bottles were injected with 100 mL of pure oxygen and methane in a 3:2 ratio. The bottles with low methane loading rates were injected with gas in the same ratio but with 50 mL instead of 100 mL. The bottles were placed on a shaker at 120 rpm at 25°C in a dark room.

The second set of batches was prepared with dAMS instead of dNMS. 70 mL of headspace was removed and 120 mL of oxygen and methane in a 2:1 ratio was injected. Apart from the media and the $O2:CH_4$ ratio, the second set of batches was prepared identically to the first set of batches.

Figure 2 depicts an illustration of the general experimental setup.

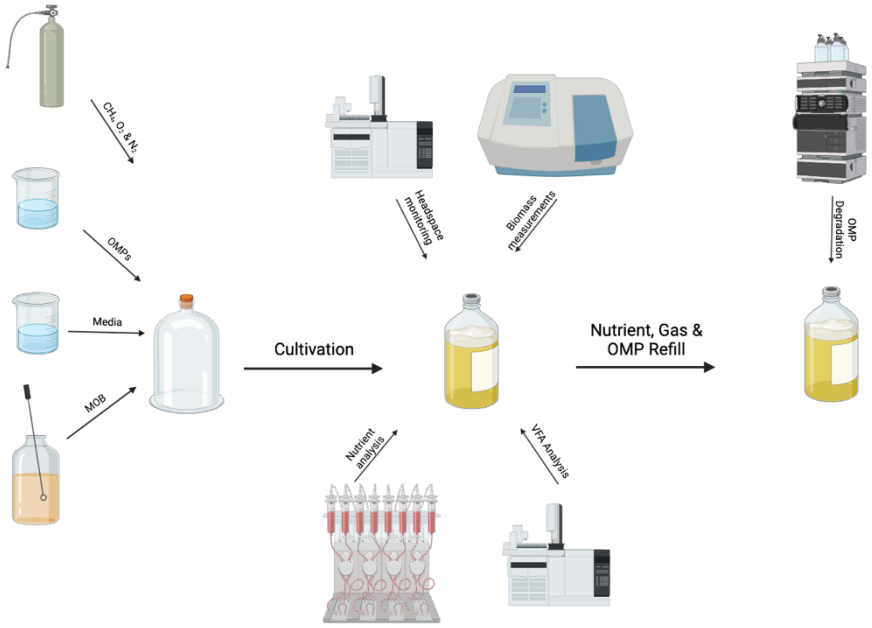


Figure 2: Sketch of the experimental setup.

2.2.1 First set of batches

The batches were run with SRTs of 3 and 15 days. To generate the proper SRTs, 5 mL and 66 mL of inoculum were used for re-inoculation respectively. The initial concentrations of gaseous methane were 3 and 6 $mg \cdot L^{-1}$. All bottles were run in duplicates, generating 16 bottles (Table 1). Biomass concentration, (See 2.4.1), headspace concentrations (See 2.4.4), nutrients (See 2.4.2) and volatile fatty acids (VFAs) (See 2.4.3) were monitored throughout.

Table 1: Summary of the different batches, all batches were run in duplicates. SS refers to short SRT (3 days), LS refers to Long SRT (15 days), LM refers to Low Methane (3 $mg \cdot L^{-1}$) and HM refers to High Methane (6 $mg \cdot L^{-1}$).

With OMPs	SS LM	SS HM	LS LM	LS HM
Without OMPs	SS LM	SS HM	LS LM	LS HM

2.2.2 Second set of batches

As 15 days of SRT provided the more easily measurable growth rates, this was kept as a constant operational parameter to facilitate measurements. Table 2 shows a clarification of the bottles used in the experiment.

Table 2: Scheme of the different batches in the second set of experiments. All batches were run in duplicates. 2:1 and 3:2 refers to the O_2:CH_4 ratios. dAMS and dNMS refers to the cultivation media.

With OMPs	dAMS 2:1 O_2:CH_4	dAMS 3:2 O_2:CH_4	dNMS 2:1 O_2:CH_4
Without OMPs	dAMS 2:1 O_2:CH_4	dAMS 3:2 O_2:CH_4	dNMS 2:1 O_2:CH_4

2.3 Organic Micropollutants

Seventeen different micropollutants were added to half of the bottles in both sets of experiments. They were added within quantification limits for analysis i.e. 2 µg/L. Table 3 shows the OMPs and a variety of their properties. The chosen OMPs, have all been present in drinking water in various countries in the European Union (Benner et al., 2013). Furthermore, they were deemed to be potentially biodegradable by MMO, based on the substrate range of MMO. The availability of the chemicals currently in stock also affected the selection of the OMPs.

Table 3: The organic micropollutants used, their abbreviations, the initial concentrations, their applications, and their CAS numbers.

Compound	**Abbreviation**	**Concentration ($\mu g \cdot L^{-1}$)**	**Application**	**CAS nr**
Alachlor Oxanilic Acid (OA)	ALA - OA	2	Herbicide	171262-17-2
Alachlor Ethanesulfonic Acid (ESA)	ALA - ESA	2	Herbicide	142363-53-9
2,6-dichlorobenzamide	BAM	2	Herbicide	2008-58-4
Atrazine	ATZ	2	Herbicide	1912-24-9
Bentazon	BTZ	2	Herbicide	25057-89-0
Benzotriazole	BTA	2	Anti-corrosion	95-14-7
Carbazole	CBZ	2	Insecticide	86-74-8
Chloridazon	CDZ	2	Herbicide	1698-60-8
Diclofenac	DFC	2	Analgesic	15307-86-5
Dimetachlor Ethanesulfonic Acid (ESA)	DMT - ESA	2	Herbicide	Metabolite of 50563-36-5

Dimetachlor Oxanilic Acid (OA)	DMT- OA	2	Herbicide	Metabolite of 50563-36-5
Dimethyl sulfide	DMS	2	Various bacterial metabolite	75-18-3
Glyphosate	GPS	2	Herbicide	1071-83-6
Isoproturon	ISP	2	Herbicide	34123-59-6
Mecoprop	MCP	2	Herbicide	93-65-2
Metoprolol	MTP	2	β-blocker	51384-51-1
Sulfamethoxazole	SMX	2	Antibiotic	723-46-6

2.3.1 Biodegradation test

48h biodegradation tests were performed to track the degradation over time and to generate biodegradation constants k_{bio} for the OMPs. An extended protocol can be viewed in Appendix 4. The biodegradation tests were run after adaptation. The MOMs were provided with 25 $mg \cdot L^{-1}$ of nitrogen, the gasses of the headspace were refilled and OMPs were added in the same concentrations as for the adaptations. Biomass (See 2.4.1), OMPs (See 2.4.5), nutrients (See 2.4.2) and headspace (See 2.4.4) were monitored throughout.

2.4 Analytical methods

2.4.1 Biomass

The biomass concentration was monitored throughout using optical density (OD) at 600 nm (Cary® 50 Bio UV-Visible Spectrophotometer, Agilent Technologies, USA). Measurements were taken each day initially and twice per day when the exponential growth phase had been initiated. Before measurements, the serum bottles were heavily shaken to ensure that no biomass had been sedimented. Media was used to zero the reader. A correlation between OD and total suspended solids (TSS) was made as a function to track the produced cell dry weight (CDW) (Figure A3). An extended protocol can be viewed in Appendix 5.

2.4.2 Nutrients

PO_4, N, NO_3, and NH_3 concentrations were monitored using a continuous-flow auto-analyzer (SKALAR San++, Netherlands). Samples were taken each day and twice during the exponential growth phase. A 0,8 mL sample was extracted and filtered using a 0,2-µg filter (Econo filter, nylon, 25 mm, 0.2 µm, Agilent

Technologies, USA) and diluted to a factor ten with distilled water. The samples were stored at -20°C and then thawed in a refrigerator before analysis.

2.4.3 Volatile Fatty Acids

VFAs were analyzed using gas chromatography (GC) (Thermo Scientific TRACE™ 1300 Gas Chromatograph) equipped with a Flame Ionization Detector (FID). Liquid samples of 1 mL were extracted and filtered using a 0,2-µg filter (Econo filter, nylon, 25 mm, 0,2 µm, Agilent Technologies, USA). 50µL of 3 M H_3PO_4 were then added to acidify the samples. 100 µL of 0,5M 4-Methylvaleric acid was added as an internal standard to all the samples. The samples were stored at -20°C and then thawed in a refrigerator before analysis.

2.4.4 Gas Monitoring

The headspace concentrations were monitored using gas chromatography (Thermo Scientific TRACE™ 1300 Gas Chromatograph) equipped with a HP-Plot/Q column (Length 15 m, diameter 0,32 mm, film 20 µm, Agilent Technologies, USA) for CH_4 and CO_2 measurements. O_2 measurements were also done using gas chromatography (Thermo Scientific TRACE™ 1300 Gas Chromatograph) using an HP-Molesieve column (Length 30 m, diameter 0.53, film 50 µm, Agilent Technologies, USA). CH_4, O_2 and CO_2 volumes were measured daily. Calibrations for CH_4 and CO_2 were made with gas mixtures of known compositions with CH_4:CO_2 content of 5:5, 40:30 and 100:0 (% v/v). Calibrations for O_2 were made with gas mixtures containing 1, 10, 25 and 100 (% v/v) of O_2.

2.4.5 Organic micropollutants

1 mL samples were extracted and filtered with a 0,2-µg filter (Econo filter, nylon, 25 mm, 0.2 µm, Agilent Technologies, USA). The bottles were heavily shaken to ensure sufficient mixing. The OMP concentrations were measured by HPLC-MS/MS (1290 Infinity II – 6470 LC-QQQ, Agilent Technologies, USA). Chromatic separation was achieved on a 2,1 x 100 mm, 2,7 micron reversed-phase column (Poroshell 120 EC-C18, Agilent Technologies). When operating in ESI+ mode, 0,1% formic acid was used as aqueous phase and acetonitrile with 0,1% formic acid was used as a mobile phase. When operating in ESI- mode, 10 mM ammonium acetate pH 4,0 was used as aqueous phase and acetonitrile:ammonium acetate pH 4,0 (90:10) was used as mobile phase. The samples were kept frozen at -20°C until analysis.

2.5 Calculations

2.5.1 Specific growth rate

The specific growth rate was calculated using equation 1.

$$\frac{dln(OD)}{dt} = \mu \tag{1}$$

Ln (OD) is the natural logarithm of the optical density of the biomass, t is time (d) and μ is the specific growth rate (d^{-1}). The slope was fitted linearly using Microsoft Excel.

2.5.2 Yields

The yield of CDW, calculated from the OD to TSS correlation (Appendix 5), produced by the removed amount of substrate is expressed under equations 2, 3 and 4. Yields of methane, nitrogen and phosphorus with respect to biomass were calculated, where the change in biomass divided with the change of substrate represented the yield.

$$Y_{CDW/CH_4} = \frac{CDW \cdot V_L}{\Delta CH_4} \tag{2}$$

$$Y_{CDW/N} = \frac{CDW}{\Delta N} \tag{3}$$

$$Y_{CDW/P} = \frac{CDW}{\Delta P} \tag{4}$$

$Y_{x/x}$ refers to the yield (g/g), CDW is the cell dry weight ($g \cdot L^{-1}$), V_L refers to the liquid volume (L), CH_4 is methane (g), N is nitrogen ($g \cdot L^{-1}$) and P is phosphorous ($g \cdot L^{-1}$).

2.5.3 Uptake rates

Uptake rates based on methane and nitrogen were determined using equations 5 and 6. The rates were estimated using linear regression in Microsoft Excel. The rates were calculated during the exponential growth phase.

$$\frac{dCH_4}{dt} = -r_{CH_4} \cdot CH_4 \tag{5}$$

$$\frac{dN}{dt} = -r_N \cdot N \tag{6}$$

CH_4 is the methane in the headspace (mg), r_{CH_4} is the methane uptake rate (mg $CH_4 \cdot d^{-1}$), N is the nitrogen in the media (mg N) and r_N is the nitrogen uptake rate (mg $N \cdot d^{-1}$).

2.5.4 Gas masses

The masses of the different gasses in the headspace were calculated as:

$$M_{gas} = \frac{\%_{gas} \cdot V_h \cdot P \cdot Mw_{gas}}{100 \cdot R \cdot T} \tag{7}$$

Where $\%_{gas}$ represents the gas percentage in the headspace, V_h is the total volume of the headspace (m^3), P is the total pressure of the headspace (atm), Mw_{gas} is the molecular mass of the gas ($g \cdot mol^{-1}$), R is the molar ideal gas constant (0,0000821 $atm \cdot m^{-3} \cdot K^{-1} \cdot mol^{-1}$) and T is the temperature (K).

2.5.5 Pressure of headspace

The total pressure of the headspace was calculated using Dalton's law (eq. 8) and the ideal gas law (eq. 9). As the ideal gas law was used, it was assumed that the gasses were ideal when calculating the pressure of the specific gasses. It was also assumed that the gasses were at standard temperature and pressure (STP) conditions (1 atm & 273 K), to be able to calculate the molar contribution to the gas mix. As 1 mole of gas is equal to 22,414 dm^3 at STP, this was utilized when calculating the pressure.

$$p_{total} = \sum_{i=1}^{n} p_i \tag{8}$$

p_{total} is the total pressure (pa) and p_i is the partial pressure of the different gasses (pa).

$$p \cdot v = n \cdot R \cdot T \tag{9}$$

p is the pressure (pa), v is the volume (m^3), n is the amount of moles, R is the molar ideal gas constant (8,314 $m3 \cdot Pa \cdot K^{-1} \cdot mol^{-1}$) and T is the temperature (K).

2.5.6 Degradation kinetics

To generate the biomass-related reaction rate constants (k_{bio}), degradations constants (k) were obtained using first-order degradation kinetics given in equation 10 (Tang et al., 2017). The degradation constants were fitted using Prism 10 (GraphPad).

$$C = C_0 \cdot e^{-k \cdot t} \tag{10}$$

C is the concentration of the OMP ($\mu g \cdot L^{-1}$), C_0 is the initial concentration of the OMP ($\mu g \cdot L^{-1}$), k is the degradation rate (d^{-1}) and t is the time (d). The k was then normalized by the average biomass concentration to give k_{bio} ($L \cdot g^{-1} \cdot d^{-1}$), to compare with literature data as well as between compounds.

2.5.7 Statistical analysis

Statistical analyses were carried out using Prism 10 (GraphPad). Means of data were compared with unpaired T-tests to determine significant differences. The data throughout the report is expressed in mean values $\pm$ standard error of mean (SEM), given in equation 11.

$$SEM = \frac{SD}{\sqrt{n}} \tag{11}$$

SD is the standard deviation and n is the number of samples.

3. Results

3.1 First set of experiments

The first set of batches was operated with SRTs of 3 vs 15 days as well as methane loading rates of 40 mL vs 60 mL. The MOMs were cultivated until two similar values of OD were obtained, meaning that the exponential growth phase had ended. Tables 4 display the specific growth rates, uptake rates for methane and nitrogen as well as the yields on methane, nitrogen, and

phosphorus. The phosphorus content of the bottles with short SRT was insufficient. Therefore, these bottles suffer from inconsistent growth and the results are considered unsustainable. This is noticeable when comparing the specific growth rate, as the SEMs differ, ranging from 0,23 to 0,33 compared with the μ for the long SRTs which had SEMs of 0,06 to 0,14 (Table 4). The first re-inoculations of short SRTs showed significant growth, with μ ranging from 0,66 to 1,05 (Figure A4). As the μ were 0 for the third re-inoculation, this is most likely due to insufficient phosphorus.

The longer SRT bottles depleted almost all the available oxygen and methane during the different inoculations resulting in yields of 0,32-0,38 g CDW·g CH_4^{-1} (figure A1; table 4). The methane uptake rates differed (P=0.0225), as the higher methane bottles had a larger methane uptake rate than the lower methane bottles (table 4). Nitrogen was present in excess (≈ 25 mg · L^{-1}) for all the batches. The yields on nitrogen and phosphorus were around twice the amount for the higher methane loading rate bottles (table 4). The nitrogen uptake rates as well as the yield on methane didn't differ significantly (P >0,05). The bottles produced malate in concentrations ranging from 0,5-2,77 g·L^{-1}. Malate, a product of methane fermentation, has been produced previously by methanotrophs in hypoxic environments (Khanongnuch et al., 2023). This further implicated the depletion of the available oxygen. CO_2 was produced in higher concentrations for the high methane loading bottles indicating greater methane oxidation (figure A2). The presence of OMPs did not have any significant effects on growth and biokinetic performance (P > 0,05), indicating that the compounds do not inhibit bacteria (Benner et al., 2014).

Table 4: Specific growth rates, methane and nitrogen uptake rates as well as yields on methane, nitrogen and phosphorus. Data are given in mean ± SEM. SS refers to short SRT, LS refers to long SRT, LM refers to low methane loading and HM refers to high methane loading.

Conditions	Specific growth rate (d^{-1})	Methane Uptake Rate (mg $CH_4 \cdot d^{-1}$)	Nitrogen Uptake Rate (mg N·d^{-1})	$Y_{CDW/CH4}$ (g CDW·g CH_4^{-1})	$Y_{CDW/N}$ (g CDW·g N^{-1})	$Y_{CDW/P}$ (g CDW·g P^{-1})	OMPs Yes/No
SS & LM	0,64 ± 0,33	3,890 ± 2,25	0,01 ± 0,01	0,25 ± 0,03	16,63 ± 5,91	-	Yes
SS & LM	0,47 ± 0,24	0,63 ± 0,63	0,08 ± 0,04	0,55 ± 0,10	6,72 ± 4,25	-	No
SS & HM	0,49 ± 0,27	5,525 ± 3,19	0,17 ± 0,15	0,40 ± 0,03	10,19 ± 0,63	-	Yes
SS & HM	0,44 ± 0,23	2,58 ± 2,58	0,13 ± 0,08	0,41 ± 0,02	10,83 ± 1,51	-	No

LS & LM	0,66 ± 0,06	13,39 ± 2,97	0,74 ± 0,08	0,33 ± 0,00	6,57 ± 0,61	36,73 ± 25,97	Yes
LS & LM	0,66 ± 0,09	14,42 ± 2,59	0,88 ± 0,20	0,32 ± 0,00	5,57 ± 0,03	36,03 ± 7,75	No
LS & HM	0,53 ± 0,14	19,33 ± 4,28	0,78 ± 0,14	0,38 ± 0,02	12,18 ± 0,24	50,09 ± 35,42	Yes
LS & HM	0,56 ± 0,06	18,25 ± 4,05	0,66 ± 0,04	0,37 ± 0,02	15,74 ± 2,20	66,12 ± 18,71	No

3.2 Second set of experiments

The second set of experiments was operated with the best performing SRT from the first set i.e. 15 days. Ammonia was used as a nitrogen source instead of nitrate and a 2:1 O_2:CH_4 ratio was equipped in the headspace with a methane loading rate of 60 mL as well as the previously used 3:2 ratio for comparison. The MOMs from the previous experiment were equipped with the new O_2:CH_4 ratio with the previous media dNMS, to examine differences. Half of the bottles were spiked with OMPs. The new set of experiments also featured insufficient amounts of phosphorus for the dNMS media. This had a presumed impact on the activity as the growth rates reduced significantly.

The different O_2:CH_4 ratios with dAMS did not display any significant differences ($P > 0,05$) with any of the measured rates and yields (table 5). The bottles containing the same O_2:CH_4 ratio with different nitrogen sources differed significantly ($P=0,0181$) with regards to methane uptake rate. The dNMS bottles (LS & HM, table 4), had a higher methane uptake rate (18,25-19,33 mg $CH_4 \cdot d^{-1}$) than the dAMS bottles (10,45-12,18 mg $CH_4 \cdot d^{-1}$; 3:2 with dAMS; table 5). The nitrogen uptake rates were also higher for the dNMS bottles ($P = 0,0236$), with rates of 0,78-0,66 mg $N \cdot d^{-1}$ (table 4) compared with 0,31-0,34 mg $N \cdot d^{-1}$ (table 5). The yields on methane, nitrogen and phosphorus did not show any significant differences ($P > 0,05$). The 3:2 bottles with dAMS produced higher concentrations of CO_2 and depleted more O_2 than the 2:1 bottles with dAMS indicating higher methane oxidation (figure A1; figure A2).

Table 5: Specific growth rates, methane and nitrogen uptake rates as well as yields on methane, nitrogen and phosphorus. Data are given in mean ± SEM. 3:2 and 2:1 refers to the O_2:CH_4 ratio. dAMS and dNMS refers to the media.

Conditions	**Specific growth rate (d^{-1})**	**Methane Uptake Rate**	**Nitrogen Uptake Rate**	**$Y_{CDW/CH4}$ (g CDW·g CH_4^{-1})**	**$Y_{CDW/N}$ (g CDW·g N^{-1})**	**$Y_{CDW/P}$ (g CDW·g P^{-1})**	**OMPs Yes/No**

		(mg $CH_4 \cdot d^{-1}$)	(mg $N \cdot d^{-1}$)				
3:2 & dAMS	0,46 ± 0,10	10,45 ± 1,15	0,31 ± 0,00	0,32 ± 0,02	9,00 ± 0,04	46,27 ± 17,02	Yes
3:2 & dAMS	0,48 ± 0,10	12,18 ± 5,13	0,34 ± 0,02	0,64 ± 0,14	13,71 ± 4,57	65,31 ± 56,03	No
2:1 & dAMS	0,43 ± 0,13	10,05 ± 2,80	0,34 ± 0,02	0,57 ± 0,16	10,02 ± 2,97	18,48 ± 1,21	Yes
2:1 & dAMS	0,40 ± 0,04	12,59 ± 0,86	0,28 ± 0,04	0,35 ± 0,06	8,98 ± 0,65	196,27 ± 145,17	No
2:1 & dNMS	0,09 ± 0,09	1,90 ± 1,07	0,13 ± 0,01	0,40 ± 0,16	11,74 ± 6,81	-	Yes
2:1 & dNMS	0,07 ± 0,07	2,13 ± 0,35	0,25 ± 0,11	0,50 ± 0,10	5,62 ± 4,32	-	No

3.3 OMP degradation

The CDZ and BTZ levels were below the detection limit as well as the DFC samples for the long SRT batches. BTA was not analyzed due to time constraints. The bacteria from the second set of experiments did not feature in the degradation test, also due to time constraints.

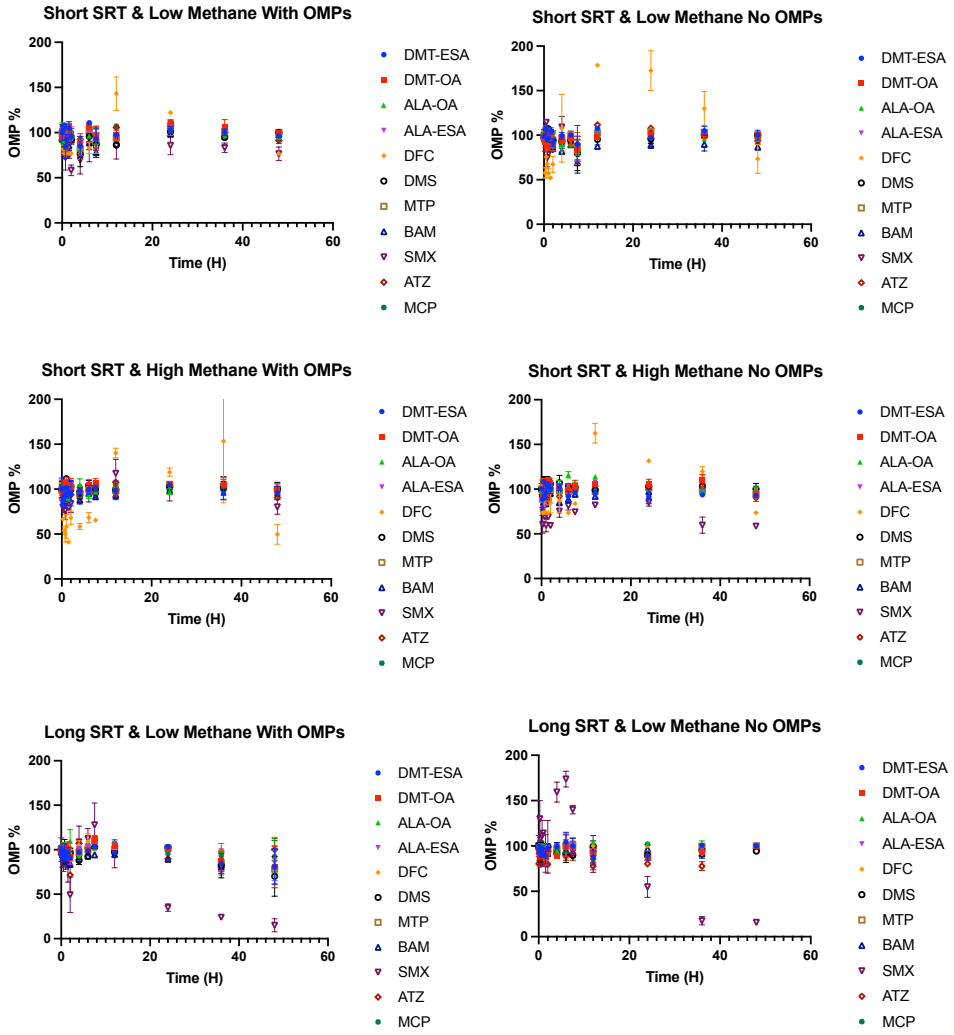
Table 6 display the removal efficiencies for the different batches. Figure 3 shows the behavior of the OMPs. The OMPs appear to enlarge in concentration for some of the batches which is most likely due to insufficient mixing of the bottles before sampling or general measurement errors. The OMPs that were not degraded very well showed small inconsistencies in concentrations compared with the OMPs that degraded well, which decayed over time.

The short SRT batches were expected to have less effective biodegradability due to the lack of phosphorous, as previously mentioned. The batches displayed low biodegradability in general, degrading only DFC and SMX above 20% (table 6).

For the long SRT bottles, there was a clear trend between the MOBs that had and had not been adapted to OMPs. The bottles without OMP adaptation failed to degrade any compound except SMX above 20% (table 6). The bottles with OMP adaptation, degraded DMS, MTP and SMX above 20% as well as DMT-ESA, DMT-OA, ALA-ESA and BAM between 10-20% (table 6).

Table 6: Removal efficiencies of the organic micropollutants, negative removal was indicated by 0%.

Conditions	DMT–ESA (%)	DMT–OA (%)	ALA–OA (%)	ALA–ESA (%)	DFC (%)	DMS (%)	MTP (%)	BAM (%)	SMX (%)	ATZ (%)	MCP (%)	OMPs Yes/No
SS & LM	0	0	4	3	27	0	0	13	1	7	1	Yes
SS & LM	4	0	5	2	23	0	0	2	23	9	3	No
SS & HM	4	0	4	1	50	0	0	1	20	9	6	Yes
SS & HM	7	6	0	5	26	0	6	5	41	9	6	No
LS & LM	19	19	8	15	0	30	22	19	85	0	8	Yes
LS & LM	0	0	0	0	0	6	0	0	84	0	0	No
LS & HM	16	12	5	10	1	27	23	4	81	0	1	Yes
LS & HM	0	0	0	0	0	3	0	0	78	0	0	No



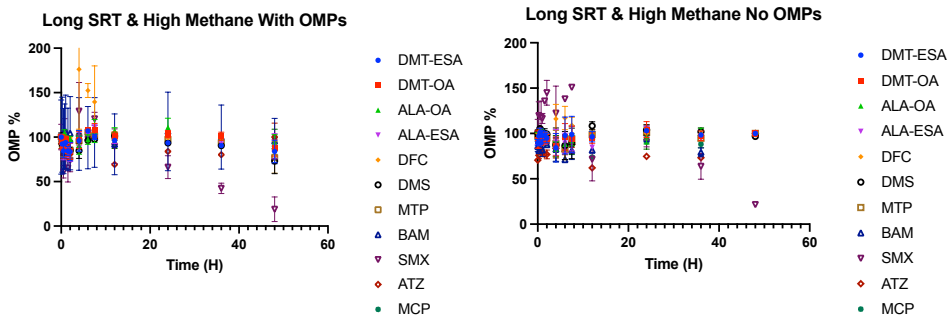


Figure 3: Degradation of organic micropollutants with the measured conditions. The micropollutants were normalized to their initial concentration given at 100 %. The data are given in mean ± SEM.

Figure 4 shows the fitted k_{bio}s. As the concentrations fluctuated throughout the 48 hours for many of the OMPs, the k_{Bio} differed significantly for some compounds when compared with the removal efficiencies (Fig. 4; table 7). The removal rates were fitted in Prism 10 and the negative k_{Bio}s were not accounted for. For most of the compounds, they were in the expected range based on their removal efficiencies (Appendix, table A4). However, there were some deviations. For DFC, as the concentrations fluctuated, the removal rate was negative as the larger concentrations exceeded the starting concentrations numerous times for the short SRT batches (Fig.3). SMX, which was degraded by all bottles except one, had some deviances regarding the degradation efficiency. Most notable were the short SRT bottles containing OMPs, which had the lowest removal efficiency and negative k_{bio} indicating that they failed to degrade SMX.

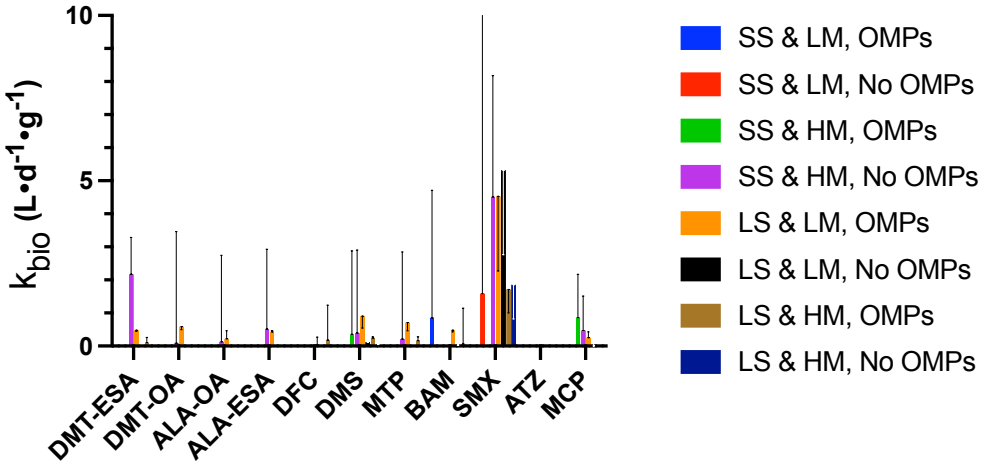


Figure 4: The k_{bio} values. The black lines indicate 95% confidence intervals.

4. Discussion

4.1 Biokinetic Characterization

4.1.1 Impact of SRT

As the short SRT bottles failed to grow, the impact of SRT is difficult to conclude.

4.1.2 Impact of methane loading rate

Both Lee et al., (2015) and Mortensen et al., (2023) reported higher biomass productivity with increased methane loading rate. When comparing the performances of the longer SRT bottles with different methane loading, the methane uptake rate as well as the yields on nitrogen and phosphorus were larger for the higher methane loading rate bottles (table 4). Valverde-Pérez et al., (2020) cultivated MOB in a bubble-free membrane aerated bioreactor (MABR), using different rates of methane loading. Higher biomass productivity was linked with a higher methane content, with increasing yields on nitrogen. The yields on methane were, however, higher with a lower methane loading of 2,9-5,6 v/v O_2:CH_4, 0,43 g CDW·CH_4^{-1} compared with 0,26 g CDW·CH_4^{-1}, for the higher methane loading of 1,5-2,9 v/v, a loading similar to this study. As yields on methane reached 0,38 g CDW·CH_4^{-1} for this study, a lower methane loading might be preferable. The increased yields on nitrogen for Valverde-Pérez et al., (2020) led to lower microbial protein accumulation which also might be the case for this study since nitrogen accumulation has been linked with a higher microbial protein content previously (Khoshnevisan et al., 2020). Unfortunately, this study does not include an amino acid analysis because of time constraints.

4.1.3 Impact of O_2:CH_4 ratio

The methane/oxygen ratio has been proven to have a significant effect on the MOB community. Studies have shown that Gammaproteobacteria generally favors environments with high concentrations of O_2 and low concentrations of CH_4 with Alphaproteobacteria preferring the contrary (Chidambarampadmavathy et al., 2017). When comparing the 3:2 and the 2:1 ratio of the dAMS bacteria, there are generally small differences in performance. The specific growth rate is slightly higher for the 3:2 ratio but there were no significant differences overall ($P > 0,05$). No other results displayed any significant differences ($P > 0,05$). This is interesting, considering that previous studies have shown that methane oxidation decreases with O_2 concentrations above 20% (v/v) (Ren et al 1997). Compared with previous

experiments, the biomass yields on methane were slightly higher for the 2:1 ratio (0,35-0,57 g CDW·g CH_4^{-1}) than for MBR experiments with a similar ratio, reaching yields of 0,39 g CDW·g CH_4^{-1}. The yields on nitrogen were also higher (8,98-10,02 g CDW·g N^{-1} compared with 5,2 g CDW·g N^{-1}), again possibly implicating a lower protein content (Valverde-Pérez et al., 2020). Considering the lack of differences in general, it would have been interesting to increase or decrease the ratio even further.

4.1.4 Impact of nitrogen source

The impact of the nitrogen source is visible when comparing the growth parameters of the 3:2 ratio with dAMS with the 3:2 ratio with dNMS.
The methane uptake and the nitrogen uptake rates were higher for the dNMS bacteria (table 4). The structural similarity of ammonia and methane has been reported to inhibit MMO in the past (Nyerges & Stein, 2009). However, inhibition decreases with methane levels above 100 ppm, and as such shouldn't inhibit MMO in the dAMS media (King & Schnell, 1994). Nitrite, a product of ammonia co-metabolism, has also been reported to inhibit methane oxidation by a yet uncharacterized mechanism (Nyerges & Stein, 2009). The nitrite levels were, however, below the detection limit for the dAMS bacteria.
Using ammonia as a nitrogen source has previously been linked with a more diverse MOB community, which could explain the slower uptake rates, as the community may consist of slower-growing bacteria (King & Schnell, 1994). The assimilatory pathways of nitrate and ammonia may also affect the growth parameters, as nitrate assimilation is less efficient than the assimilation of ammonia (Stein et al., 2006). Since dNMS displayed faster uptake, this might be because of the re-inoculation of MOMs. Since the MOMs were re-inoculated from the previous dNMS media to dAMS, traces of nitrate were still present. This is visible when looking at the different growth curves, as the bacteria grew faster after the second re-inoculation (appendix 7). As nitrate wasn't reduced significantly in comparison with ammonia, the competition of N source and the overall adaption of the MOM community might be responsible for the slower uptake rates.

The growth rates were lower than in previous studies, with growth rates of 1,11 d^{-1} being reported for dAMS bacteria and 1,06 d^{-1} for dNMS bacteria, compared with 0,46-0,48 d^{-1} for dAMS in this study and 0,53-0,56 d^{-1} for dNMS (Goonesekera et al., 2022). The same study showed larger nitrogen assimilation for dAMS bacteria than for dNMS, contrary to this study. As nitrate was reduced to ammonium and depleted at the end of the experiment, Goonesekera et al., (2022) deduced that it is the main metabolic pathway.

However, only low concentrations of ammonium (<1,2 mg/L) were present in the dNMS bottles.

4.2 Biodegradation of organic micropollutants

The MOMs in the short SRT batches were expected to display less effective biodegradability due to the lack of activity. Generally, it was difficult to conclude any trends as the MOMs with short SRT depicted a large variety in degradation performance. DFC and SMX were the only compounds degraded above 20% (table 6). As there was generally low activity, this aligns with slow metabolization and low nutrient uptake.

For the long SRT bottles, there was a clear trend between the MOMs that had and had not been adapted to OMPs. The bottles without OMP adaptation failed to degrade any compound except SMX above 20%.

The highest removal efficiencies were within the long SRT bottles, adapted to OMPs. DMS, MTP and SMX were all degraded above 20% with SMX displaying the highest removal efficiency of 81-85%. Interestingly, the MOMs with lower methane loading displayed slightly higher removal efficiencies (table 6) which is contradicted in previous reports (Mortensen et al., 2023). The same report also mentions k_{bio}s being unaffected by a larger methane loading, apart from SMX, furthermore indicating that degradation of SMX is benefitted by a larger methane loading. This was interesting, considering that the bottles with a higher methane loading displayed a higher methane uptake rate i.e. higher methane oxidation. This may indicate heterotrophic degradation. The MMO activity was also notable. pMMO has been reported to benefit from higher O_2 concentrations and would therefore be present to a higher extent in the high methane loading bottles (McDonald, 1997). As SMX is an aromatic compound, sMMO is the only enzyme that should be able to degrade SMX. As the Cu^{2+} concentration in the media was higher (0,12 $\mu mol \cdot L^{-1}$) than the reported inhibitory concentration for sMMO (0,1 $\mu mol \cdot L^{-1}$), this indicates that pMMO might be capable of degrading aromatic compounds which has been suggested in previous studies by Benner et al., (2014). The same report also suggests partial biodegradation by heterotrophs for water samples spiked with OMP concentrations below 10 $\mu g \cdot L^{-1}$, which can be applied in this thesis as the concentrations were aimed to be about 2 $\mu g \cdot L^{-1}$.

DFC was unfortunately below the detection limit for most of the long SRT samples. This was concerning since the OMPs were procured from the same stock solution. As DFC was moderately degraded for the short SRT bottles (table 6), it would have been interesting to get an accurate measurement for the

long SRT. The k_{bio}s were negative for DFC for all the bottles. This is most likely due to measurement errors as the DFC concentrations were far above and below the starting concentrations at different time intervals (figure 3).

DMS showed moderate degradability, with removal efficiencies of 27-30% (table 6). Wang et al., (2023) have previously reported DMS degradation by *Methylobacter, Methylocystis* and *Crenothrix.* Valverde-Pérez, B. *et al.* (2020) discovered the presence of *Methylocystis* as part of the same MOM culture used in this experiment, however, the presence was only significant after 65 days i.e. after a longer time than for this experiment. MTP also showed moderate degradability, 22-23% (table 6).

The k_{bio}s for most of the compounds were below 0,1, indicating removal below 20% (table A4). However, there were some deviations compared with the removal efficiencies. Most notably, DMT-ESA had a k_{bio} of 2,17 for short SRT and high methane loading (figure 4), while only being degraded by 7% (table 6). The k_{bio} for MCP was also notably high (0,86; figure 4) indicating a removal of above 20% while only being degraded 6% by the MOM with short SRT and high methane loading (table 6).

SMX had k_{bio}s of 4,5-5,3 (figure 4) for the bottles with the highest removal efficiencies (table 6). The k_{bio}s are higher than for previous studies, but with lower removal efficiencies as SMX has been reported to display >97% removal efficiency for MABR treatment and MOB degradation (Mortensen et al., 2023; Benner, J. *et al.* 2014). Wobeser Broedsgaard, (2023) reported removal efficiencies of 71% for an integrated MBR with activated sludge with k_{bio}s of 0,1-0,3. The SMX removal for this experiment had higher k_{bio}s than previous experiments, indicating higher biomass efficiency but with removal efficiencies in slightly lower ranges than for MABR and other MOB degradation.

4.3 Outlook

For future work, examining the effects of SRT would be necessary with enough phosphorus in the media. When studying the effect of the nitrogen source, using uncultivated MOM would facilitate the analyses as there were traces of the previous media in the new re-inoculations. Two different nitrogen sources were in other words present which might have affected the growth and productivity.

A microbial community analysis was not part of the project. It would have been interesting to establish the compositions of the consortiums to conclude how the parameters affect the selection. As low methane loading had higher

removal efficiencies of OMPs, a complete analysis of the different compositions of the high and low methane MOMs would facilitate proper conclusions.

In future experiments, varying the copper concentration would be interesting to examine the effects on OMP removal. The copper concentration is the most important factor governing MMO formation. To examine the substrate ranges and effects of pMMO and sMMO with copper concentrations below 0,1 $\mu mol \cdot L^{-1}$ and above 5 $\mu mol \cdot L^{-1}$ would have been necessary as these are the reported inhibitory concentrations (Stanley et al., 1983). Furthermore, the effects of different temperatures were not tested. Different cultivation temperatures might have improved biomass productivity as well as OMP removal.

Lastly, the degradation products were not analyzed which might have been necessary as they could be potentially harmful to health and the environment.

5. Conclusions

Four different operational parameters were alternated during the inoculations: SRT, methane loading rate, O_2:CH_4 ratio and the nitrogen source. Only SRT and methane loading were tested in 48h biodegradation tests due to time constraints. 15 days SRT, 60 mL methane loading and nitrate were the highest performing operational parameters. For cultivation, the results highlights that a high initial biomass concentration is needed for successful growth as well as a high supply of methane. Nitrate as a nitrogen source was preferred, contrary to previous reports. This might partly be due to slow adaptation as the bacteria was re-inoculated from dNMS to dAMS, leaving traces of nitrate, with two possible nitrogen sources. Redoing the experiments with uncultivated bacteria might reveal more justifiable results.

When comparing the OMP removal, the long SRT bacteria degraded OMPs to a larger extent than the short SRT bacteria. However, the lower CH_4 loading had higher removal efficiencies than the higher loading, of which only MTP was degraded further (23 instead of 22 %). Generally, the OMPs displayed low to moderate biodegradability apart from SMX which was degraded 81-85% with long SRT. DMT-ESA, DMT-OA, DMS, MTP, DFC and BAM were degraded between 19-50 %, displaying moderate biodegradability which might be improved with alternative cultivation

parameters. The biodegradation test also highlighted the importance of adapting the MOBs to OMPs as the bacteria without OMPs revealed low biodegradability.

Finally, the bacteria grown in the second set of experiments are expected to have a slightly lower biodegradation than the long SRT bacteria from the first experiment. The growth performance was slightly lower and thus the rate of co-metabolism is expected to be lower as well. However, further research is needed to establish how MOB communities and their growth parameters affect OMP removal more precisely.

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APPENDIX

1. Media recipes

Table A1: dNMS recipe, adapted from (Whittenbury, et al., 1970). (25 g NO_3/L)

Item	**Chemical**	**Amount**
Stock A	$MgSO_4$ x $7H_2O$	10 g
	$CaCl_2$ x $2H_2O$	1,5 g
	KNO_3	9,4 g
	Distilled water	1 L
FeNaEDTA	FeNaEDTA	0,5 g
	Distilled water	0,1 L
Na_2HPO_4 Stock	Na_2HPO_4 x $2H_2O$	35,6 g
	Distilled water	1 L
KH_2PO_4 Stock	KH_2PO_4	27,2 g
	Distilled water	1 L
Trace solution	Na_2EDTA x $2H_2O$	0,5 g
	Fe SO_4 x $7H_2O$	0,2 g
	H_3BO_3	0.03 g
	$CoCl_2$ x $6H_2O$	0.02 g
	$ZnSO_4$ x $7H_2O$	0.01 g
	$MnCl_2$ x $4H_2O$	0,003 g
	Na_2MoO_4 x $2H_2O$	0,003 g
	$NiCl_2$ x $6H_2O$	0,002 g
	$CuSO_4$ x $5H_2O$	0,025 g
	Distilled water	1 L
dNMS	Stock A	40 mL
	Distilled water	1,6 L
	FeNaEDTA Stock	2 mL
	Trace solution	2 mL
	Dissolve and bring to 2 L	
Sterilization	Autoclave for 20 min and allow to cool down to room temperature	
pH adjustment to 6,8	KH_2PO_4 Stock	10 mL
	Na_2HPO_4 Stock	10 mL

Add until desired pH is reached		

Table A2: dAMS recipe, adapted from (Whittenbury, et al., 1970). (25 g NH_4/L)

Item	**Chemical**	**Amount**
Stock A	$MgSO_4$ x $7H_2O$	10 g
	$CaCl_2$ x $2H_2O$	1,5 g
	NH_4CL	5 g
	Distilled water	1 L
FeNaEDTA	FeNaEDTA	0,5 g
	Distilled water	0,1 L
Na_2HPO_4 Stock	Na_2HPO_4 x $2H_2O$	35,6 g
	Distilled water	1 L
KH_2PO_4 Stock	KH_2PO_4	27,2 g
	Distilled water	1 L
Trace solution	Na_2EDTA x $2H_2O$	0,5 g
	Fe SO_4 x $7H_2O$	0,2 g
	H_3BO_3	0.03 g
	$CoCl_2$ x $6H_2O$	0.02 g
	$ZnSO_4$ x $7H_2O$	0.01 g
	$MnCl_2$ x $4H_2O$	0,003 g
	Na_2MoO_4 x $2H_2O$	0,003 g
	$NiCl_2$ x $6H_2O$	0,002 g
	$CuSO_4$ x $5H_2O$	0,025 g
	Distilled water	1 L
dAMS	Stock A	40 mL
	Distilled water	1,6 L
	FeNaEDTA Stock	2 mL
	Trace solution	2 mL
	Dissolve and bring to 2 L	
Sterilization	Autoclave for 20 min and allow to cool down to room temperature	
pH adjustment to 6,8	KH_2PO_4 Stock	25 mL
	Na_2HPO_4 Stock	25 mL
Add until desired pH is reached		

2. Protocol for OMP stock solution

Protocol for micropollutants stock solution

Equipment: milliQ water, mother stock solution

Terminology:

C_mother = concentration of micropollutant in mother stock solution

C_stock = concentration of micropollutant in stock solution

V_stock = volume of stock solution

V_media = volume of culture media

V_spike = volume to be spiked into serum bottles

C_mp = Target concentration of micropollutant in serum bottle

Values:

C_mother = 1 g/L

C_stock = 200 µg/L

V_stock = 500 mL

V_media = 100 mL

V_spike = 1 mL

C_mp = 2 µg/L

Calculations:

Target concentration is 2 µg/L . This needs to be converted based on our culture media volume:

C_mp = 2 µg/L = 0.2 µg/ 0,1 L = **0,2 µg**/ 100 mL

0,2 µg is the mass of micropollutant that we want to add every time that we spike our V_spike. Therefore the mass contained in V_spike has to be 0,2 µg/L.

From this we can calculate the concentration needed for our stock solution. Indeed 0,2 µg/1mL multiplied to reach our target volume for the stock, will return 0,2 µg * 500 /1mL*500 = **100 µg/ 500 mL**

Now, the mother stock has a concentration of 1 g/L and based on previous calculations we only need 100 µg for each micropollutant.

So, 1 g/L = 1 000 000 µg/1000mL = **100 µg/ 0,1 mL**

In 0,1 mL of mother stock solution, we have the mass necessary to prepare our stock solution.

Procedure:

- Pour 0,1 mL of mother micropollutant stock in a small beaker under a ventilated fume hood.
- Let methanol evaporate.
- Add milliQ water and mix/stir solution.
- Add solution to a graduated flask (500 mL)
- Fill flask to 500 mL with milliQ water

Storage:

If stored in methanol, store at -20 degrees. Store mother stock solutions of individual micropollutants in methanol at -20 degrees.

Store stock solution in fridge covered with aluminum.

3. O_2 & CO_2 concentrations

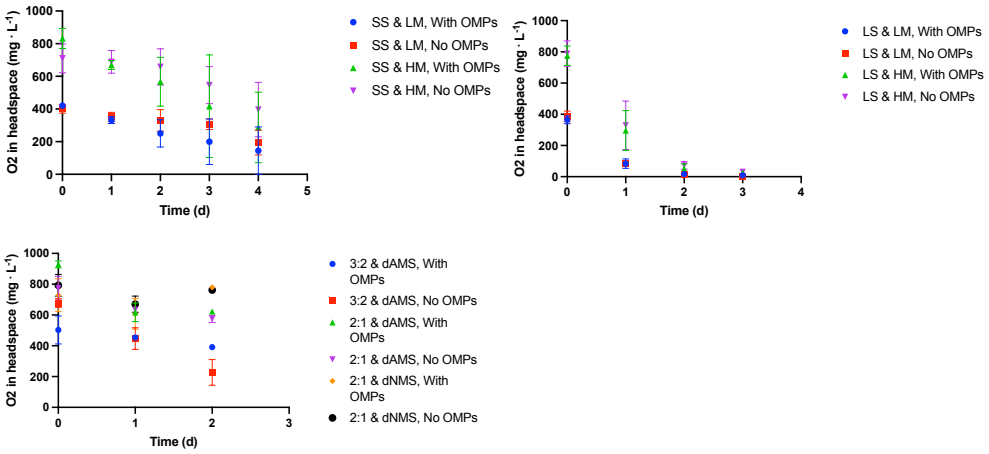


Figure A1: Oxygen depletion in headspace (mg/L). Data are given in mean +/- SEM.

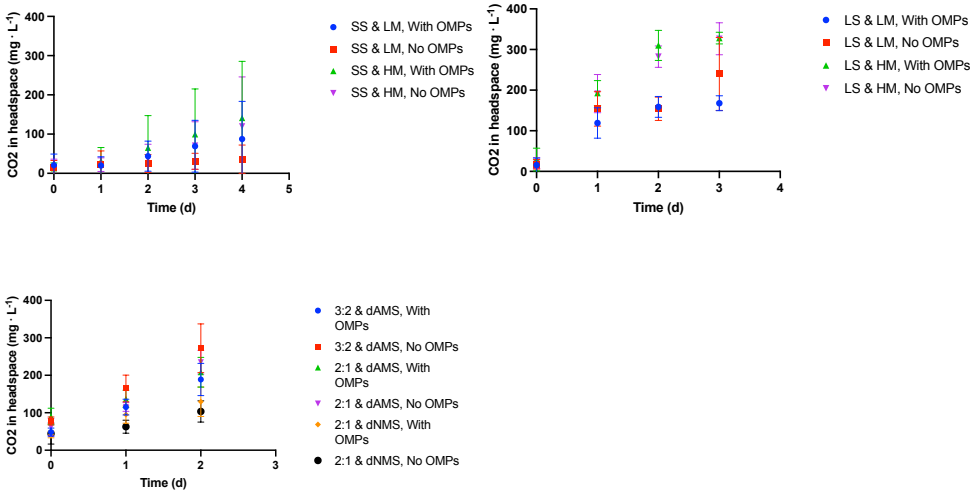


Figure A2: CO2 production in headspace (mg/L). Data are given in mean +/- SEM.

4. Protocol for 48H batch test

48h batch test - Experimental plan

Objective
OMP degradation

Materials

- 2 x 4 SKALAR tubes + caps + 0,2µm filters + 3 mL syringes + needles
- 2 x 14 glass vials for OMP analysis + Methanol/Acetonitrile + 0,2µm filters
- 2 x 2 Cuvettes for OD + 3 mL syringes + needles
- 2 serum bottles + rubber stoppers + caps
- 200 mL media
- 2 mL OMP stock solution
- 2 x 3 mL adapted MOBs
- Pipettes + pipette tips (1 mL, 5 mL, 10 mL)
- CH4, O2, N2 gas
- 60 mL syringe + stopper

Sampling

- Take 14 samples for OMPs with different time intervals
- Three samples for nutrients (SKALAR)
- Two samples for OD + GC
- 1 min interval between bottle 1 and bottle 2

Experimental conditions

- Two bottles with same conditions
- High Methane ratio. 40 mL CH4 and 60 mL O2 in headspace + 100 mL N2
- 3 mL adapted inoculum (one from each CH4 ratio) + 97 mL media + 1 mL OMP stock solution

Before 48h Batch Test

- Prepare serum bottles
- Before first sample give it 1 minute to mix
- Add 2,35 mL of NaNO3 stock of 8 g/L N to each bottle.

Sampling Schedule

Table A3**:** Sampling schedule for 48h batch test.

Clock	**Time (h)**	**Sample Volume (mL)**	**Analysis**
8:00	0	0,8 + 1 + 1,5	SKALAR + OD + OMP + GC (prior)
8:15	0,25	1	OMP
8:30	0,5	1	OMP
8:45	0,75	1	OMP
9:00	1,0	1	OMP
9:30	1,5	1	OMP
10:00	2	1	OMP
12:00	4	1	OMP
14:00	6	1	OMP
15:30	7,5	1	OMP
20:00	12	0,8 + 1	SKALAR + OMP
8:00	24	0,8 + 1	SKALAR + OMP + GC
20:00	36	1	OMP
8:00	48	0,8 + 1,5 + 1	SKALAR + OD + OMP + GC

5. TSS Measurement

The TSS measurement was done by following a standard protocol developed by DTU Sustain.

Protocol for TSS and VSS measurements:

TSS and VSS quantification is based on gravimetric analysis, i.e. weighing of filters before and after filtration of known amount of wastewater and

- Heating at 105 °C to evaporate residual water
- Heating at 550 °C to "burn" organic matter (=volatile solids)

Equipment

- Filtration unit
- Glass fiber filters (0.7 µm or similar pore size)
- Non-filtered samples of influent and effluent
- Influent wastewater: 20—50mL
- Effluent wastewater: 100—250mL

Procedure

a. Pre-condition glass fiber filters with distilled water

b. Insert wet filters in oven at 105 °C for ≥ 1 h

c. Remove filters from the oven and let them reach ambient temperature

d. Weigh dry filters (**A**)

e. Insert dry filters in the filtration unit, and filter known volumes (**V**) of influent and effluent wastewater

f. Insert wet filters in oven at 105 °C for ≥ 1 h

g. Remove filters from the oven and let them reach ambient temperature

h. Weigh dry filters (**B**)

i. Insert dry filters in muffle furnace at 550 °C for ≥ 15 min

j. Remove filters from the oven and let them reach ambient temperature

k. Weigh dry filters (**C**): the residual amount on the filter is inorganic ash

l. Repeat the procedure to run analysis in triplicate

Calculations

(B-A)*1000 / V= TSS [g/L]

(B-C)*1000 / V= VSS [g/L]

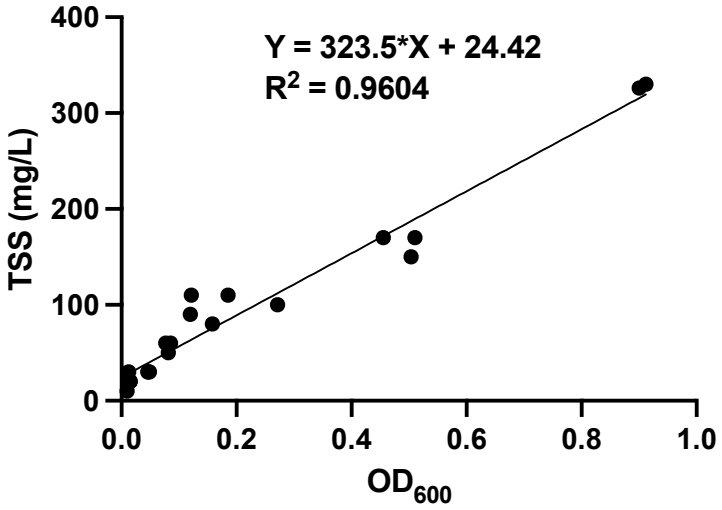


Figure A3: TSS correlation to OD measurements.

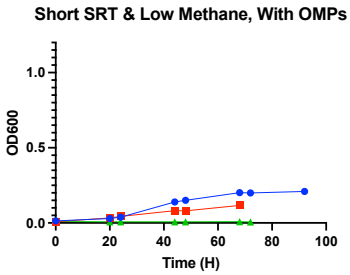
6. Characterization of biodegradability

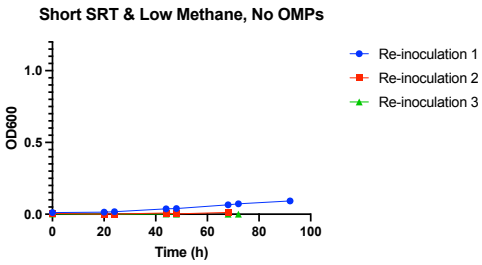
Table A4: Characterization of biodegradability based on literature data. (Joss et al., 2006)

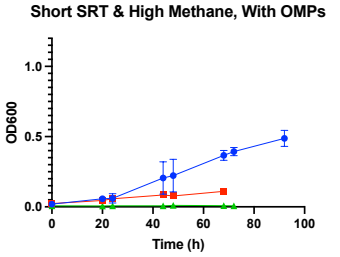
Biodegradability	**k_{bio} ($L \cdot g^{-1} \cdot d^{-1}$)**	**Removal (%)**
Low	<0,1	<20%
Moderate	0,1 – 10	20 – 90%
High	>10	> 90%

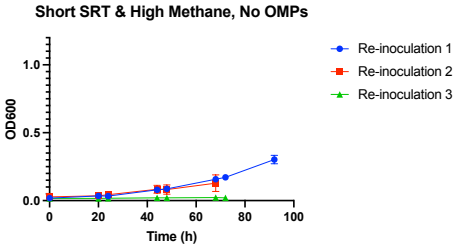
7. Growth curves

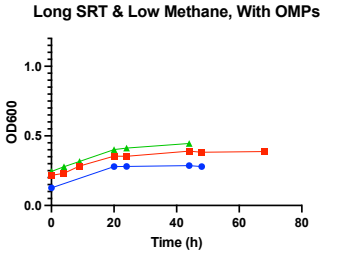
Figure A4 display the growth curves for the different sets of bacteria.

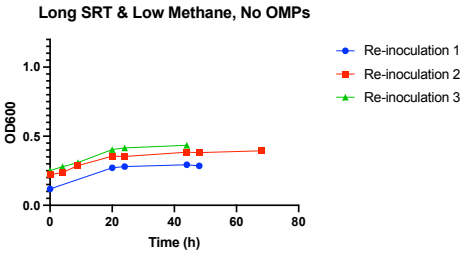

Short SRT & Low Methane, With OMPs
OD600
Time (H)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3

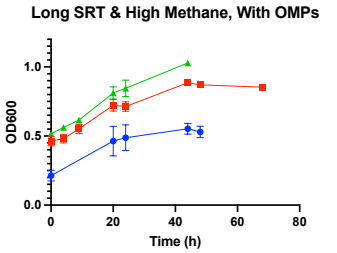

Short SRT & Low Methane, No OMPs
OD600
Time (h)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3

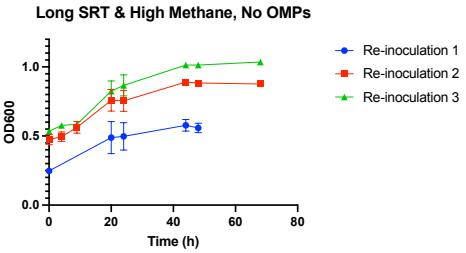

Short SRT & High Methane, With OMPs
OD600
Time (h)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3

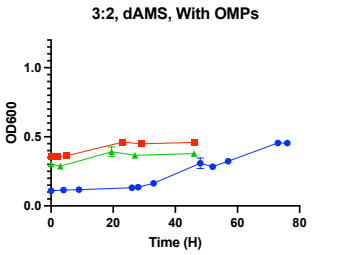

Short SRT & High Methane, No OMPs
OD600
Time (h)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3

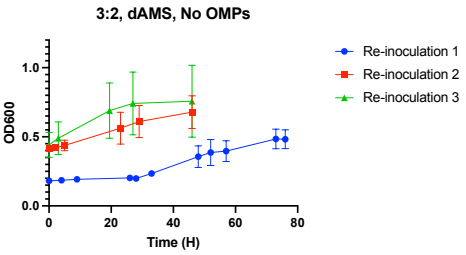

Long SRT & Low Methane, With OMPs
OD600
Time (h)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3


Long SRT & Low Methane, No OMPs
OD600
Time (h)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3


Long SRT & High Methane, With OMPs
OD600
Time (h)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3


Long SRT & High Methane, No OMPs
OD600
Time (h)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3


3:2, dAMS, With OMPs
OD600
Time (H)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3


3:2, dAMS, No OMPs
OD600
Time (H)
Re-inoculation 1
Re-inoculation 2
Re-inoculation 3

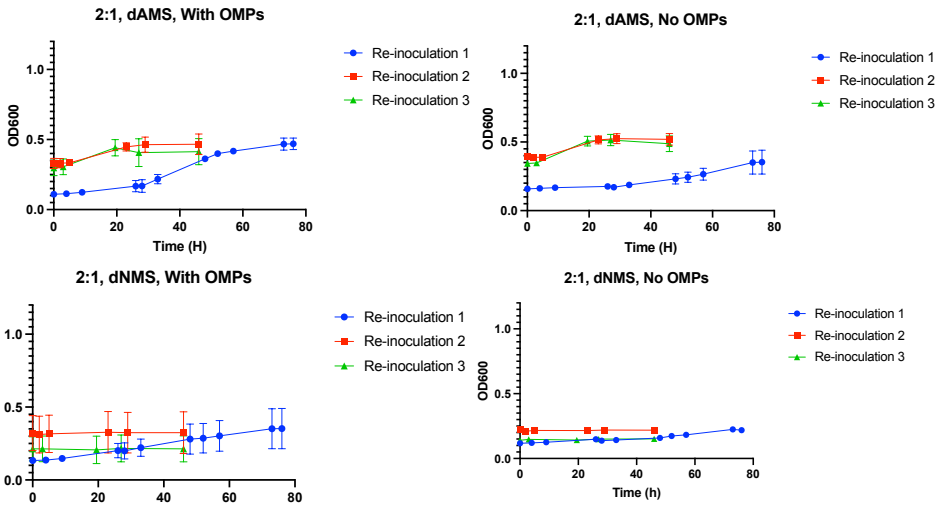


Figure A4: The different growth curves during the three inoculations.