Norwegian University of Life Sciences

Master's Thesis 2018 60 ECTS
Faculty for chemistry, biotechnology and food science

Measuring growth of denitrifiers in digestate using gas kinetics

Måling av vekst hos denitrifiserende bakterier i digestat ved hjelp av gasskinetikk

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Biotechnology

Acknowledgements

I would firstly like to thank my supervisor Lars Bakken, for helping me and encouraging me throughout the whole process, with amongst other things wonderful quotes from Bjørnstjerne Bjørnson, and for being so understanding about all the struggles I have had during this time. I could never have done this without all the wonderful help I got from him, and I am very grateful!

I would also like to thank Daniel Mania for all the help and support with the robot, and all the couching in the lab. He has always been happy to answer all my questions, I would never have been able to do it without him, and his support. He has listened to me, comforted me and understood me. He is truly one of the nicest people I have ever met.

Thank you to Kjell-Rune Jonassen for transporting digestate from VEAS for my experiment, giving me tips in the lab, discussing my experiments and providing me with the Dechloromonas for my last experiment.

Thank you to Lars Molstad, who has showed up at the lab both during a party and in afternoons, when we had technical difficulties with the robot.

To the rest of the NMBU nitrogen group, thank you for bacterial cultures, answering questions and interesting discussions.

It is no secret that I have been struggling with illness during my time working with this master's degree, and I could never have done it without the support of my family and friends.

Thank you mom and dad, for supporting me and believing in me even though times have been tough. Thank you for the emotional and financial support to be able to finish this. Thank you for bringing me home when I needed to go home. I could not have done this without you.

Thank you Anneli, Helene and Bjarne for the long talks, all the understanding, love and support.

Abstract

Denitrification is a microbial, anaerobic process reducing nitrogen oxyanions to gaseous products in a stepwise reaction that leads to a release of nitrous oxide gas to the atmosphere. Denitrification has four steps; NO_3 - NO_2 – NO - N_2O - N2, catalysed by four reductase enzymes NAR/NAP, NIR, NOR and N_2OR, which are coded by the genes nar, nir, nor and nosZ. The intermediate N_2O is a greenhouse gas that escapes the soil and enters the atmosphere. This is unfortunate, because Nitrous oxide has 200 times the potency of CO_2.

A big contributor to the release of nitrous oxide to the atmosphere is agricultural practices, which is why it is important to find a way of mitigating the release from agriculture. The only nitrous oxide sink is soil, so it would be ideal to find a method of using the nitrous oxide before it has a chance to be released to the atmosphere.

Agricultural emissions of N_2O might be mitigated if the bacterial composition in the soil can be swayed to contain more fully fledged denitrifiers, or denitrifiers who express the *nosZ* gene. A thought is to inoculate these bacteria in the fertilizers used in agricultural farming.

NMBU Nitrogen Group started to explore such options a year ago, working with the digestates from anaerobic digestion of wastewater sludge from Vestfjordens Avløpsselskap (VEAS). Their first approach was to enrich N2O reducing bacteria in the digestates by anaerobic digestion with N2O in the headspace, and found that within 2-300 hours of incubation, the digestates became dominated by a *Dechloromonas* strain (identified by metagenomics). Although this strain is a full-fledged denitrifier, it has a strong expression of nosZ, because the digestates enriched with this strain induced very little N2O emission from soil, compared to untreated digestates.

This thesis aimed to try and get denitrifiers to grow in the VEAS digestate in an attempt to find a method to reduce nitrous oxide emissions. This was done by a series of experiments with inoculum of denitrifiers in pure culture, different treatments of digestate and different dilutions of digestate.

Denitrifiers were grown anaerobically in media, before being added to a crimp sealed, He – washed flask with the different digestate treatments. They were then added to an auto sampling robot, where the bacterial growth was measured through gas chromatography.

There was no success with any of the added denitrifiers, but the digestate indigenous Dechloromonas reduced nitrous oxide in an efficient manner, both in fresh, heated and autoclaved digestate, although at a somewhat lower rate in the last two, it was still successful.

Sammendrag

Denitrifikasjon er en mikrobiell, anaerob prosess som reduserer nitrogen oxyanioner til ulike gasser gjennom en stegvis reaksjon som fører til utslipp av nitrogenoksid-gass til atmosfæren. Denitrifikasjon foregår i fire steg; NO_3 - NO_2 – NO - N_2O - N2, katalysert av fire reduktaseenzymer; NAR/NAP, NIR, NOR og N_2OR, som er koded for av genene nar, nir, nor og nosZ. Den intermediate gassen N_2O er en drivhusgass som unnslipper jordsmonnet og stiger opp i atmosfæren. Dette er uheldig, da nitrogenoksid er en 200 ganger mer potent drivhusgass enn karbondioksid.

Jordbruk er en av de største bidragsyterne for utslipp av nitrogenoksid, og det er derfor det er så viktig å finne en måte å redusere utslippet fra jordbruk. Den eneste sinken for nitrogenoksid er jord, og derfor ville det vært ideelt å finne en måte å bruke opp nitrogenoksid-gassen før den har en sjanse til å stige opp i atmosfæren.

Utslipp av N_2O gass kan bli dempet hvis den bakterielle komposisjonen av jorda kan bli suksessfult endret til å inneholde flere fully fledged denitrifiserende bakterier, eller denitrifiserende bakterier som utrykker *nosZ*-genet. En tanke er å inokulere disse bakteriene i gjødsel brukt i jordbruk.

NMBU nitrogen-gruppen startet utforskningen av denne muligheten for et år siden, gjennom å jobbe med digestat fra avløpsselskapet VEAS, Vestfjordens Avløpsselskap. Deres første tilnærming var å tilsette nitrogenoksid-reduserende bakterier i digestatet med N_2O i headspace, og fant ut av innen 2-300 timer av inkubasjon ble digestatet dominert av en Dekloromonas stamme (identifisert gjennom metagenomikk). Selv om denne stammen er en full-fledged denitrifiserende bakterie, så har den en sterk ekspresjon av nosZ, fordi digestatene tilsatt denne stammen induserte veldig lite nitrogenoksid-utslipp from jord, sammenliknet med ubehandlet digestat.

Denne oppgaven hadde som mål å få denitrifiserende bakterier til å vokse i VEAS digestat, i et forsøk på å finne en måte å redusere nitrogenoksid-utslipp. Denne ble gjort gjennom en serie av eksperimenter med inokulum av denitrifiserende bakterier i renkultur, ulike behandliger med digestat og ulike fortynninger av digestat.

Denitrifiserende bakterier ble dyrket anaerobt i media, før de ble tilsatt I en crimpet, He – vasket flaske med ulike digestat-behandlinger. De ble deretter satt i en robot som automatisk tar gassmålinger fra flaskene. Bakterieveksten blir overvåket ved hjelp av gasskromatografi.

Det var ingen suksess med noen av de eksternt tilsatte denitrifiserende bakteriene, men digestatets opprinnelige Dekloromonas-stamme reduserte nitrogenoksid på en effektiv måte, bade i fersk, varmebehandlet og autoklavert digestat. Selv om raten for reduksjon var noe lavere i de to siste behandlingene, var det fortsatt effektivt.

CONTENTS

1. Introduction

1.1 ENVIRONMENTAL ASPECTS

The annual input of reactive nitrogen to the biosphere has more than doubled with human interference. A big part of that nitrogen is types of reactive nitrogen like NO_X and NH_X, stemming from emissions from agricultural activity, and byproducts of fuel combustion. The elevated levels of N has a direct impact on natural systems, like changes in biogeochemical processes, diversity and the trophic system of species (Elser et al. 2009). Anthropogenic N ends up in the atmosphere as N_2, N_2O or NO, because of red-ox reactions of mineral nitrogen by various microbes (Bakken et al. 2012).

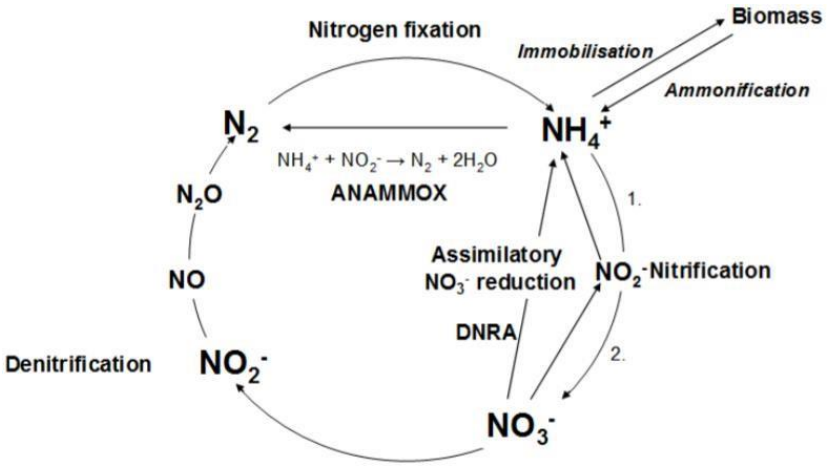


***Figure 1 – The nitrogen cycle**, adopted from (Bergaust 2009).*

In response to oxygen depletion, denitrifying bacteria switch to anoxic respiration, and uses nitrogen instead of oxygen as a terminal electron acceptor to be able to sustain their respiratory metabolism (Nadeem et al. 2013). They do this through the process of denitrification; reducing nitrogen oxyanions to gaseous products in a stepwise reaction that leads to a release of nitrous oxide gas to the atmosphere (Canfield et al. 2010). Denitrification has four steps; NO_3 - NO_2 – NO - N_2O - N2, catalysed by four reductase enzymes NAR/NAP, NIR, NOR and N_2OR (figure 2), which are coded by the genes nar, nir, nor and nosZ (Bakken et al. 2012).

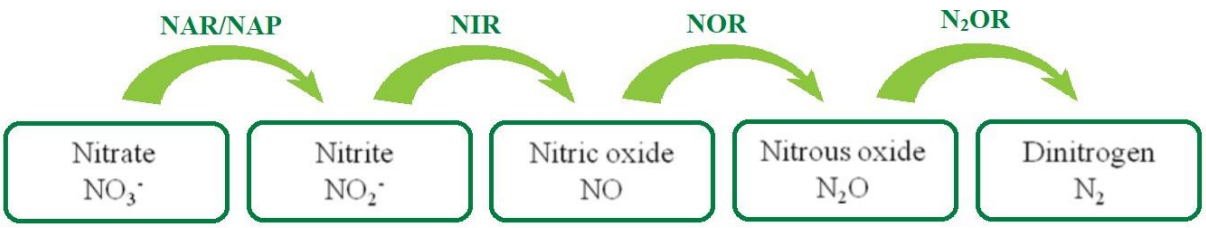


***Figure 2 – Denitrification**. Step 1, reduction of nitrate to nitrite using the reductases NAR: membrane bound nitrate reductase/ NAP: periplasmic nitrate reductase. Step 2, Reduction of nitrite to nitric oxide using the reductase NIR: nitrite reductase. Step 3, reduction of nitric oxide to nitrous oxide using the reductase NOR: nitric oxide reductase. Step 4, reduction of nitrous oxide to the harmless gas dinitrogen using the reductase N_2OR: nitrous oxide reductase.*

Several soil microbial pathways produce N_2O. Nitrous oxide is a by-product of nitrification, and an obligate intermediate in denitrification. Out of the total N_2O in the atmosphere, 70% stems from soil (Mosier 1998). If one compares N_2O and CO_2 as greenhouse gases, nitrous oxide has a 300 times greater warming potential per molecule (Forster et al. 2007). N_2O is also the main factor depleting the ozone layer (Ravishankara et al. 2009). Since both nitrification and denitrification produce N_2O as an intermediate, when looking at global N_2O emissions, agriculture is a big contributor with a quarter of the nitrous oxide emissions (Canfield et al. 2010). There is however only one sink for N_2O that is known; reduction of N_2O to the harmless gas N_2 by organisms that have and express the nitrous oxide reductase (N_2OR) encoding gene *nosZ* (Juhanson et al. 2017).

More than 60 genera of microorganisms carry out denitrification, and the ratio between the gases resulting from the denitrification process is hypothetically corresponding with the taxonomic composition of the denitrifying community. This is because the organisms have different regulation of the denitrification gene expression, and because some organisms have a

"truncated" denitrification process where they lack one to three of the denitrification genes encoding enzymes necessary for different steps of the denitrification. These organisms can be a problem if they lack the functional gene to reduce N_2O to N_2. "Full-fledged" denitrifiers (denitrifiers who are able to reduce NO_3 completely to N_2) have different patterns for regulating the different genes, which results in the genes being expressed at different times, and the intermediates accumulate at different times. (Nadeem et al. 2013). An example is the genus Thauera, that have a significant transient production of N_2O when switching to anoxic conditions, because the gene encoding N_2OR lags behind in expression compared to NIR and

NOR (Liu et al. 2013). A denitrifying community dominated by organisms with a very strong expression of nosZ (such organisms exist; Paracoccus denitrificans for instance) will have an inherently lower N_2O/N_2 product ratio than a community which is dominated by organisms with weak expression of nosZ. Likewise, domination by organisms which lack the nosZ genes will have higher inherent N2O/N2 product ratios than communities dominated by organisms equipped with only nosZ (lacking nar, nir and nor) (Qu et al. 2016).

1.2 AIM OF THIS THESIS

Agricultural emissions of N_2O might be mitigated if the bacterial composition in the soil can be swayed to contain more fully fledged denitrifiers, or denitrifiers who express the *nosZ* gene. A thought is to inoculate these bacteria in the fertilizers used in agricultural farming.

This study tries to get fully fledged denitrifiers to grow in biorest.

In theory, this could be achieved by inoculating such bacteria to soils. To have any effect, however, the amount of inoculum would have to be within the same order of magnitude as the number of indigenous denitrifying denitrifying bacteria in the soil, which is 10^7-10^8 cells g^{-1} soil, based on Lycus et al (2017) (17% of the culturable aerobes were denitrifiers). For an inoculum to have an effect on the gas kinetics, one would thus have to add $2*10^{12}$ -10^{13} cells m^{-2} soil surface (assuming 200 kg soil m^{-2}). This would be extremely costly as a stand-alone operation, requiring establishment of large scale fermenter systems and adequate pipelines to the farmed soil. We would not need such investments however, if we could exploit anaerobic digestion technology as a platform:

Anaerobic digestion is a technology for producing biogas from wastes such as urban organic waste, wastewater sludge and animal manure, and within the next decade, this technology will be implemented at large scale throughout the developed countries. The digestates (i.e. the organic material remaining) from this industry is destined for agricultural soils as fertilizers. In theory, these digestates contain sufficient amounts of organic carbon to sustain the growth of respiratory organisms to reach high cell densities. If so, the marginal costs of enriching the digestates with N2O-reducing bacteria would be marginal.

NMBU Nitrogen Group started to explore such options a year ago, working with the digestates from anaerobic digestion of wastewater sludge from Vestfjordens Avløpsselskap (VEAS). Their first approach was to enrich N2O reducing bacteria in the digestates by anaerobic

digestion with N2O in the headspace, and found that within 2-300 hours of incubation, the digestates became dominated by a *Dechloromonas* strain (identified by metagenomics). The number of cells reached ~10^9 mL^{-1}, and the strain was subsequently isolated. Although this strain was a full-fledged denitrifier, it evidently had a strong expression of nosZ, because the digestates enriched with this strain induced very little N2O emission from soil, compared to untreated digestates (Jonassen et al, unpublished).

NMBU nitrogen group has for long studied such full-fledged denitrifiers with strong nosZ expression, and their model strain *Paracoccus denitrificans* is of particular interest because it has a conspicuous *bet- hedging* strategy: when experiencing declining oxygen concentrations, all the cells express nosZ, while only a minority express the other denitrification genes. As a result, the population becomes a strong sink for N2O, thus potentially quenching the N2O emission from soil.

To be able to study this bet hedging, they constructed a strain in which the gene for the fluorescent protein mCherry was coupled to the gene for nitrite reductase (*nirS*) . This mCherry-nirS strain was used to verify the bet hedging hypothesized from modelling (Lycus et al. 2018).

2. Materials

2.1 Digestate

To secure identical digestates for all experiments, a large volume of digestate was collected at one occasion, and froze in portions. The digestate was collected from the wastewater treatment plant VEAS, Vestfjorden Avløpsselskap on the 10th of October 2017. It was sanitized by warming it evenly up to 55 °C for 3 hours, and frozen in portions of 250 mL at 20 °C. For each experiment, a portion was thawed overnight at 4 ºC prior to further treatments. The characteristics of the digestate is shown in table 1.

Figure 3: The digestate was collected from this tank at the wastewater treatment plant.

Table 1 Parameters for the digestate. *A – average values for 2017, B – values for our sample 10.10.17.*

[a] Amounts expressed as percentage of wet weight. [b] Amounts expressed as percentage of dry weight. [c] Determined by titration. [d] Determined by titration. [e] FOS = Flüchtige Organischen Sühren = VFA.

A

	Digestate characteristics						
Parameter:	pH:	TS (%)[a]:	VS (%)[b]:	Total alkalinity (mekv/l)[c]:	VFA (mekv/l)[d]	FOS/TAK[e]	N-NH_3/NH_4^+ (mg-N/L);
Mesophilic digestate (T = 37 °C)	7,61 ± 0,06	3,84 ± 0,11	54.22 ± 1.40	185 ± 6	16.1 ± 0.53	0.087 ± 0.004	1824 ± 46

Operational parameters		
Specific gas production (Nm^3/kg VS·d) :	HRT (d):	VS loading rate (kg VS/m^3·d):
1,03 ± 0,27	24.38 ± 6,34	2,41 ± 0,76

B

	Digestate characteristics						
Parameter:	pH:	TS (%)[a]:	VS (%)[b]:	Total alkalinity (mekv/l)[c]:	VFA (mekv/l)[d]	FOS/TAK[e]	N-NH_3/NH_4^+ (mg-N/L);
Mesophilic digestate (T = 37 °C)	7,59	3,82	54,95	172	14	0,081	1838

Operational parameters		
Specific gas production (Nm^3/kg VS·d) :	HRT (d):	VS loading rate (kg VS/m^3·d):
1,15	22,6	2,3

2.2 Laboratory equipment

Laboratory equipment	**Supplier**
Glass flask 50 mL	Matriks
Aluminium caps	Matriks
Rubber septa	Matriks
Automatic pipettes of different volumes	Thermo
Parafilm	
Petri dishes	
Sterile filters, 0.20 µm pore size	Sarstedt
Centrifuge tubes, 1.5, 15 and 50 mL	
Glass gas syringes with pressure lock, 5 mL	VICI Precision Sampling
Plastic syringes of different volumes	BD Plastipak
CryoTubes	
Different glass equipment	
Aluminium cap closer and opener	
Glass Blue cap bottles 500 mL and 1L	
Magnetic stirrer	
Stirring magnets	

Instruments	**Supplier**

Autoclave	
Sterile bench	Thermo Fisher
Vortexer	VWR
Autosampling robot	
Gas Chromatograph AgilentTG Technologies, 7890A GC systems	Agilent
Gas evacuation and filling system	
Stirring water bath	
Centrifuges Kubota 3500 Eppendorf minispin microsentrifuge	 Kubota Eppendorf
Nitric Oxide analyser NOA 208i (Out of order during my experiments)	Sievers
Delta 320 pH metre	Mettler
WPA Spectrawave s800 Diode array Spectrophotometer	

2.3 Gas standards for the robot

Standards	**Components**

High GC Standard	150 ppm N_2O 1% CO_2
	1% CH_4
Low GC standard	5 ppm N_2O 100 ppm CH_4 5 ppm H_2 250 ppm Sulphur Hexafluoride, F_6S 2000 ppm CO_2
NO standard	N_2 containing 25 ppm NO

2.4 Media

A modified Sistrom's media (Sistrom 1960) was used as growth media to cultivate *P. denitrificans*.

34.8 g/l	K_2HPO_4
1.95 g/l	NH_4Cl
40 g/l	Succinic acid
1 g/l	L-Glutamic acid
0.333 g/l	L-Aspartic acid
5 g/l	NaCl
2 g/l	Nitrilotriacetic acid

3 g/l	$MgSO_4 * 7 H_2O$
0.34 g/l	$CaCl_2 * 2 H_2O$
0.02 g/l	$FeSO_4 * 7 H_2O$
1 ml/l	Trace elements solution 10,000x
1 ml/l	Vitamins solution 10,000x

TSB (Tryptic Soy Broth) media was used to grow Bacillus vireti

30 g	TSB
5.9 g	$Na_2HPO_4 * 2H_2O$
5.4 g	$NaH_2PO_4 * H_2O$
Fill up to 1 L	Distilled water

3. Methods

3.1 Freezer

1. The different bacterial strains used was first isolated as a single colony on a petri dish with ideal agar, and then made into a glycerol stock in cryo-tubes stored in the -80 ºC freezer
2. The digestate used was all sampled the same day and frozen In portions to create a uniform baseline for all the experiments.

3.2 Digestate treatments

A large amount of digestate was sampled at one time. Most of the digestate sampled want through a sanitation process of being heated at 55 ºC for 3 hours before being frozen. A small portion was frozen directly while still fresh.

These are the different treatments of digestate used in this experiment:

1. Fresh
   This is the only digestate not collected at the same time as the rest of the digestate used in the experiments, because it had to be freshly collected from VEAS and used directly in the experiment.

2. Frozen fresh
   This is from the original digestate, but it was not heated to 55 ºC before freezing, and had just been frozen.

3. 55 ºC heated
   This digestate had just gone thrugh the 55 ºC heating for 3 hours, frozen, and thawed for the experiment.

4. 70 ºC heated
   Digestate that was first heated to 55 ºC, then frozen, thawed, and heated again at 70 ºC for 3 hours.

5. Autoclaved
   Digestate that was first heated to 55 ºC, then frozen, thawed, and autoclaved.

3.3 Preparation for experiment

The digestate to be used in any experiment was either thawed at 4 ºC overnight, or thawed in a shaking water bath and heated in the same water bath to 70 ºC, and then kept at that temperature for 3 hours.

120 mL flasks were autoclaved either containing water, digestate, or media. The flasks containing a mix of the different things were autoclaved separately, so as to not react with each other during the autoclaving process, and then pipetted into a sterile flask in a sterile bench.

The flasks to be used for digestate that is not autoclaved, or a mix of media and digestate, were autoclaved containing just enough water to create steam in the bottles in order to sterilize them. When the increase in pH in digestate from autoclaving was discovered, the digestate was autoclaved in a separate blue cap bottle, and transferred into sterile flasks after being pH regulated.

The digestate was pH regulated using HCl.

The inoculums for the experiment were grown in autoclaved media in a water bath at room temperature. The inoculums were grown until they had an OD above 1, so they could be dialuted to and OD of 1. This was done so that the different inoculums could be easily compared to one another.

3.4 He – washing

The flasks are autoclaved with aluminium foil covering the opening. They are moved to a sterile (UV – light before use) flow bench where the rubber septa are put on the flasks. They are then crimped tightly shut.

We are now able to control the gasses in the vials completely. We do this by He – washing the flasks through a series of vacuum followed my helium being pumped into the flasks. Several cycles makes sure that we can suck almost all of the gasses from the liquid phase out of the flasks as well. This is ideally done the day before the experiment, to make sure there is equilibrium between the phases, not to create a false positive from just leakage of gas from the liquid.

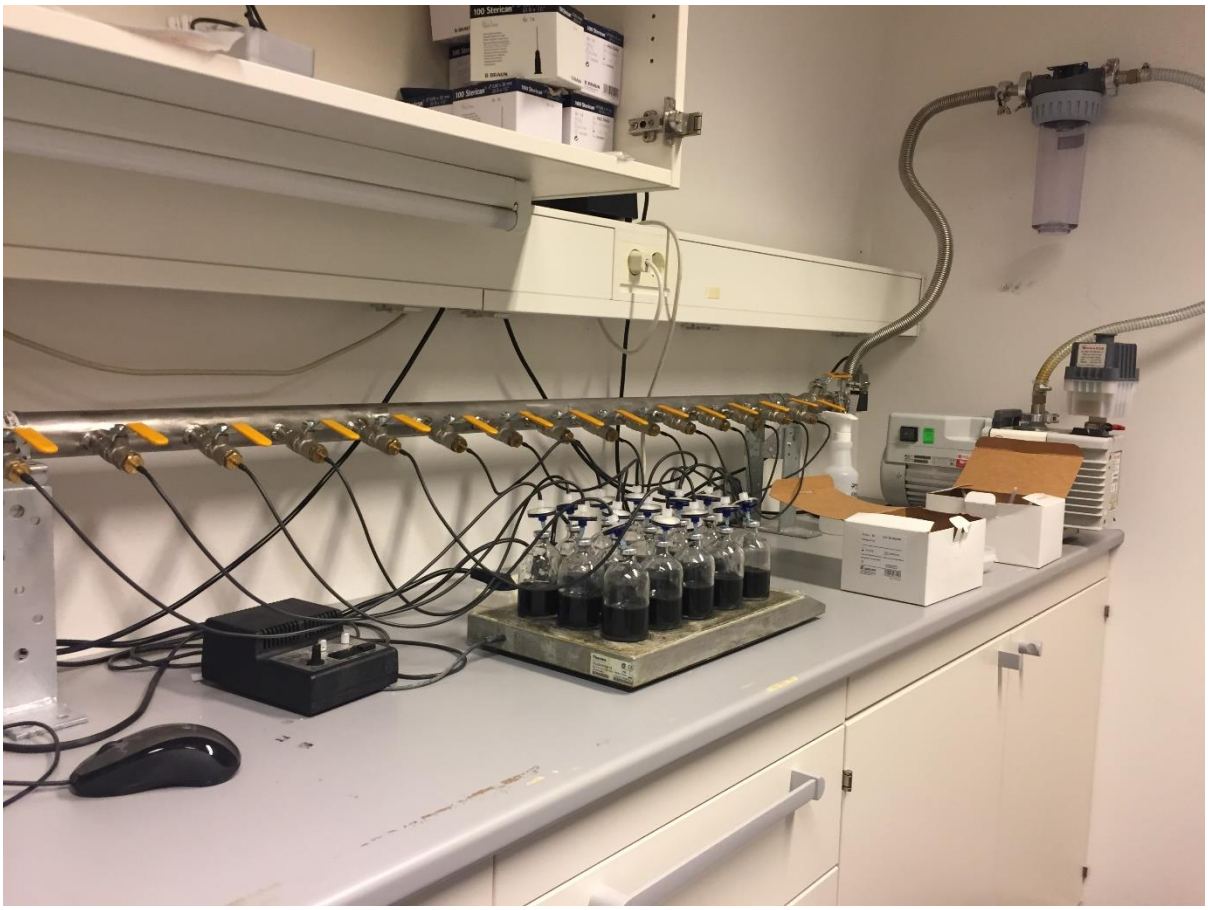


Figure 4: He - washing

He – washing for media:

3, 180, 30, 40; 3 cycles, 180 sec vacuum, 30 sec He-filling. The last 40 are He-filling after the 3 cycles are done, to ensure that the bottles are pressurized. This is important because in ensures that there is no leakage of lab air into the flasks.

The He – washing is the same for the digestate, but here the He – washing is done in 6 cycles, because of the higher viscosity of the digestate.

3.5 Getting ready for the robot

The flasks were placed in the robot, and a syringe without the piston filled with alcohol or filtrated water was used to release the over pressure. The liquid in the syringe would bubble as long as there was overpressure, and this method would prevent lab air from entering the flasks. An air tight gas glass syringe was then used to remove the same amount of He from inside the bottle that was going to be added of another gas with the same syringe right afterwards. The syringe was filled with the designated gas, and the overpressure was released by opening the air lock while the syringe was submerged in alcohol until no more bubbles were released. Then the gas was injected into the flask.

Now nitrite or nitrous oxide was added, if it was used in the flask, using a syringe. Finally the inoculum was injected, and the robot was started through the programme Python.

3.6 ROBOTIZED INCUBATION SYSTEM FOR GAS MONITORING

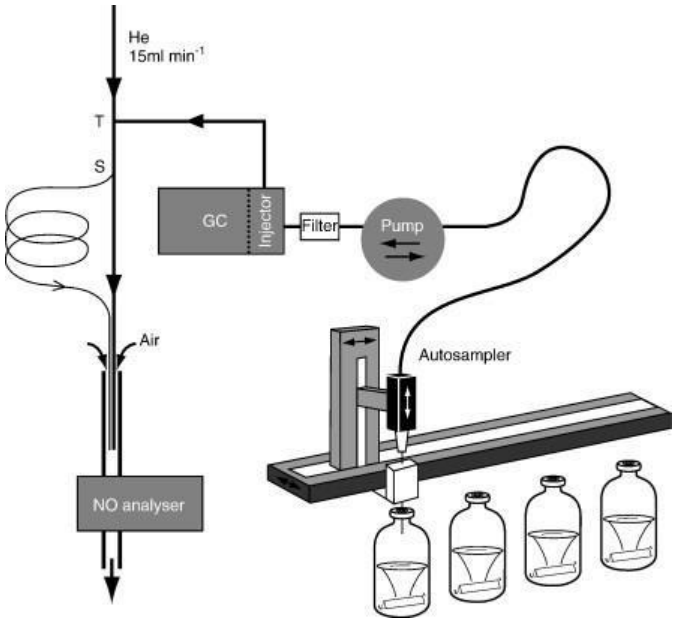


Figure 5 *– This figure is adapted from (Molstad et al. 2007). The robotized incubation system samples gases from the closed flasks via a needle using He as a carrier gas, and transfers them into a gas chromatograph and an NO analyser.*

The robotized incubation system contains of a water bath with room for 30 crimp sealed flasks with magnetic stirring. All my experiments were done at 20 ºC, with rpm of 400 on the magnetic stirrer. There are 3 more spots in the water bath with no stirring, where we can place the gas standards (see methods).

Figure 6: The robot set up. This is a picture of the old robot with only 15 stirring positions. There is a total of 3 robots today.

The robot uses the gas standards with known concentrations to calculate the raw data from the experiment coming from the NO-analyzer and Gas Cromatograph. A Gilson 222 autosampler and a peristaltic pump was responsible for headspace sampling through the rubber septa. The concentrations of N2O, N2, O2 and CO2 was analysed in a Varian CP4900 micro GC, with two columns. It has a thermal conductivity detector (TCD). An electron capture detector (ECD) and a flame ionization detector (FID). The GC was connected to the auto sampler, that was controlled by a programme that was written in Python, and the GC was controlled through EZchrome elite (Molstad et al. 2007).

This enables us to get accurate and real-time results.

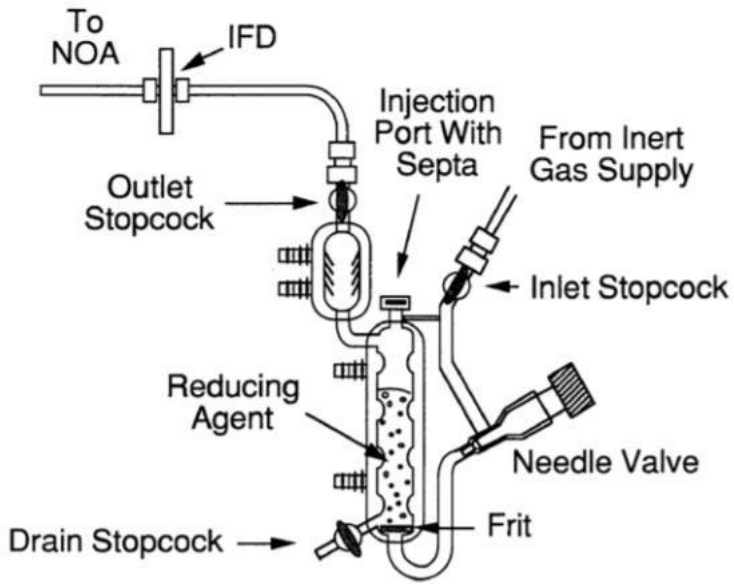


Figure 7 *– Figure sourced from the Sievers Nitric Oxide Analyzer NOA 280i Operation and Maintainance Manual, kp. 109.*

4. Results

4.1.1 Experiment 1: Experiment 1: O_2 and N_2O consumption, and CO_2 production in autoclaved digestates with and without inoculum (Paracoccus denitrificans mCherry nirS strain)

Liquid	Gas	Bacteria	Extra Gas
Autoclaved digestate	1 mL O_2	-	4 mL
Autoclaved digestate	1 mL O_2	Paracoccus denitrificans	4 mL
Autoclaved digestate	4 mL N_2O	Paracoccus denitrificans	-
70 °C heated digestate	4 mL N_2O	-	-
70 °C heated digestate	4 mL N_2O	Paracoccus denitrificans	-
Sistrom's	4 mL N_2O	Paracoccus denitrificans	-

4.1.2 Experiment 1: O_2 and N_2O consumption, and CO_2 production in autoclaved digestates with and without inoculum

(Paracoccus denitrificans mCherry nirS strain)

This experiment was meant as a first crude approach to test if Paracoccus was metabolically active (and growing) in digestate, and whether autoclaving the digestate had a negative effect, and whether or not heating the biorest to 70 °C for three hours would be enough to kill the indigenous bacteria in the biorest. We wanted to use consumption of O_2 and N_2O as measure of metabolic activity, hence autoclaving would be desirable to avoid confounding of metabolic activity of indigenous organisms and that of Paracoccus. In theory, autoclaving the digestate could result in the formation of toxic substances, hence we included a milder heat treatment (70^{o}C) as an alternative (presumably killing most of the potentially respiring organisms in the digestate without forming toxic substances).

Another point was to look at the oxygen reduction in the biorest. Another experiment was conducted before this, where TSB and Sistrom's media was added to 70 °C heated biorest, but there were too many technical difficulties with the robot and integration of the data to use it. We did however find the oxygen reduction of the digestate to be surprisingly high, leading to the addition of the flasks with added oxygen in this experiment.

After heat treatment (autoclaving, and 70 ºC treatment), the digestate was portioned into 120 mL serum vials (50 mL in each + 50 µ mole NO_3) with a Teflon coated magnetic stirrer. The vials were crimp sealed with butyl rubber septa and He-washed (Molstad et al 2007). One set of the vials were supplemented with 0.35 mL pure O2 (=> 0.5 vol % O2 in headspace) and another set of the vials were supplemented with 3 mL N2O (=> 3 vol % in headspace after reaching equilibrium with the liquid; N2O is very soluble in water: 0.028 mol L^{-1} atm^{-1} at the temperature used (20.0 ºC)). For both sets, half of the vials were inoculated with Paracoccus denitrificans mCherry nirS. The flasks were then incubated at 20 ºC in the incubation robot

(all vials were stirred), monitoring the gas kinetics by frequent sampling. A second dose of O2 was given to the vials after 50 h.

All the different bottles were done in triplets, but due to the high degree of similarity between them, only one table for each is shown here.

All the graphs for all the experiments show N_2-N and N_2O-N. This means that instead of showing the concentrations of the molecules, we show the concentration of nitrogen atoms.

N_2-N and CO_2 are accumulated values compensated for sampling from the headspace by the robot.

Oxygen was quickly consumed in autoclaved digestates, independent of the inoculation, suggesting abiotic oxygen consumption by the autoclaved digestate (Fig 5 A). Thus, any oxygen consumption by Paracoccus was effectively “drowned” in abiotic oxygen consumption. However, the complete lack of NO-production after oxygen depletion suggested that the organism was not active (Fig 5 B). There was some apparent production of N2, but equal amounts were apparently produced in the autoclaved digestate without P denitrificans (Fig 5C).

As would be expected, based on the foregoing, the vials with N_2O in the headspace showed no sign of active N_2O reduction by Pa denitrificans (Fig 6). The N_2O detection in these experiments was suboptimal, however, causing substantial noise.

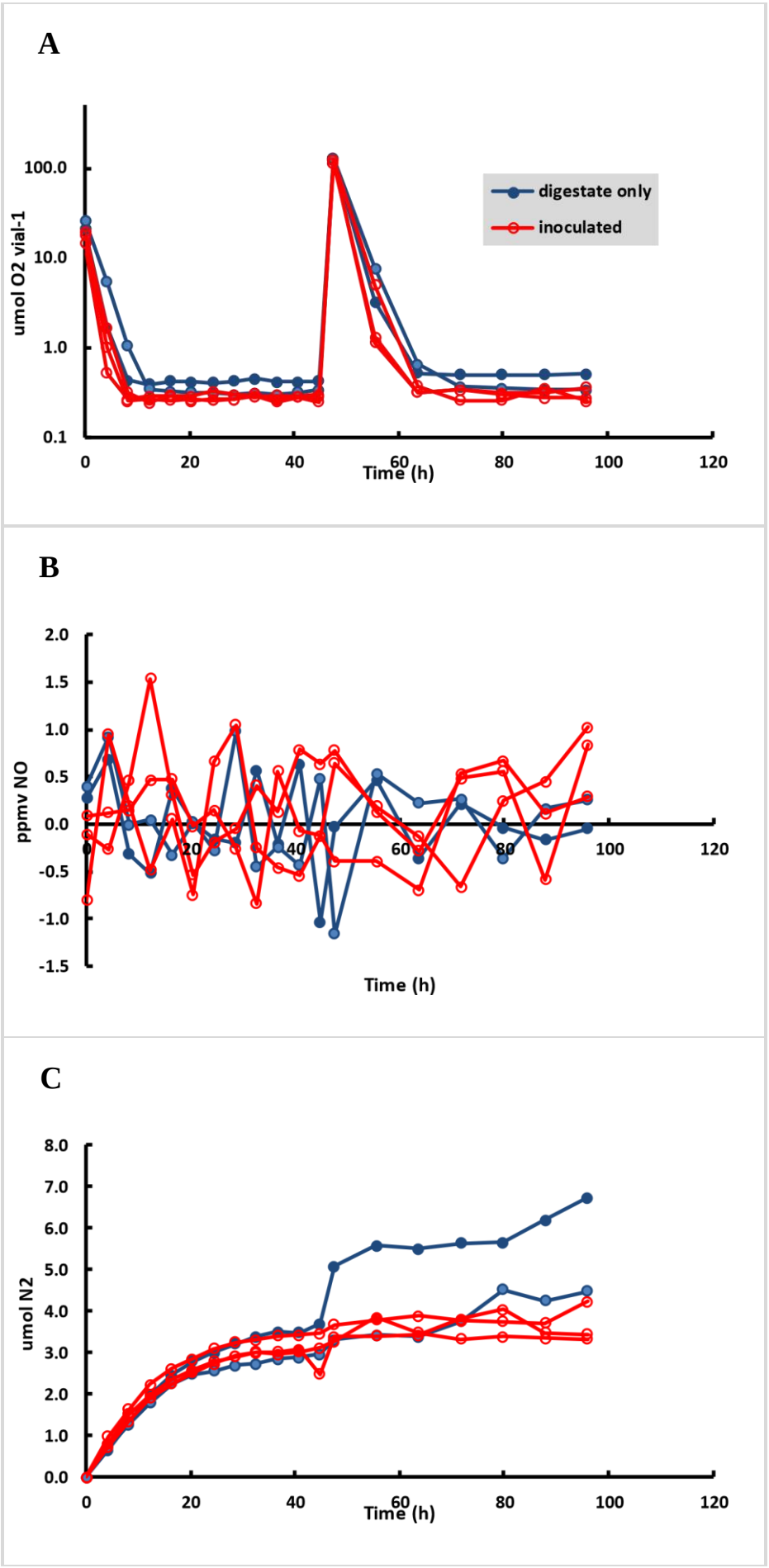

A
100.0
10.0
1.0
0.1
umol O2 vial-1
digestate only
inoculated
0
20
40
60
80
100
120
Time (h)
B
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
ppmv NO
Time (h)
C
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
umol N2
Time (h)

Fig 8. Oxygen consumption, NO and N2 production in autoclaved digestates, with and without P denitrificans. Evident abiotic oxygen consumption was observed (panel A), effectively hindering the use of oxygen consumption as a measure of metabolic activity of P denitrificans. Since the digestate was supplemented with NO3-, however, anaerobic respiration (denitrification) would result in NO accumulation, but there was no indication of this (panel B: NO was essentially below detection limit of the system, resulting in negative peak integrations in a number of cases). The apparent production of N2 (panel C, showing estimated cumulative N2 production) was identical for vials with and without P denitrificans

(the deviation for one of the un-inoculated vials was caused by injection of N2 together with O2 after 45 h). This apparent N2 production is most probably due to gradual release of residual N2 within the matrix (not removed by the He-washing).

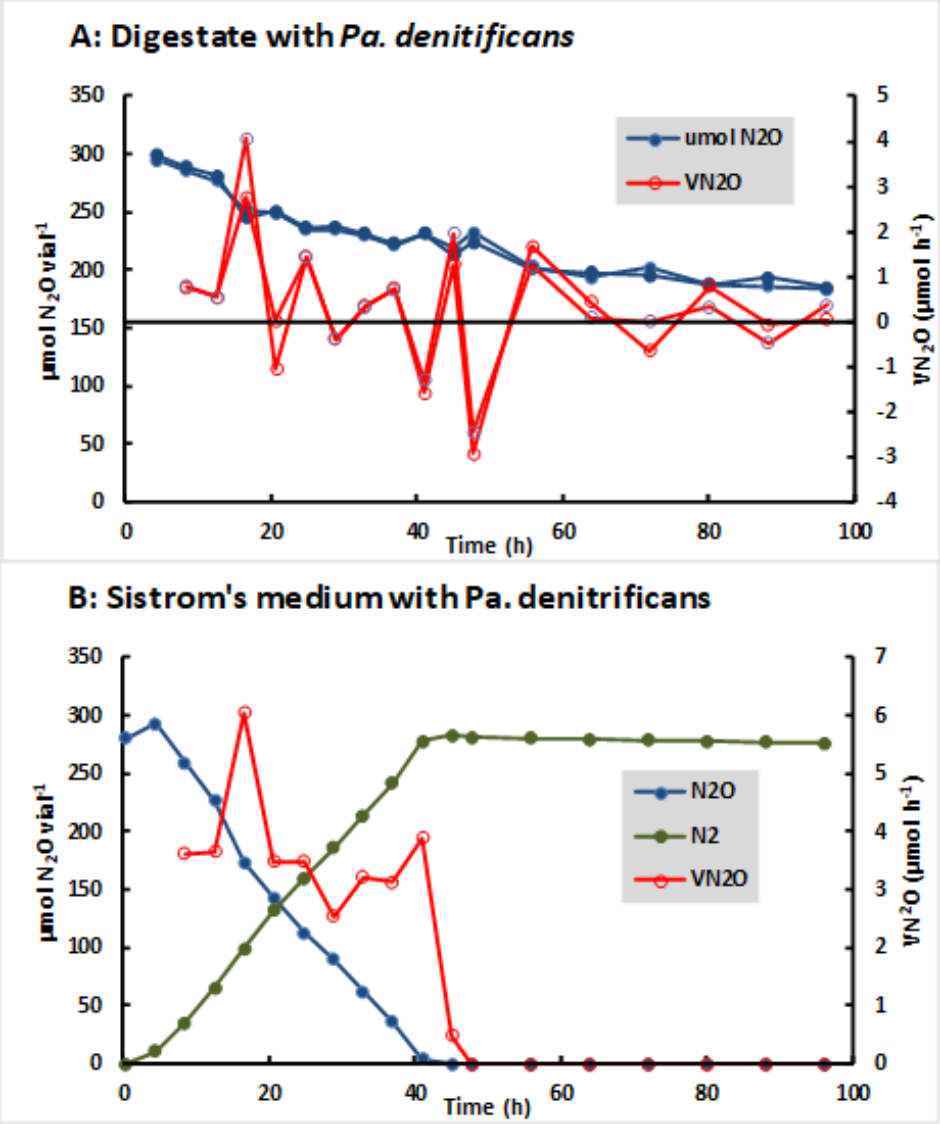


Fig 9: N2O consumption by Pa denitrificans in autoclaved digestate? The panels show the measured N2O and the estimated rates of N2O reduction for each time increment between two measurements in inoculated digestate (Panel A) and Sistrom's medium (Panel B). The results suggest some minor N2O reducing activity to begin with, but most of the decline in measured N2O is due to sampling (the estimated rates fluctuate around zero). In contrast, Pa denitrificans effectively depleted all the N2O within 50 h when growing in Sistrom's medium.

The results for the digestate heated to 70 C is shown in Figure 7, showing essentially the same result as for the autoclaved digestate. The inclusion of inoculated digestate in this case (panel B) suggest that the apparent initial N2O-reduction was due to instabilities in the quantification of N2O in this experiment.

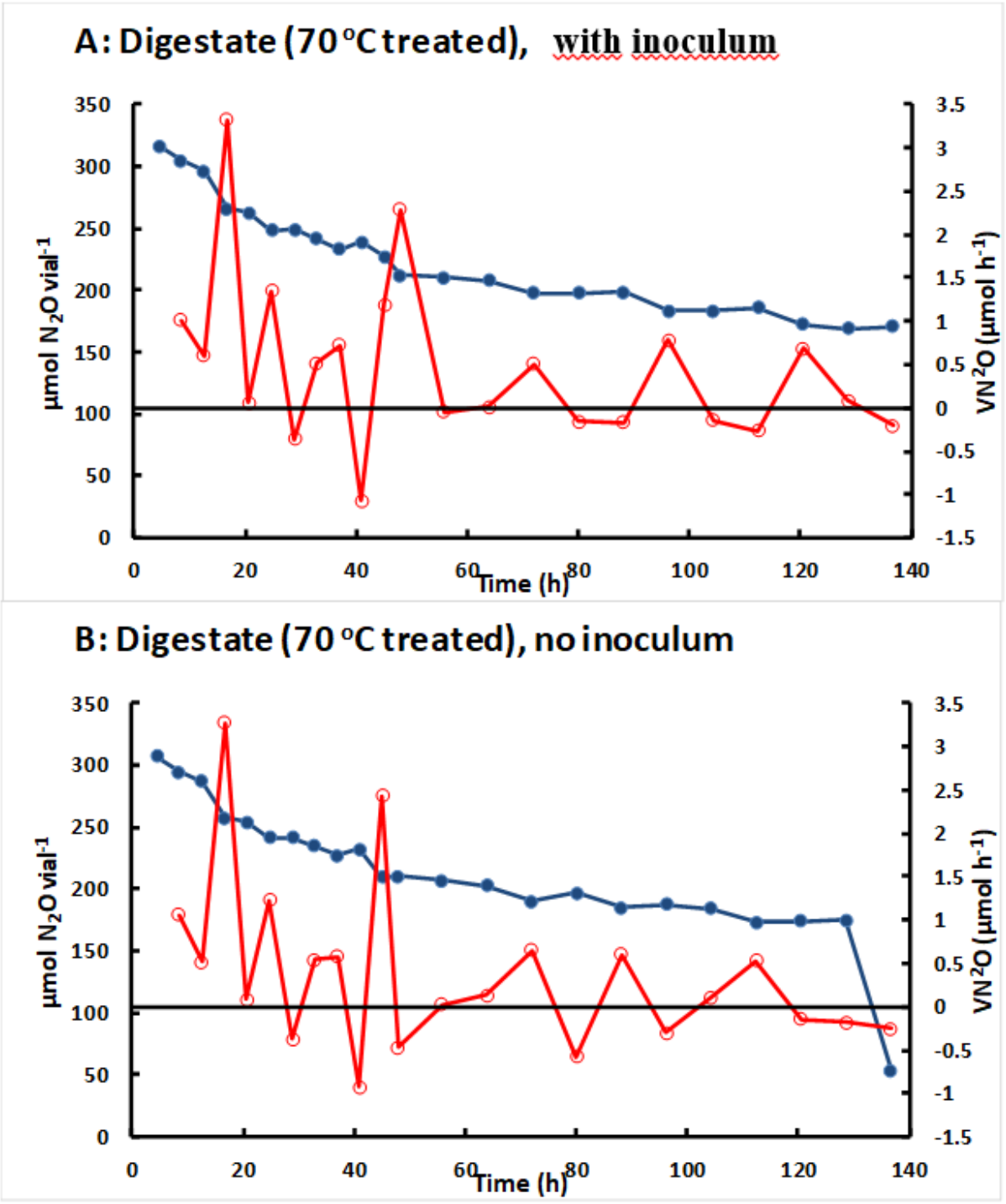


Fig 10. N2O reduction in 70 oC tretated digestate? The panels show the measured N2O and the estimated rate of N2O reduction for vials with digestate inoculated with Pa denitrificans (Panel A) and without inoculum (panel B).

The apparent N2 accumulation in all treatments (except Sistrom's with Pa denitrificans) were essentially identical to that shown for autoclaved digestate (Fig 5 Panel C).

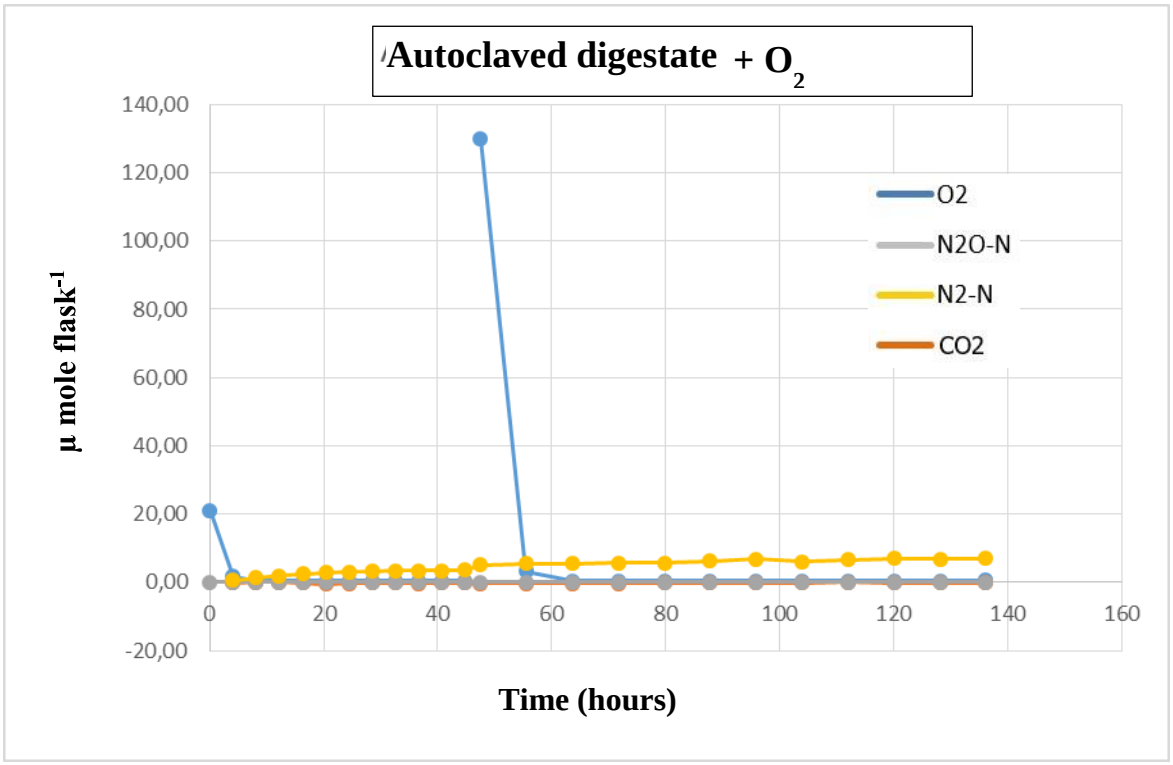


Figure 11 - The reduction rate of oxygen in the autoclaved biorest was calculated to 46.2 µmol * $vial^{-1}$ * h^{-1}.

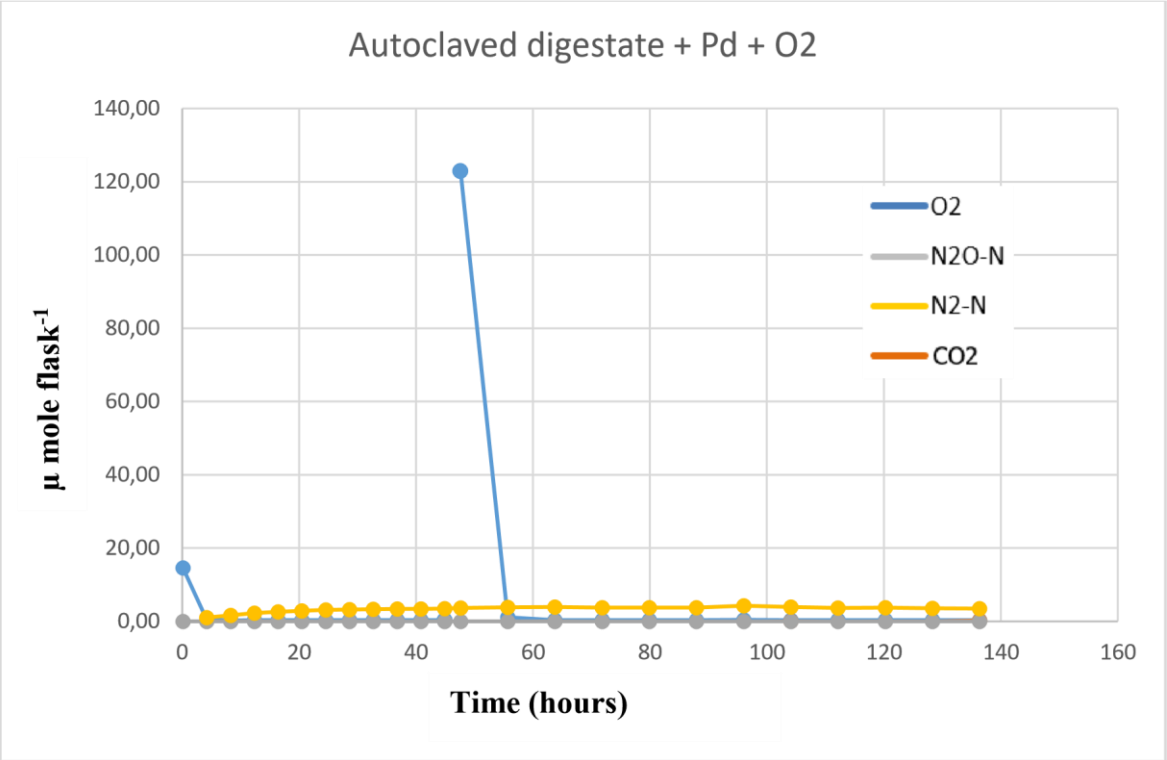


Figure 12 - 1 mL oxygen was first added to the bottles, and another 4 mL was added 48 hours into the experiment. That is why there is a sudden jump in oxygen concentration in the bottles with autoclaved biorest and O_2.

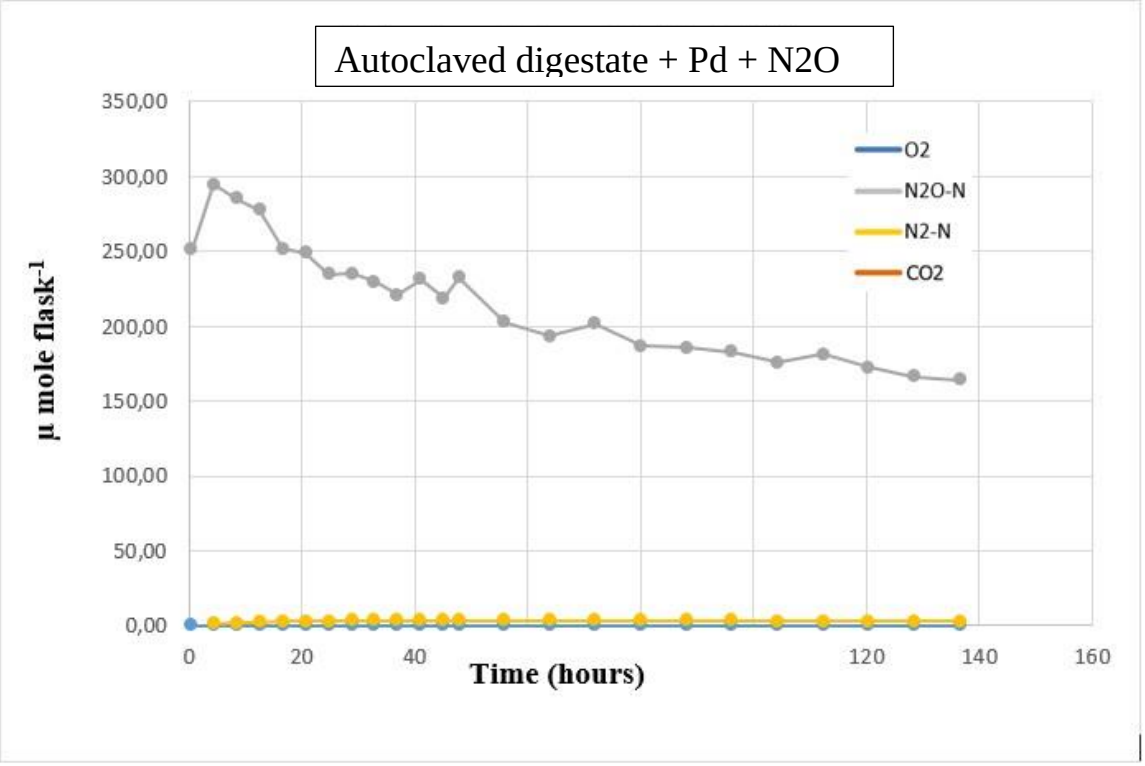


Figure 13

There is no CO_2 production in any of the flasks with autoclaved biorest, but there is CO_2 production in all the bottles containing biorest heated to 70 °C for three hours. This suggests that heating to 70 °C was insufficient to kill the indigenous bacteria.

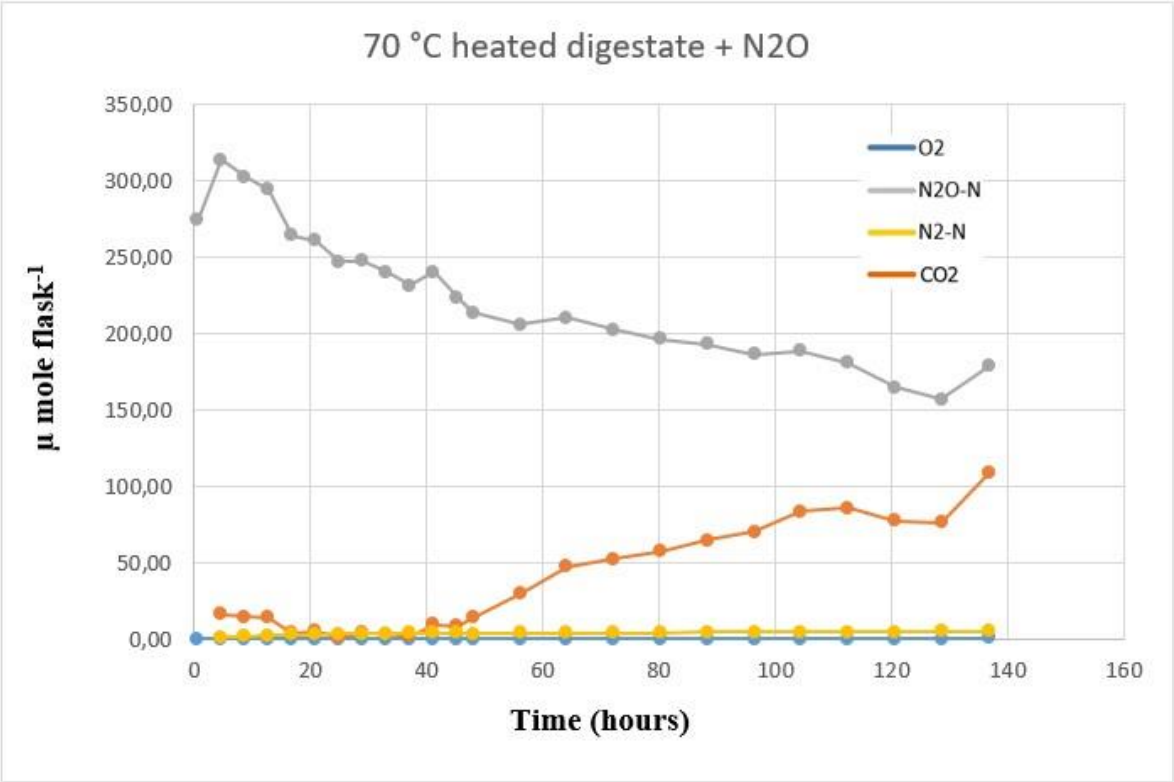


Figure 14 - When N_2O measurements follow CO_2 measurements in the way that we can see here, a possible explanation is that the N_2O is wrongly integrated, and might contain some of the CO_2 measurements.

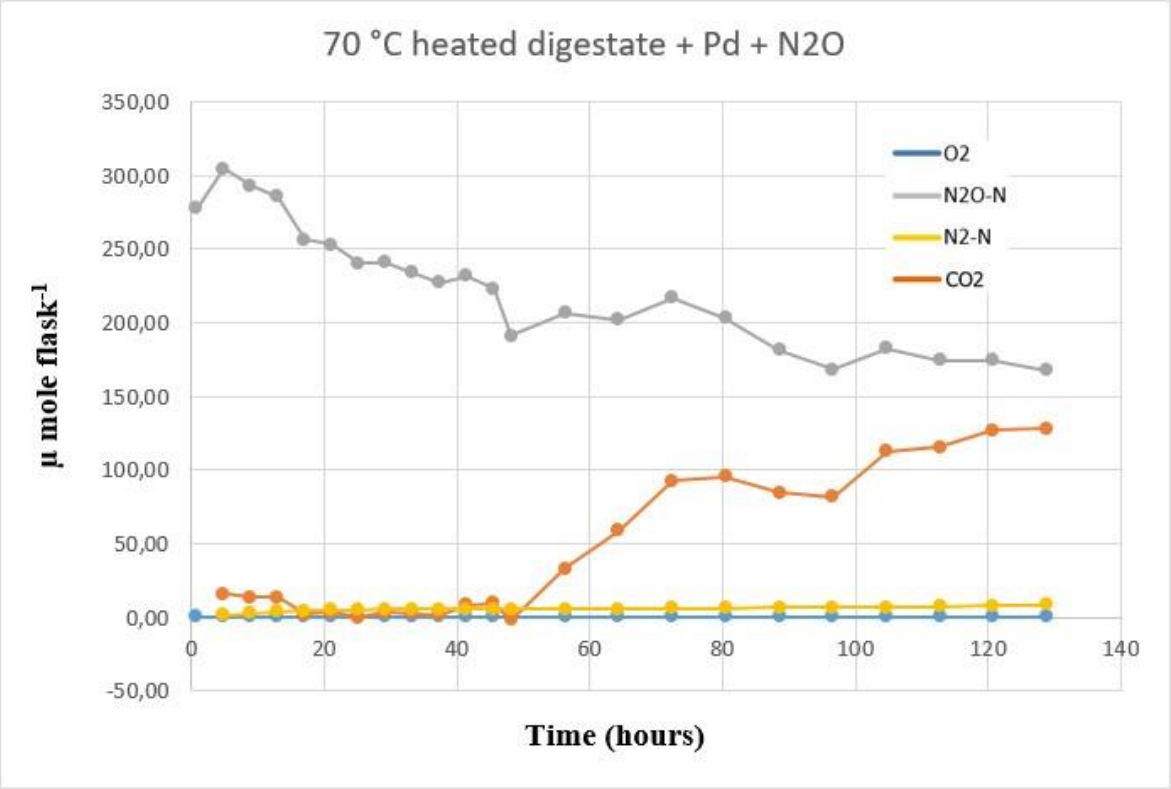


Figure 15 - Because the N_2O measurements are so similar in the autoclaved and the 70 °C heated biorest, I am assuming that the decrease in N_2O is dilutions from sampling by the robot, and not biological reduction of N_2O.

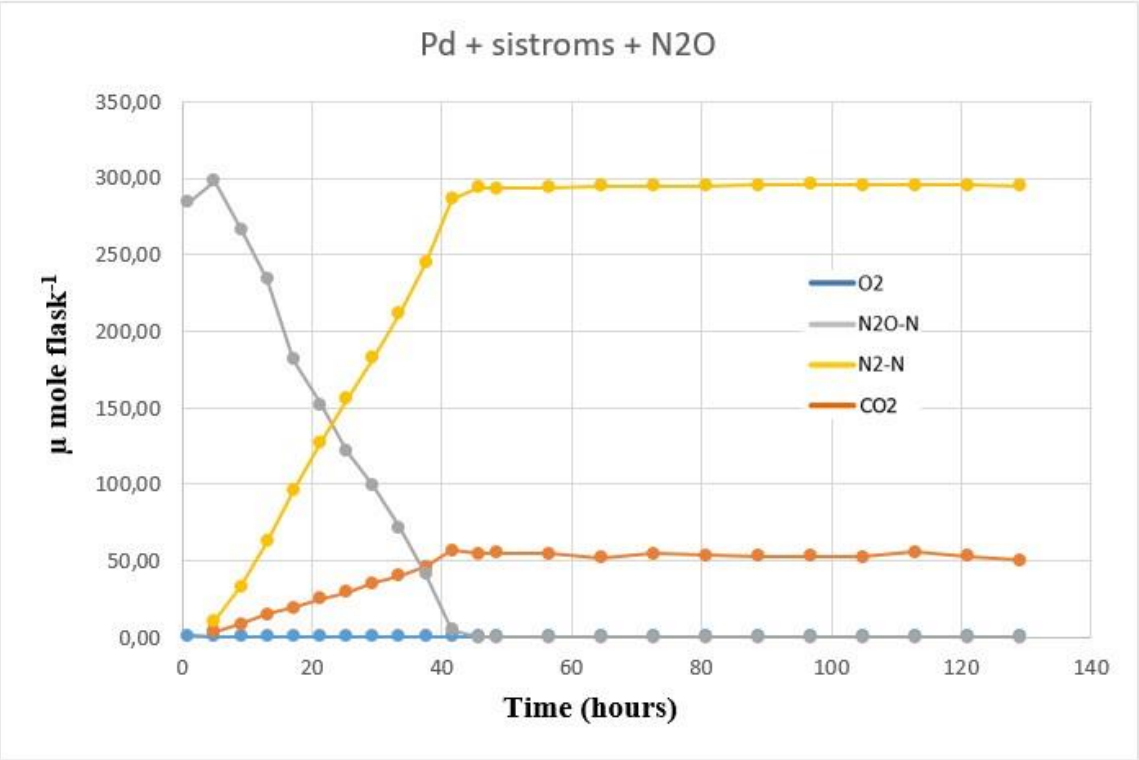


Figure 16 - This experiment was executed on the oldest of the robots. There is a lot of noise in the nitrous oxide measurements and rate calculations, because of an old column in the gas chromatograph.

From these results it looks like Paracoccus denitrificans is not contributing to anything in the samples. This might be because of inhibition of the bacteria, or it might be because they did not survive in the biorest.

4.2.1 Experiment 2: Different treatments of digestate with oxygen

Liquid	Gas	Bacteria	Extra Gas
Autoclaved digestate	15 mL O_2	-	15 mL O_2

70 °C heated digestate	15 mL O_2	-	15 mL O_2
55 °C heated digestate	15 mL O_2	-	-
Frozen fresh digestate	15 mL O_2	-	-
Fresh digestate	15 mL O_2	-	-

4.2.2 Experiment 2: Different treatments of digestate with oxygen

This experiment was conducted to test the oxygen reduction in different treatments of biorest.

The magnetic stirrer was forgotten in this experiment, and was turned on 227 hours in to the experiment.

15 mL O_2 was added to all the bottles before the experiment, and another 15 mL was added after 268 hours.

All the bottles in this experiment contain only different treatments of biorest, and added oxygen.

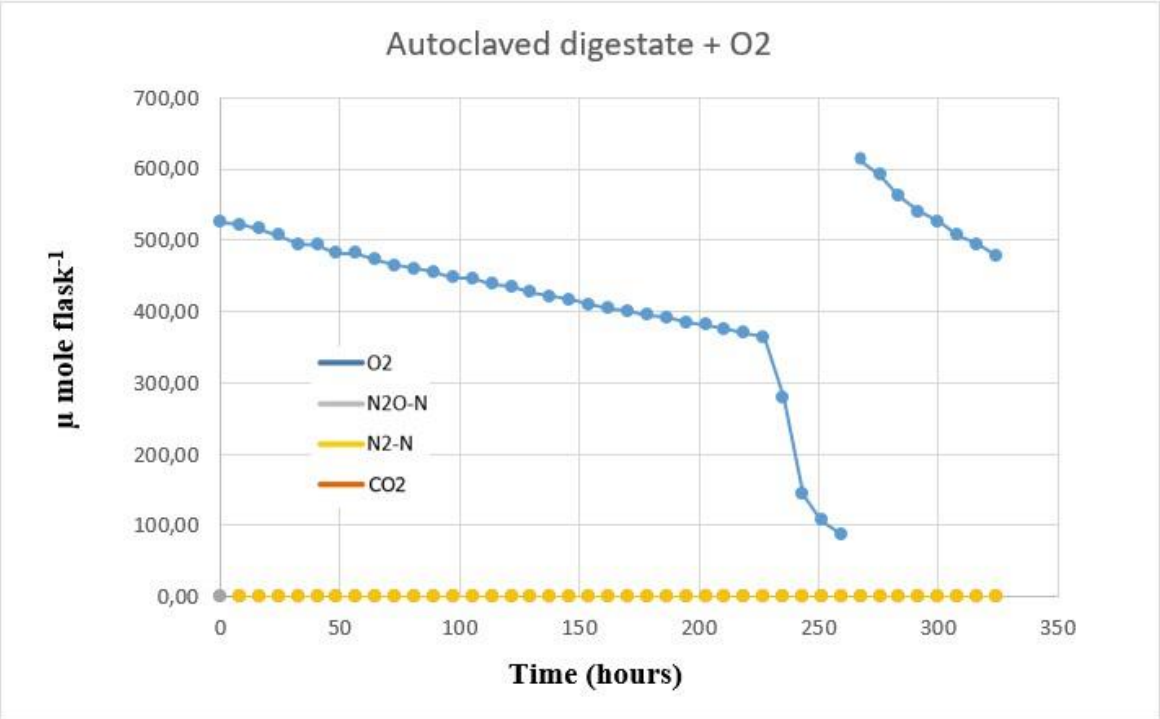


Figure 17 – The gap and sudden jump in oxygen is due to extra added oxygen

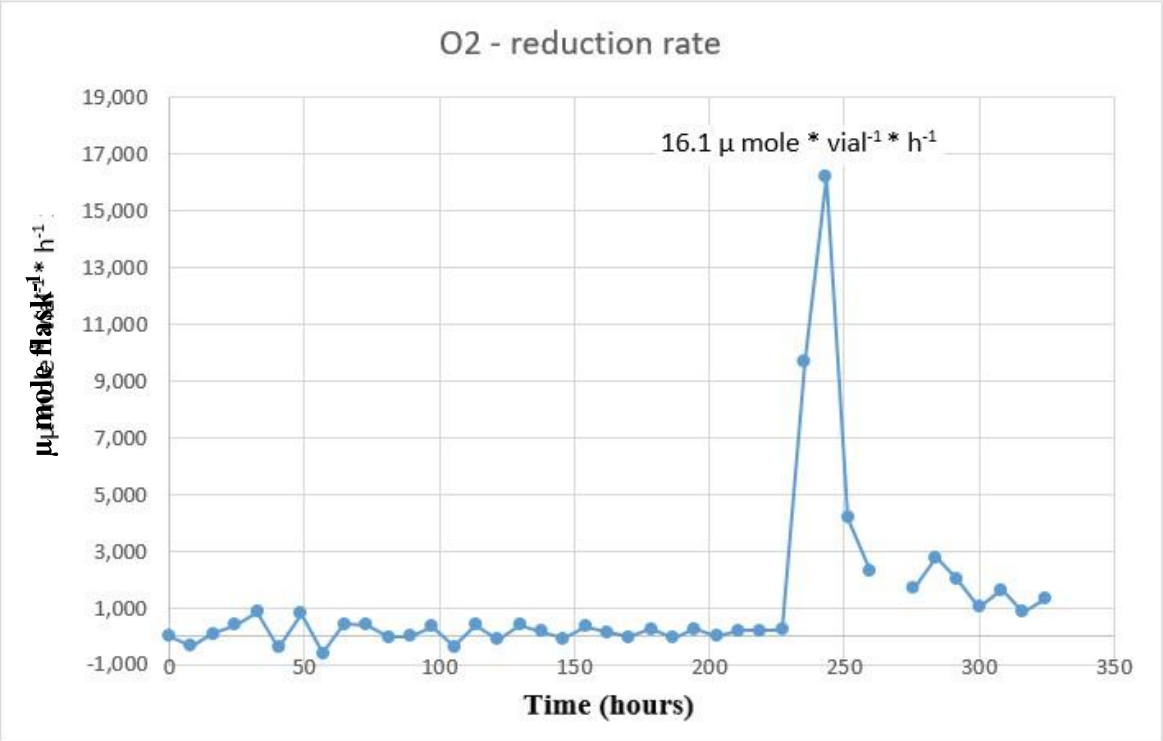


Figure 18 - The max oxygen reduction rate is 16.1 µ mole * $vial^{-1}$ * h^{-1} in the autoclaved biorest.

The gap in this table is there because I removed the injection of oxygen, seeing as how this is not a rate. The reduction rate fluctuates around 0 before stirring was turned on, which shows that the reduction in N_2O at the start of the experiment was purely sampling from the robot.

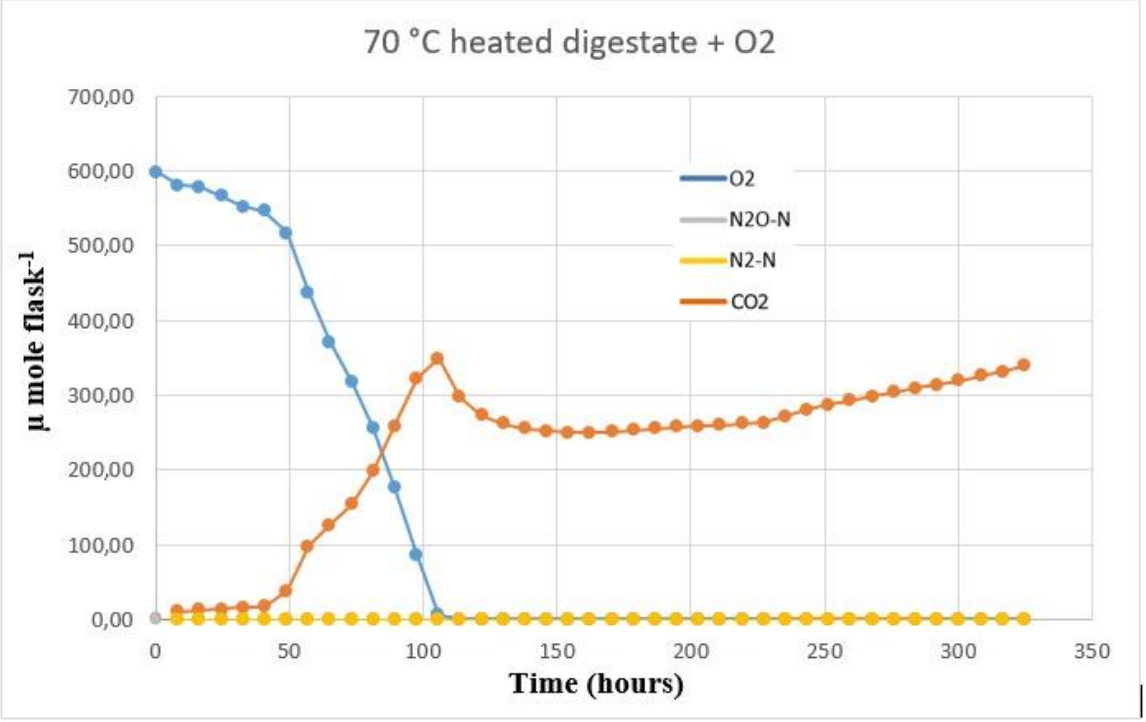


Figure 19

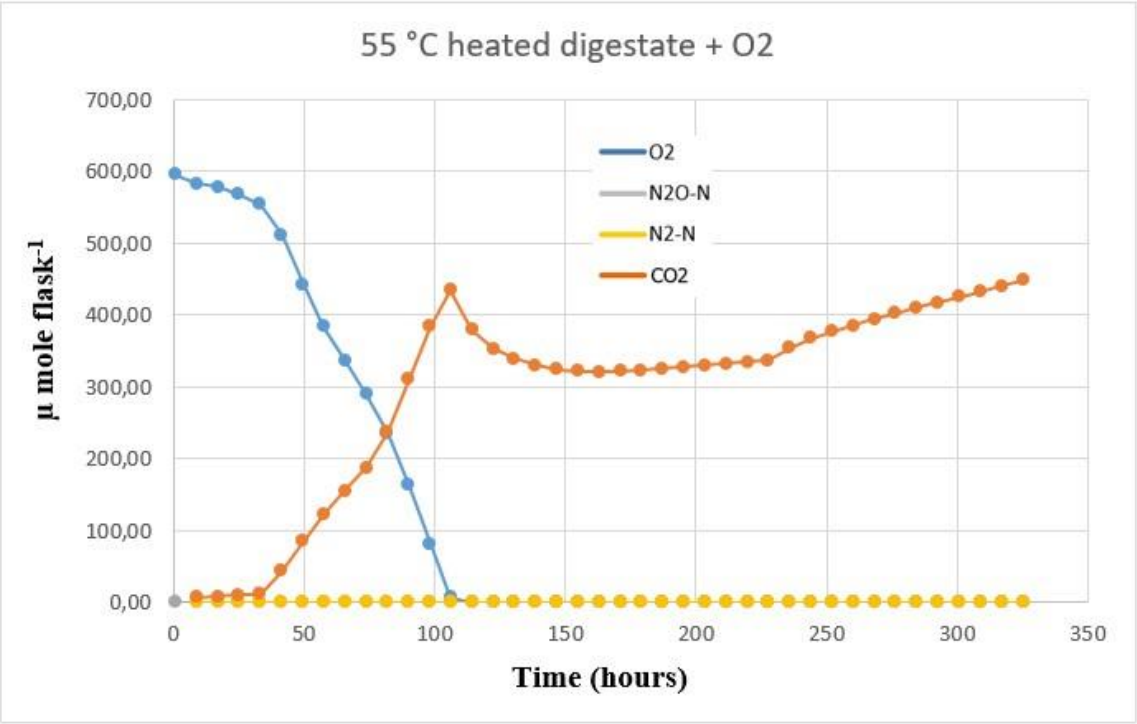


Figure 20

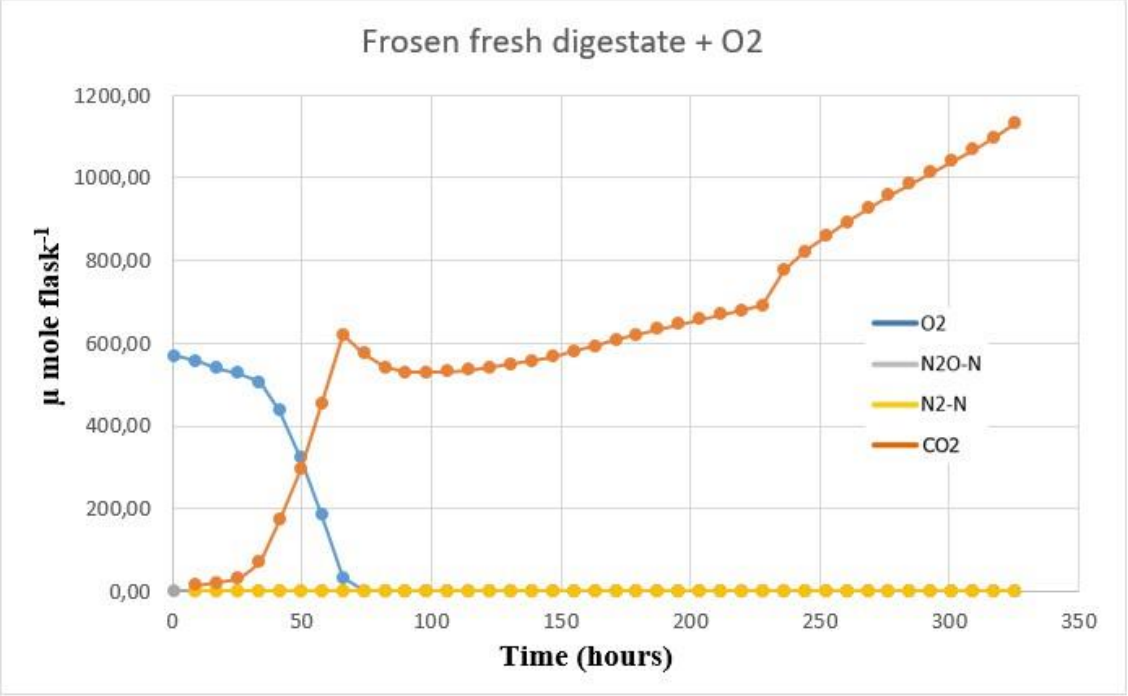


Figure 21

The less the heat treatment, the steeper oxygen reduction, unless the biorest is completely fresh, where the oxygen reduction is a bit slower. A possible explanation for that might be the difference in consistency between biorest that has been frozen, and fresh biorest. Fresh biorest

has a very thick consistency, like heavy cream. After freezing it goes a lot less viscous, and becomes a more water like texture with loose bits floating in it. It might therefore be a less viscous consistency that is the cause of why the different treatments of biorest reduce oxygen so much more efficiently than fresh.

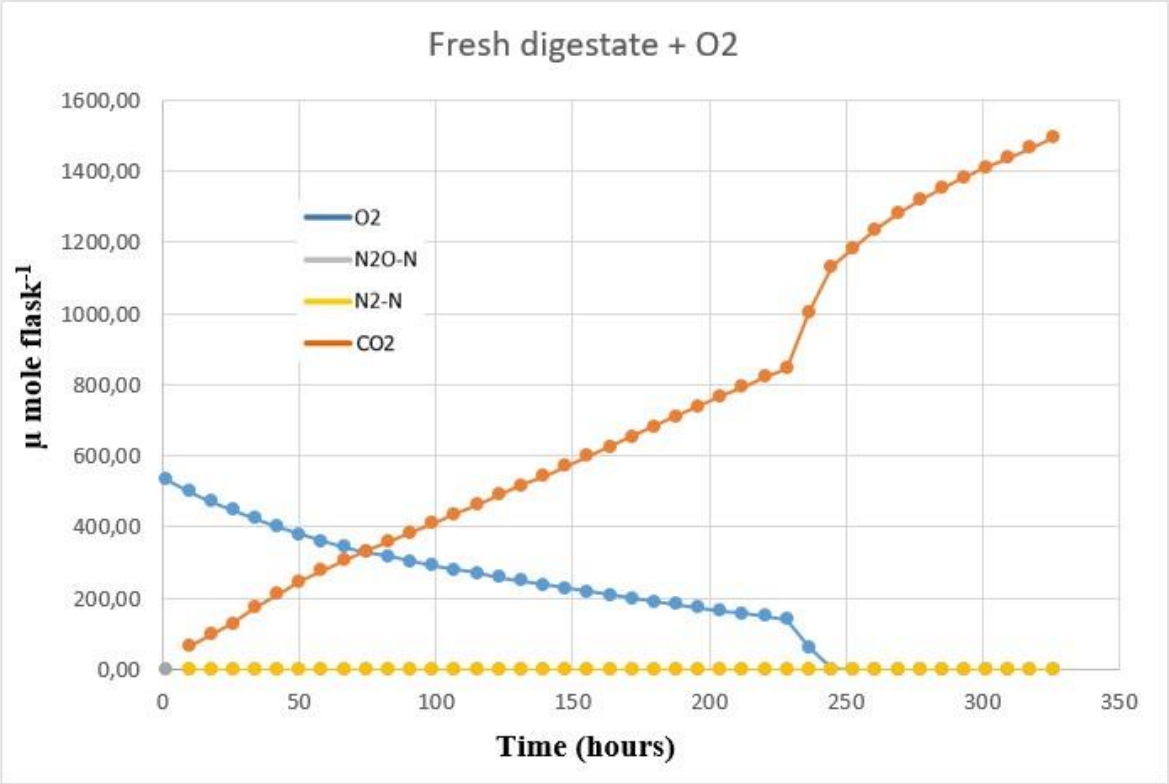


Figure 22

Similar to experiment 4.1, there is CO_2 production in all of the biorest treatments except autoclaved, which suggests that this CO_2 production in biotic.

In all the bottles we can see that the CO_2 increases even if the oxygen is gone, and even more after the stirring is turned on, which would suggest that fermentation is happening in the bottles. If the oxygen is hindered, because of consistency, from diffusing into the liquid in fresh biorest, this would produce an environment with less respiration and more fermentation.

4.3.1 Experiment 3: Paracoccus in autoclaved and 70°C heated digestate with N2O

Liquid	Gas	Bacteria	Extra Gas
Autoclaved digestate	4 mL N_2O	-	-
Autoclaved digestate	4 mL N_2O	Paracoccus denitrificans	-
70 °C heated digestate	4 mL N_2O	-	-
70 °C heated digestate	4 mL N_2O	Paracoccus denitrificans	-
Sistrom's	4 mL N_2O	Paracoccus denitrificans	-

4.3.2 Experiment 3: Paracoccus in autoclaved and 70°C heated digestate with N2O

The lacking metabolic activity by Paracoccus denitrificans in the digestate (Experiment 1) could be due to toxic substances, or lack of a suitable carbon source in the digestate. To explore this, we ran an experiment in which autoclaved digestate was diluted and supplemented with Sistrom's medium (10%). All vials were supplemented with 100 µmol

NO3- (concentration = 2 mM), and were inoculated with Pa denitrificans (no controls without Pa denitrificans were needed because sterile sludge does not reduce NO3-). As for the previous experiment, the experiment included vials with Sistrom's medium without digestate. The results (N2 and N2O production, Fig 8&9) shows that digestate is clearly toxic to Pa denitrificans: N2 production was insignificant at all digestate concentration ≥20%, and only

marginal at 10% digestate: the rate was practically constant at ~ 5 nmol N2-N h-1 . In comparison, the initial rate of N2 production in 100 % Sistrom (5-15 h) 100 nmol N2-N h-1 (in 100 % Sistrom's, the rate increased exponentially due to growth).

N2O reductase was apparently more inhibited than metabolism as such: The treatments with 10 and 20% digestate accumulated more N2 than 100% Sistrom's (Fig 9).

To judge if the digestate lacked suitable carbon sources, the experiment also included a set ov vials with the same concentrations of digestates as those shown in Figure 8&9, but without

Sistrom's. A comparison of results with and without Sistrom's (to judge the effect of carbon source in the digestate) is meaningless for all digestate concentrations ≥20% because there was essentially no metabolic activity in these (for the treatments with 10% Sistrom's). But in the treatments with 10% D +10% Sistrom's, there was a slight production of N2. The contrast (N2- and N2O-production with and without Sistrom's, 10% D) are shown in Fig 10. While

N2-production was miniscule in the absence of Sistrom's (the amounts equal that at higher digestate concentrations shown in Gig 8 & 9), there was indeed a significant N2O production towards the very end of the incubation.

The interpretation would be that in the absence of Sistrom's, Pa denitrificans was starved for carbon (miniscule N2 production), and under such starvation conditions, N2O-reductase is not competing well for electrons.

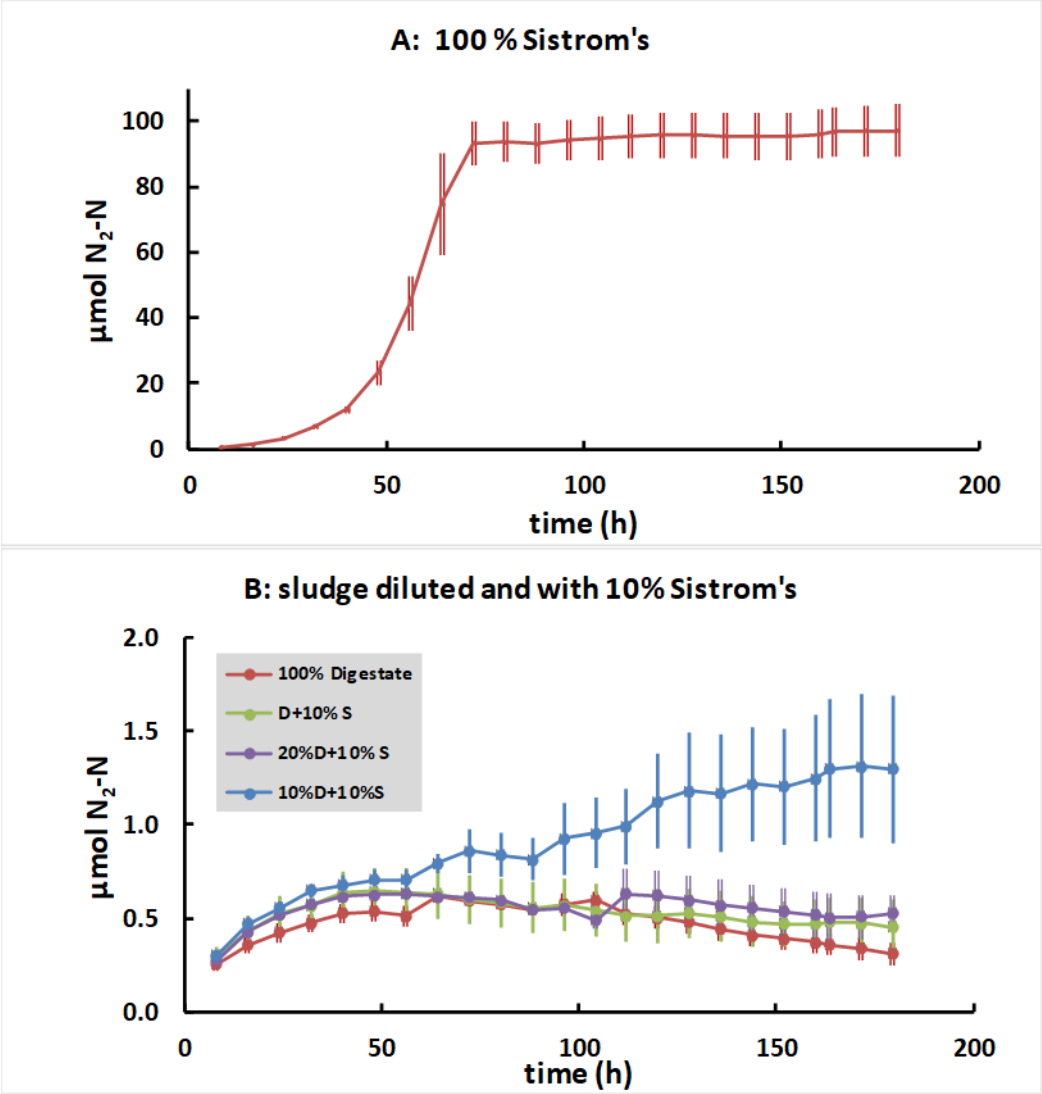


Fig 23. Toxicity of digestate; N2-production. The two panels show cumulated N2-production in vials with 100% Sistrom's medium (panel A) and in vials with different concentrations of digestates, all supplemented with 10 % Sistrom's medium (panel B). The unit is µ mole N2-N per vial. Standard error is shown as vertical bars (n=3 for all treatments)

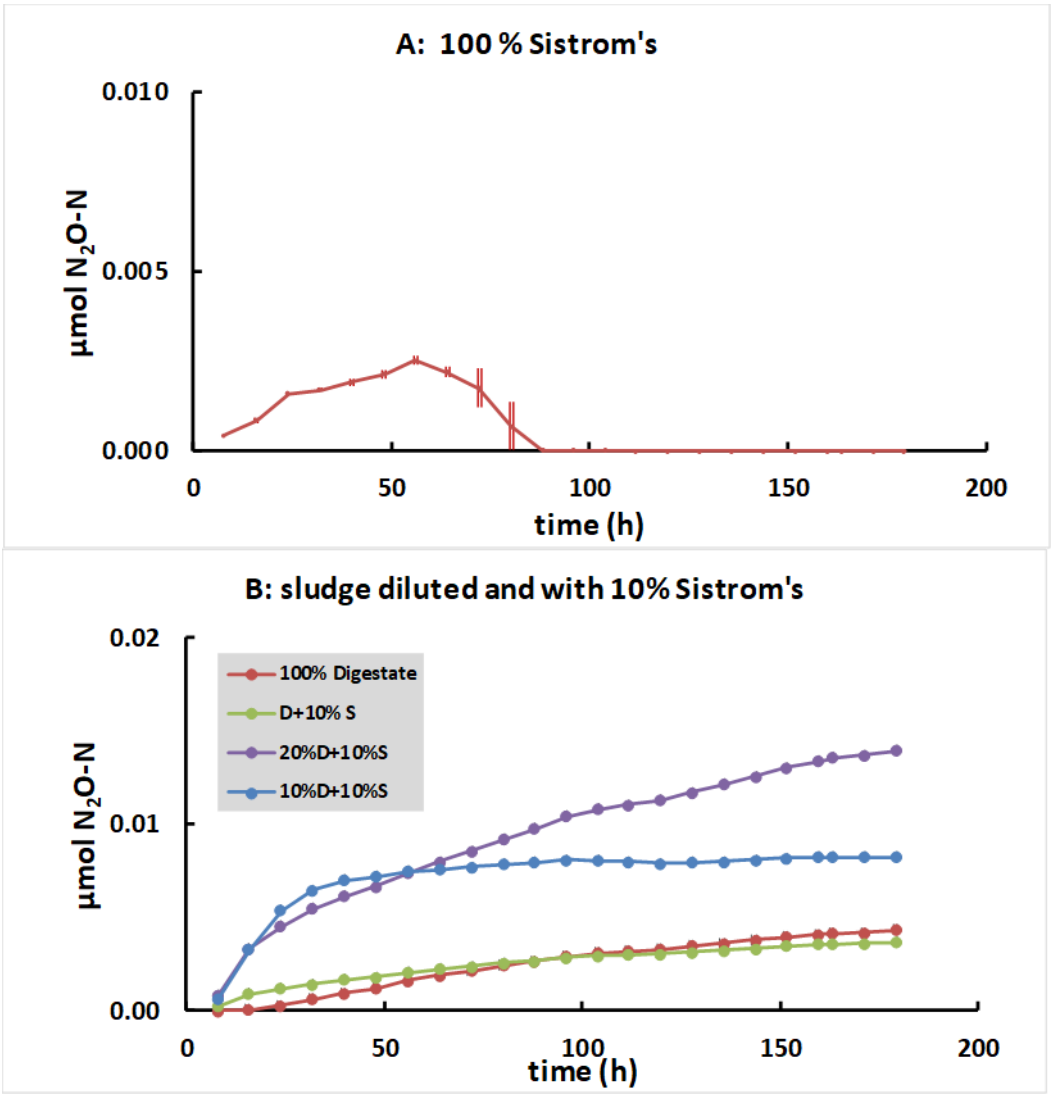


Figure 24. Toxicity of digestate; N2O production. The two panels show measured N2O in vials with 100% Sistrom's medium (panel A) and in vials with different concentrations of digestates, all supplemented with 10 % Sistrom's medium (panel B). The unit is µ mole N2O-N per vial. Standard error shown as vertical bars (n=3 for all treatments)

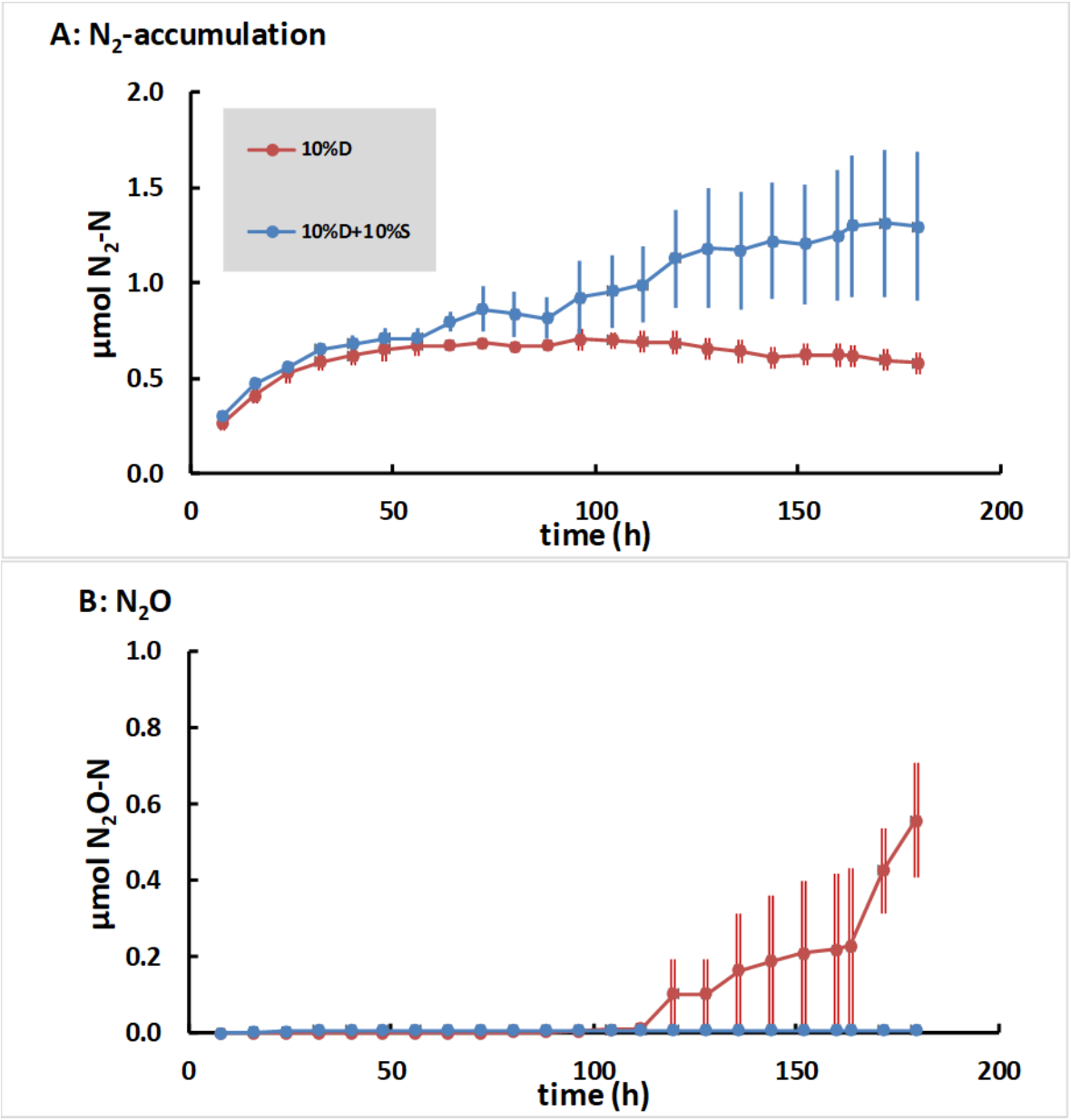


Figure 25. Carbon starvation in digestate? The panels show cumulated N2 (Panel A) and measured N2O (Panel B), in vials with 10% digestate +/- Sistrom's. Unit is µ mole N2- and N2O-N per vial. Standard error shown as vertical bars.

It does not make sense to look at the N_2O reduction rate in these bottles. Because I added to nitrous oxide right before the experiment started, there is more N_2O diffusing in to the liquid to form equilibrium, than there is P. denitrificans reducing it.

CO_2 had a very similar pattern to experiment 4.1 and 4.2, with the exact same conclusions, and therefore it is not included here as well.

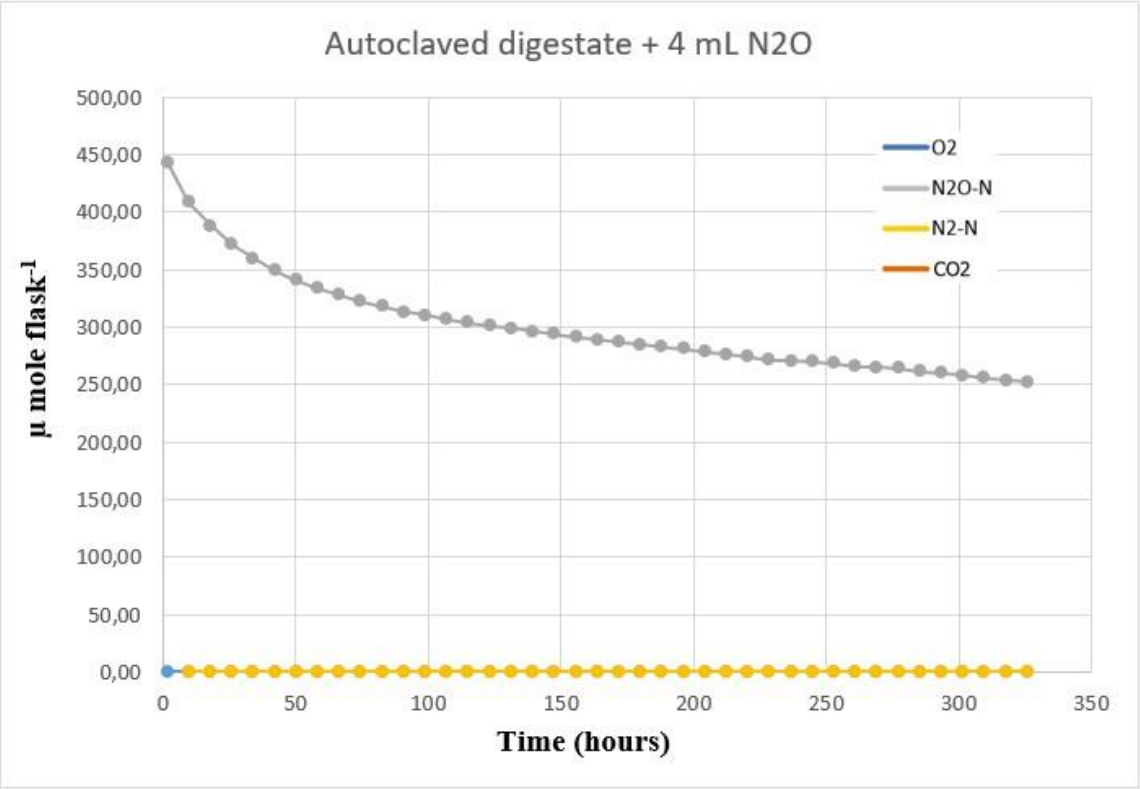


Figure 26

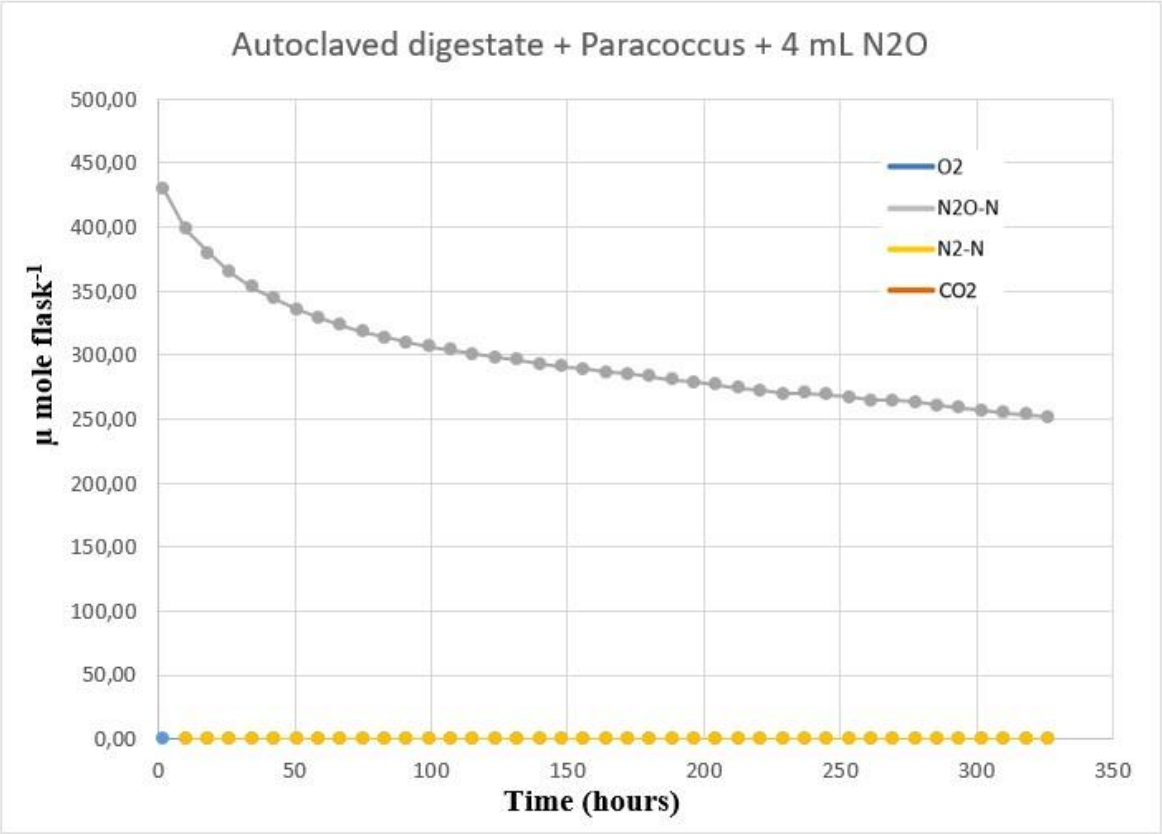


Figure 27 - The P. denitrificans in the autoclaved sludge is similar to the previous experiments either inhibited or dead, and they do not seem to be contributing to any gas changes in the bottles.

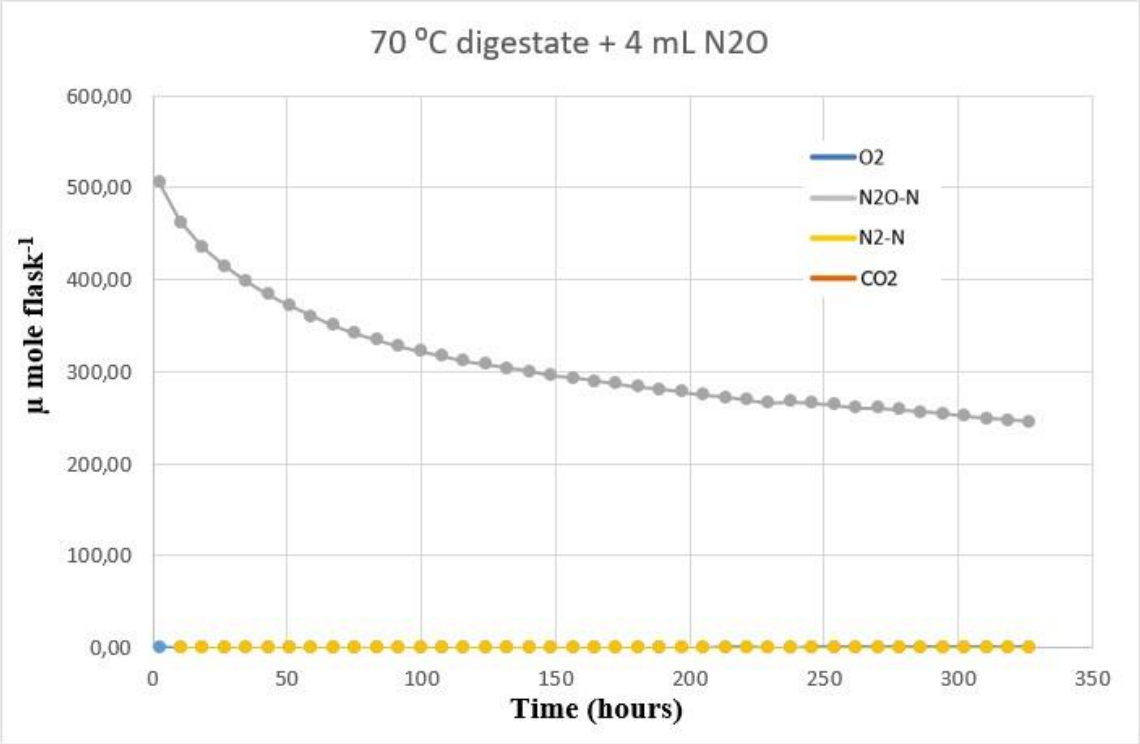


Figure 28 - The reason why the rate of nitrous oxide reduction seems to be higher at the start of the experiment is because nitrous oxide is diffusing into the liquid to form an equilibrium between the biorest and headspace in the bottles. The rest after that is sampling dilution from the robot.

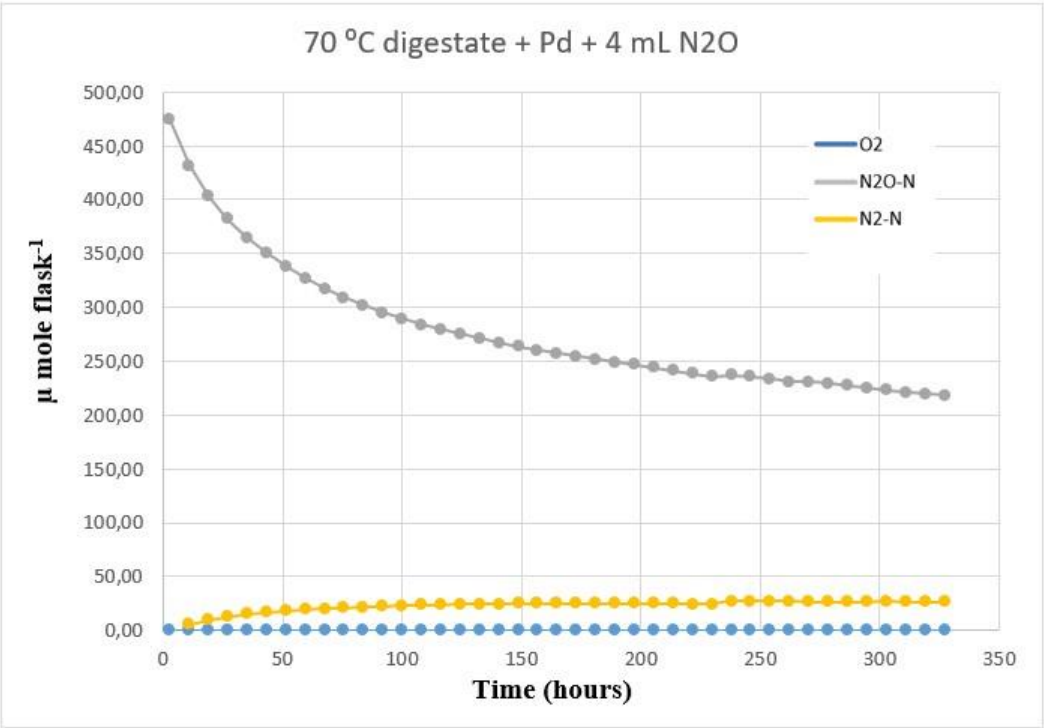


Figure 29

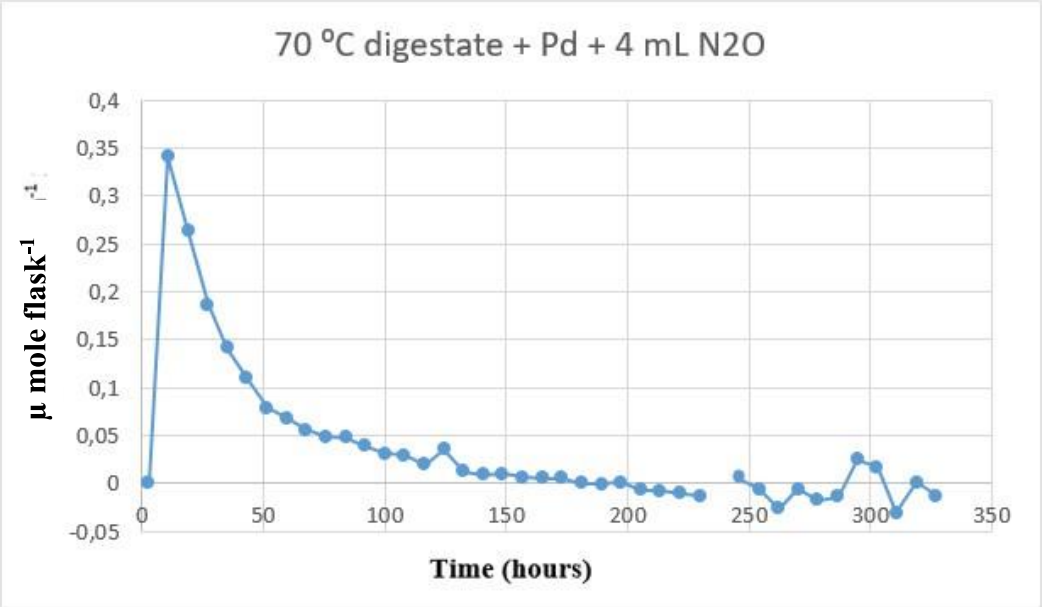


Figure 30 - In this bottle there is a production of N_2 within the first 100 hours, that is higher than without the added P. denitrificans. We still have a nitrous oxide reduction with a very low rate, and the production of nitrogen is still so small that it is difficult to look at the nitrous oxide reduction rate, but we can trust the production of N_2.

It looks as though we might have captured Paracoccus denitrificans right in the moment of death in this bottle.

One data point in the graph showing the N_2 production rate is deleted, because this data point was an outlier. There might have been a problem with the needle, or some other technical difficulty at this specific measurement.

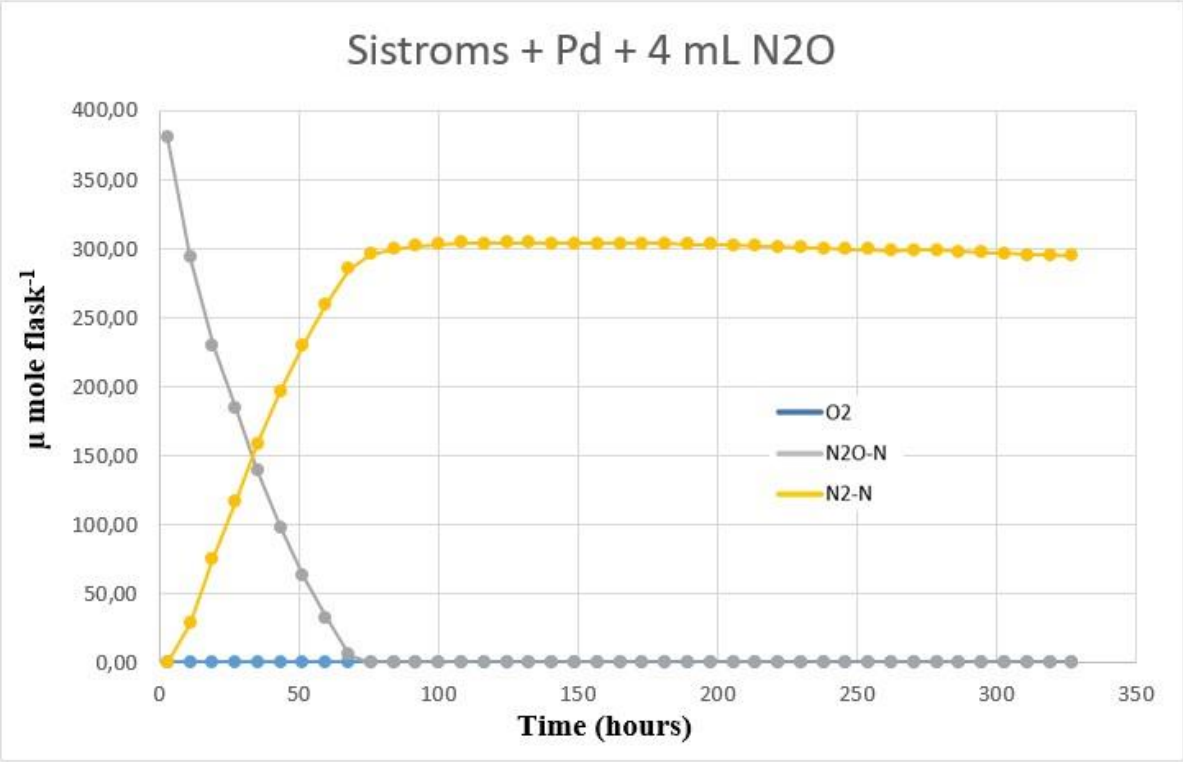


Figure 31

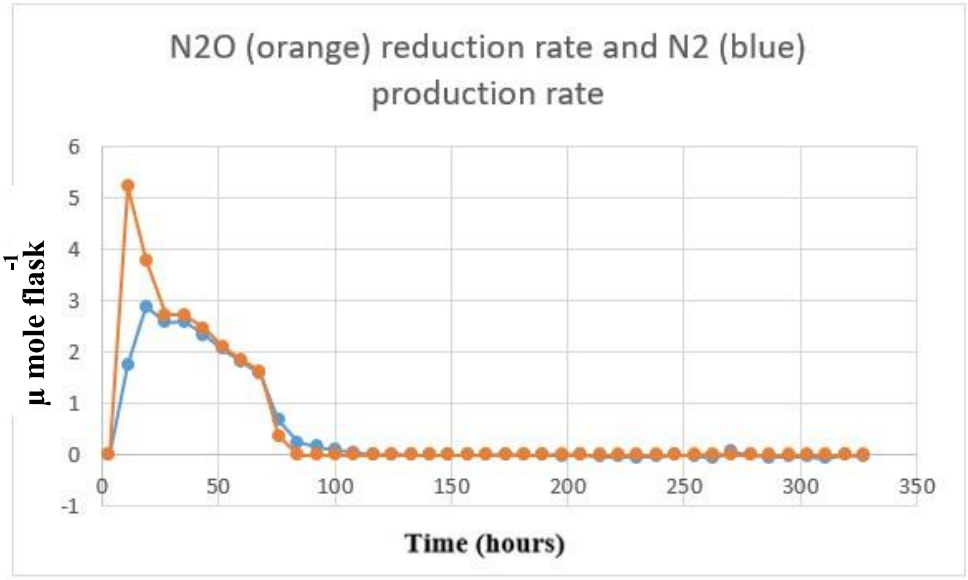


Figure 32 - The N_2O reduction rate is a mix between bacterial reduction and diffusion into the liquid at the start of the experiment. After 27 hours, the rates of nitrous oxide reduction and nitrogen production are very similar, suggesting that from there on out all of the nitrous oxide reduction is biological reduction.

4.4.1 Experiment 4: Paracoccus in autoclaved digestate dilutions with water and/or Sistrom's

Liquid	Gas	Substrate	Bacteria
Autoclaved digestate	O_2	-	-
Autoclaved digestate	-	2 mM KNO_3	Paracoccus denitrificans
45 mL autoclaved digestate + 5 mL 10x Sistrom's	-	2 mM KNO_3	Paracoccus denitrificans
10 mL autoclaved digestate + 35 mL	-	2 mM KNO_3	Paracoccus denitrificans
water + 5 mL 10x Sistrom's			
5 mL autoclaved digestate + 40 mL water + 5 mL 10x Sistrom's	-	2 mM KNO_3	Paracoccus denitrificans
Sistrom's	-	2 mM KNO_3	Paracoccus denitrificans

45 mL autoclaved digestate + 5 mL water	-	2 mM KNO_3	Paracoccus denitrificans
10 mL autoclaved digestate + 40 mL water	-	2 mM KNO_3	Paracoccus denitrificans
5 mL autoclaved digestate + 45 mL water		2 mM KNO_3	Paracoccus denitrificans

4.4.2 Experiment 4: Paracoccus in autoclaved digestate dilutions with water and/or Sistrom's

This experiment was done to see whether P. denitrificans problems with growing in biorest was due to inhibition of some sort by the biorest, or if it was lack of substrates to grow on.

We thus did a series of dilutions of the biorest with water and Sistrom's.

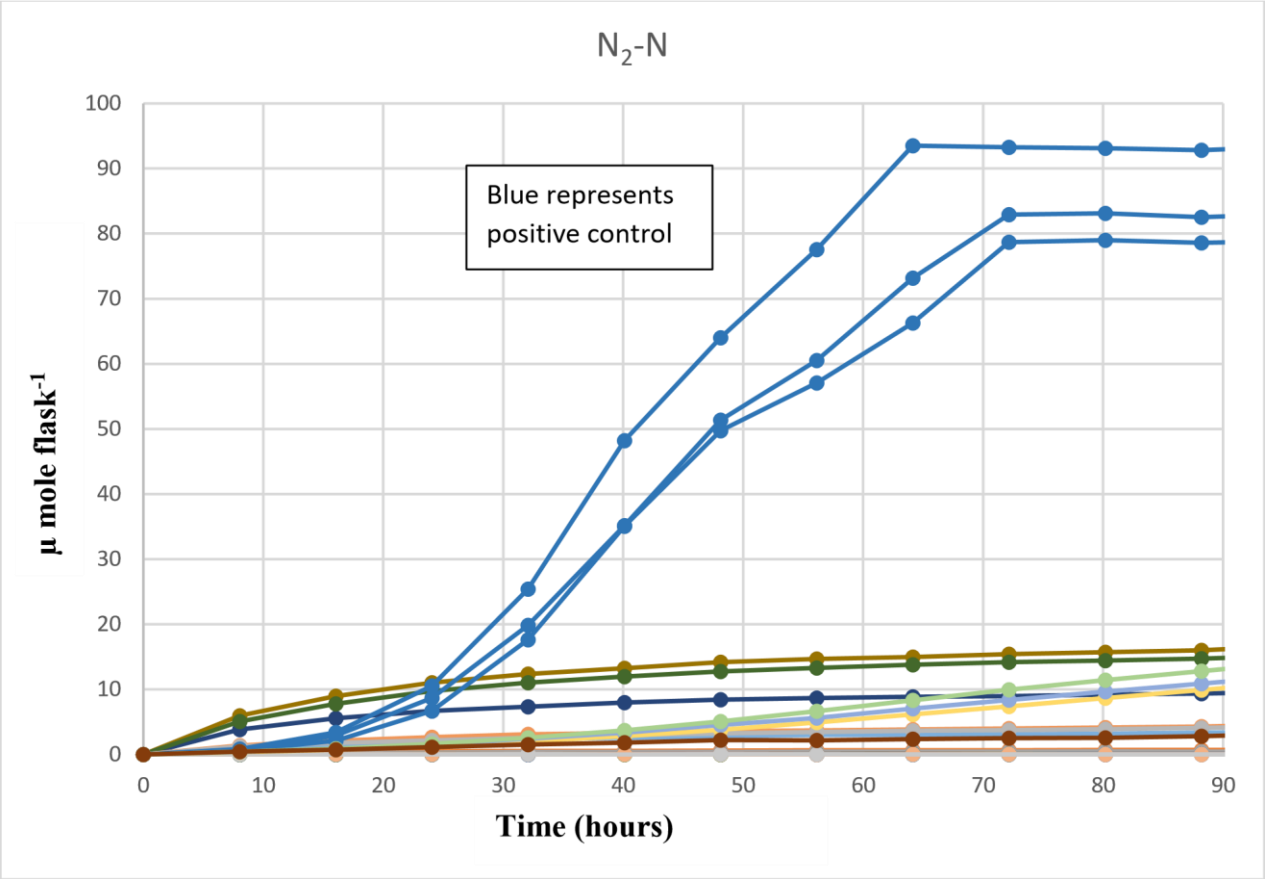


Figure 33 - The blue represents the three positive controls, which are producing nitrogen as expected. This shows us that the lack of nitrogen production in the other bottles are not due to a problem with the actual bacteria itself, but due to a factor related to the biorest. All the other bottles contained less than 14 µ mole flask^{-1}, whereas the positive control had a maximum of 93.5 µ mole flask^{-1}

All the biorest used in the experiment was autoclaved, and similar to the autoclaved biorest in the other experiments, there was no difference between the bottles containing P. denitrificans and the ones that did not. It is not possible to say whether they are dead or not, but we can say that they are at least not doing denitrification.

The N_2O-N production in all the bottles were less than 20 nano moles flask^{-1}. This little production of nitrous oxide tells us that it is not only the nitrous oxide reductase that was inhibited, but the bacteria itself.

We do not know whether or not anything was reduced to nitrite or NO, because we were not supposed to measure the nitrite concentration, and there was a problem with the NO-analyser.

This is however not likely.

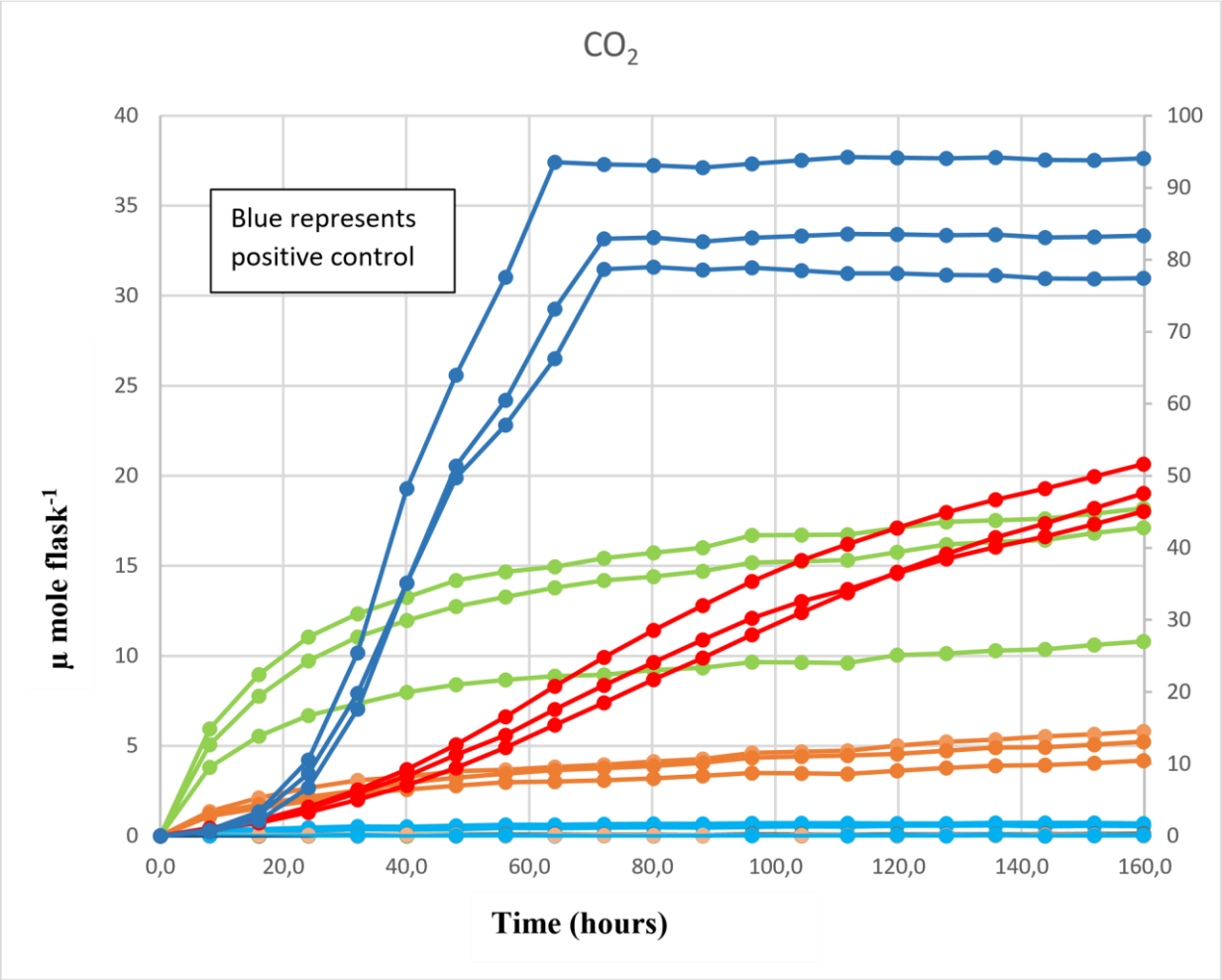


Figure 34

It is hard to look at the CO_2 in this experiment, because of the high pH of the biorest. The higher the pH, the more CO_2 gets dissolved into the liquid, making the concentration in the headspace lower, and the other way around. If the pH changes throughout the experiment, it can thus look like CO_2 production, and might just be CO_2 released from the liquid to create equilibrium.

10, 11, 12 □ The increase in the beginning looks like CO_2 coming from the biorest because it increases fast, and then stagnates to an almost horizontal line. It is hard to say whether or not this comes from biological activity. The same might count for 13, 14 and 15.

28, 29, 30 □ Paracoccus denitrificans in water. In these bottles there is biological activity. The max CO_2 concentration at the end of the experiment was 20.6 µ mole flask^{-1}, compared to 159.8 µ mole flask^{-1} in the positive control.

There is no significant activity in any of the other bottles. This is not a quantitative analysis.

We have seen that there is biologican activity in the positive control as expected, and this points to possible but very little activity in the other bottles. It is the bottles with the least biorest that have the most “growth”.

4.5.1 Experiment 5: Paracoccus, Bacillus and Dechloromonas in fresh and autoclaved digestate

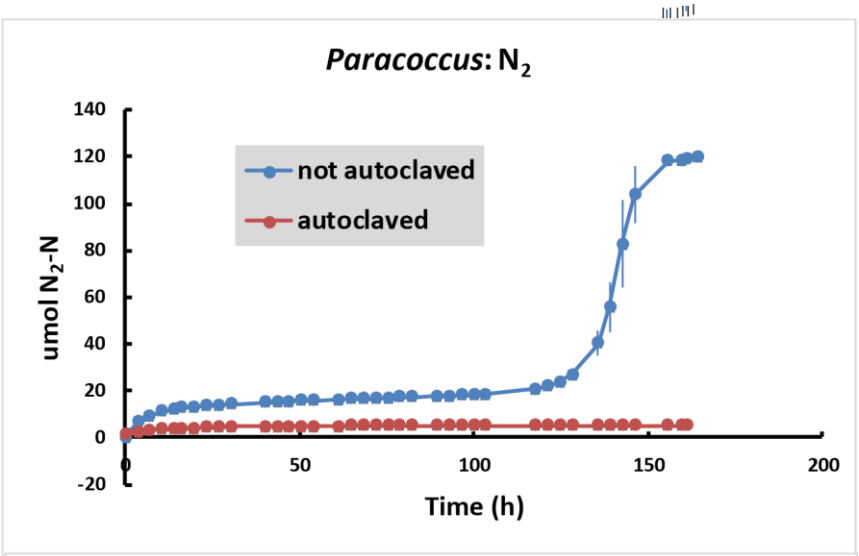
Liquid	Gas	Bacteria
Fresh digestate	5 mL N_2O	-
Fresh digestate	5 mL N_2O	Decholormonas from single cell colony
Autoclaved digestate	5 mL N_2O	Decholormonas from single cell colony
Fresh digestate	5 mL N_2O	Decholoromonas mix
Autoclaved digestate	5 mL N_2O	Dechloromonas mix
Fresh digestate	5 mL N_2O	Paracoccus denitrificans
Autoclaved digestate	5 mL N_2O	Paracoccus denitrificans
Fresh digestate	5 mL N_2O	Bacillus vireti
Autoclaved digestate	5 mL N_2O	Bacillus vireti

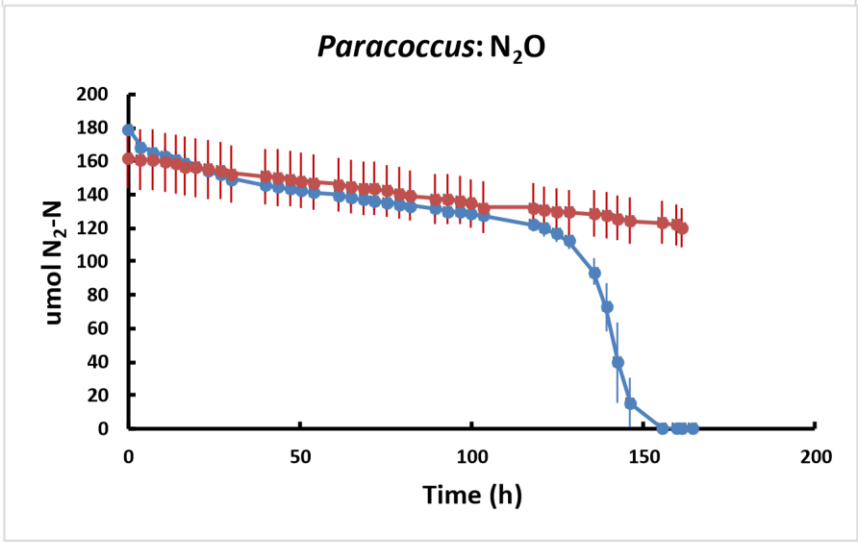
4.5.2 Experiment 5: Paracoccus, Bacillus and Dechloromonas in fresh and autoclaved digestate

The foregoing experiments showed severe inhibition of Paracoccus denitrificans in autoclaved digestate. It turned out however, that this could be due to pH, which increased from 6.95 to 9.35 by autoclaving, most likely due to removal of CO2. In order to find out a final experiment was designed, where fresh digestate was compared with autoclaved digestate which was pH adjusted to the same pH as the fresh digestate, pH 6.95 (by adding HCL). The two substrates were inoculated with three bacteria: Paracoccus denitrificans (as before), Bacillus vireti (Mania et al 2014), and a Dechloromonas sp isolated by enriching denitrifiers in the digestate (Kjell Rune Jonassen, pers com). After anaerobization of the vials, N2O was injected prior to inoculation. The vials with Bacillus vireti were also supplemented with 2 mM NO3- to secure expression of N2O reductase in this strain (Mania et al 2014).

The Dechloromonas was grown anaerobically on petri dishes. In this experiment we have flasks inoculated with Dechloromonas grown from both a single cell colony on a digestate agar (product from Kjell Rune Jonassen), and an entire plate flush of all colonies growing on Sistrom's agar.

The results for Paracoccus are shown in Figure 11. The complete inhibition by the autoclaved digestate is clear, while Paracoccus was able to sustain a minimum of metabolic activity in the fresh digestate. This shows that a) digestate is clearly toxic to Paracoccus denitrificans and b) autoclaved digestate is more toxic than fresh digestate.


Paracoccus: N_2
not autoclaved
autoclaved
umol N_2-N
Time (h)


Paracoccus: N_2O
umol N_2-N
Time (h)

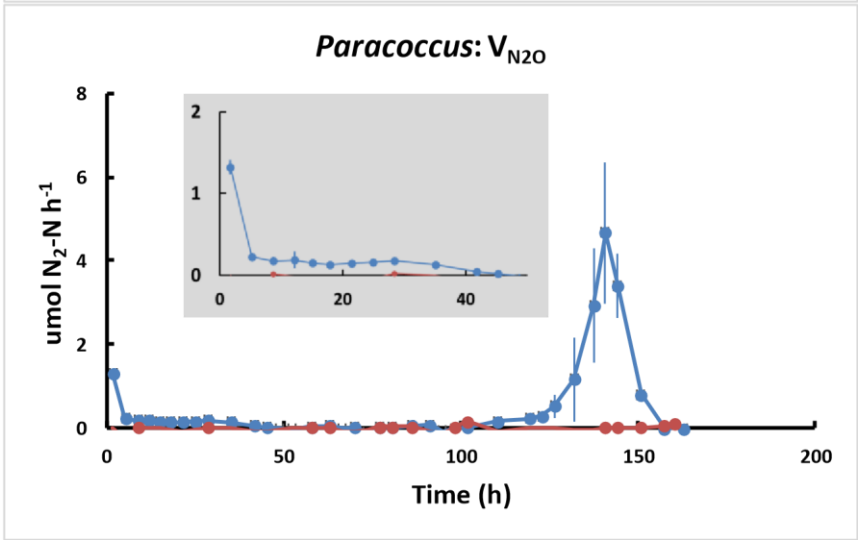
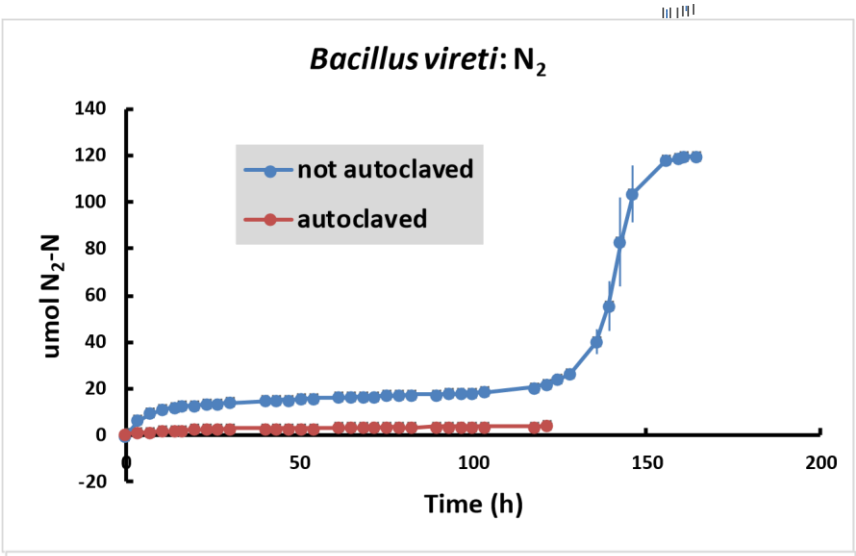
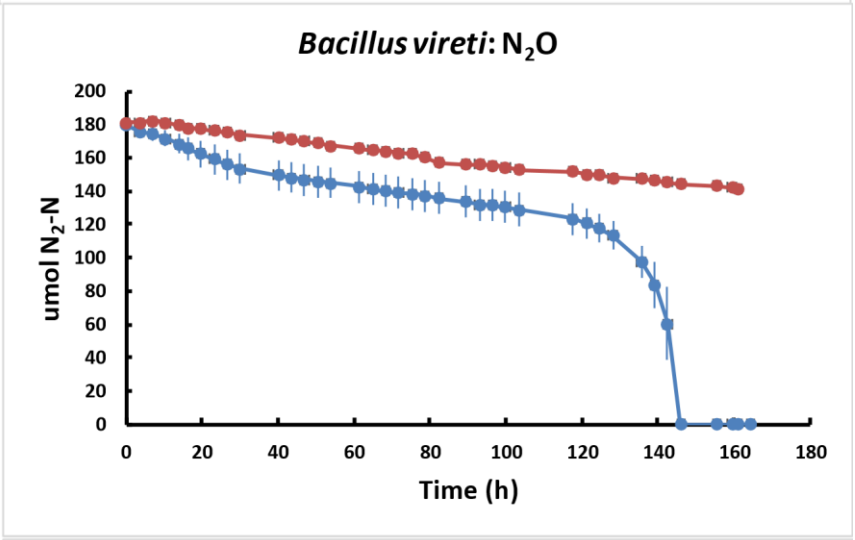

Paracoccus: V_{N2O}
umol N_2-N h^{-1}
Time (h)

Fig 35. N2O-reduction by Paracoccus denitrificans in fresh and autoclaved (pH adjusted) digestate. The top panel shows cumulated N2, the mid panel shows N2O (both as µmol N vial-1) and the bottom panel shows the estimated rate of N2O-reduction (µmol N vial-1) for each time increment (inserted panel: results for the first 50 h enhanced). Standard errors (n=3) are shown as vertical bars.

The fresh digestate contains a low population (~2E3 mL^{-1}) of N2O reducing bacteria (dechloromonas) which growth throughout, and whose metabolism becomes significant after ~100 h, Kjell Rune Jonassen). The initial N2O reduction, declining gradually during the first 50 hours, can be ascribed to Paracoccus.


Bacillus vireti: N2
not autoclaved
autoclaved
umol N2-N
Time (h)


Bacillus vireti: N2O
umol N2-N
Time (h)

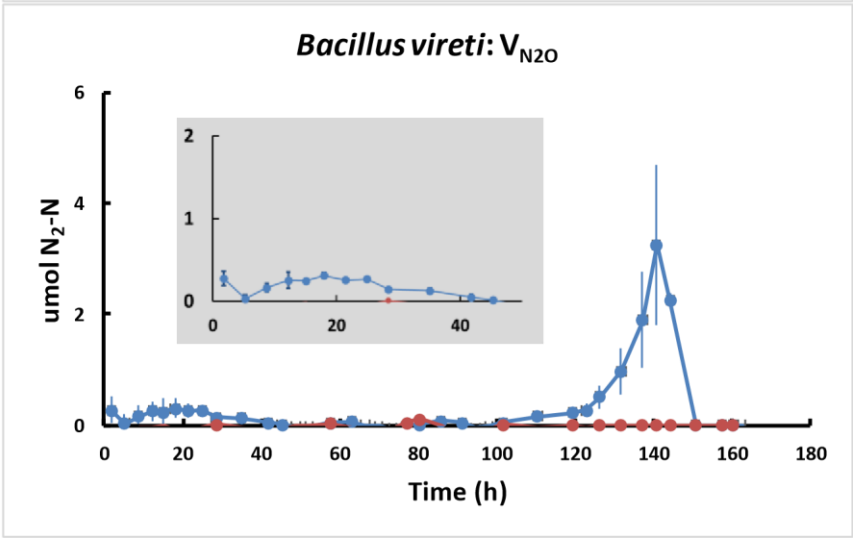
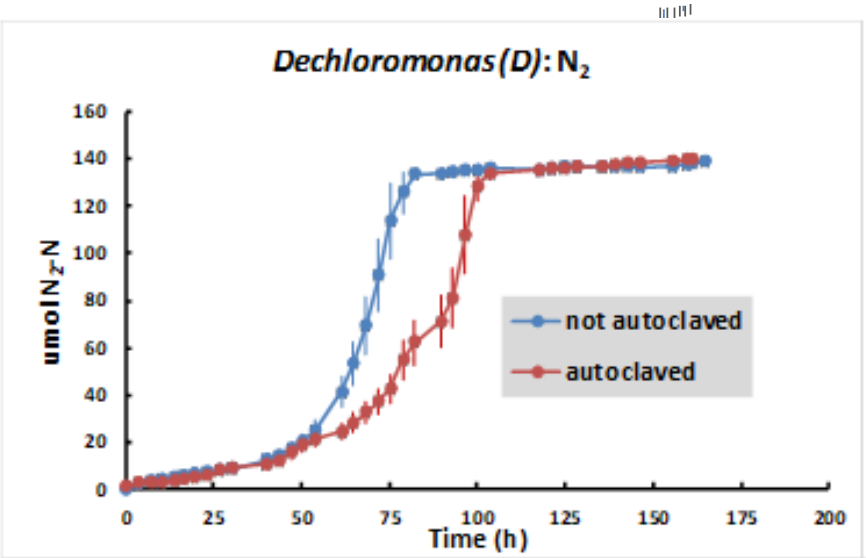
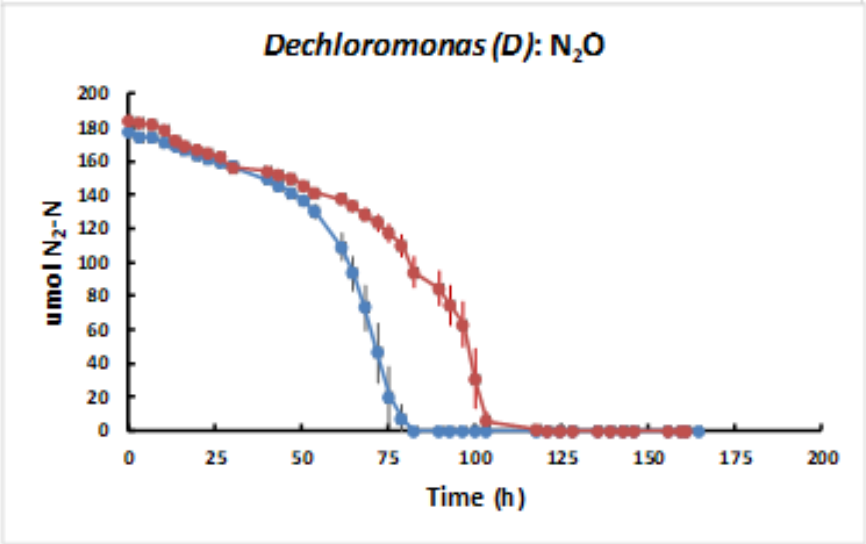

Bacillus vireti: VN2O
umol N2-N
Time (h)

Figure 36. N2O-reduction by Bacillus vireti in fresh and autoclaved (pH adjusted) digestate. The top panel shows cumulated N2, the mid panel shows N2O (both as µmol N vial-1) and the bottom panel shows the estimated rate of N2O-reduction (µmol N vial-1) for each time increment (inserted panel: results for the first 50 h enhanced). Standard errors (n=3) are shown as vertical bars.

The fresh digestate contains a low population (~2E3 mL^{-1}) of N2O reducing bacteria

(dechloromonas) which growth throughout, and whose metabolism becomes significant after ~100 h, Kjell Rune Jonassen). The N2O reduction, during the first 50 hours, can be ascribed to Bacillus vireti.


Dechloromonas (D): N2
umol N2-N
Time (h)
not autoclaved
autoclaved


Dechloromonas (D): N2O
umol N2-N
Time (h)

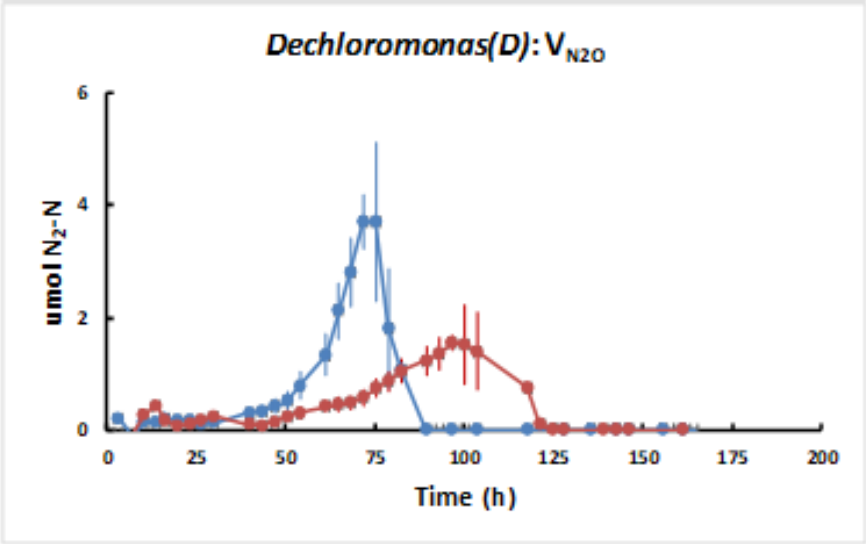

Dechloromonas(D): VN2O
umol N2-N
Time (h)

Figure 37. N2O-reduction by dechloromonas in fresh and autoclaved (pH adjusted) digestate. The top panel shows cumulated N2, the mid panel shows N2O (both as µmol N vial-1) and the bottom panel shows the estimated rate of N2O-reduction (µmol N vial-1) for each time increment. Standard errors (n=3) are shown as vertical bars.

The N2O reduction rates in Dechloromonas (Fig 13) clearly demonstrate that this organism is able to grow both in the fresh digestate and autoclaved digestate, albeit slower in the latter. The apparent growth rate can be estimated by fitting an exponential function to the measured data for the period with exponentially increasing rate of N2O-reduction (i.e. prior to growth being restricted by declining N2O concentrations). This is shown in Fig 14, where the growth rate is estimated for individual vials, providing three independent estimates of the growth rate for each substrate (i.e. autoclaved and fresh digestate).

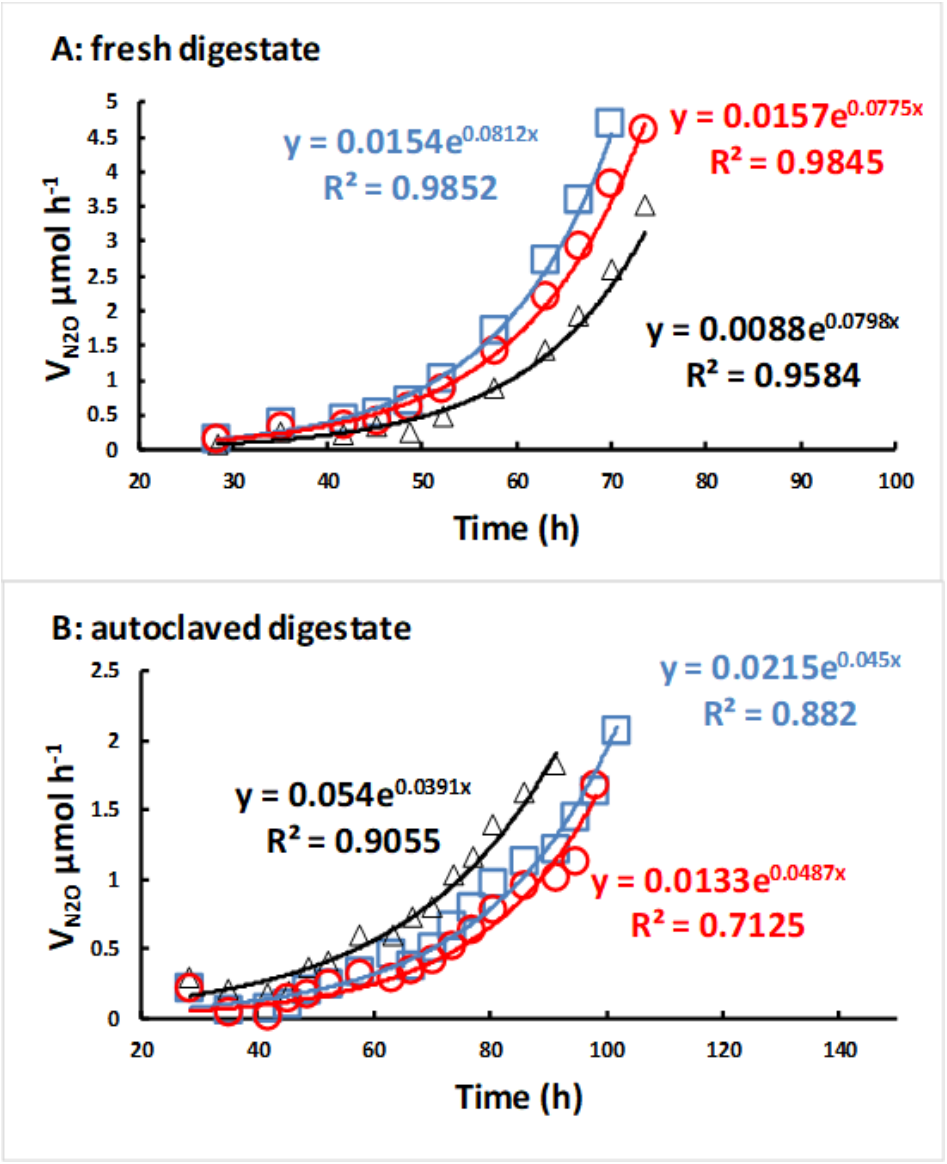


Figure 38 - Apparent growth rate of Decloromonas in fresh and autoclaved digestate. The apparent growth rate is estimated by fitting exponential functions to observed rates of N2O production. The panels show the measured rates for individual vials with fresh digesstate (panel A) and autoclaved digestate (Panel B), with fitted exponential functions. This provides

three independent estimates of growth rate for each substrate. The average growth rate is 0.079 (stdev 0.002) h-1 for fresh digestate and 0.044 (stdev 0.005) h-1) for autoclaved digestate.

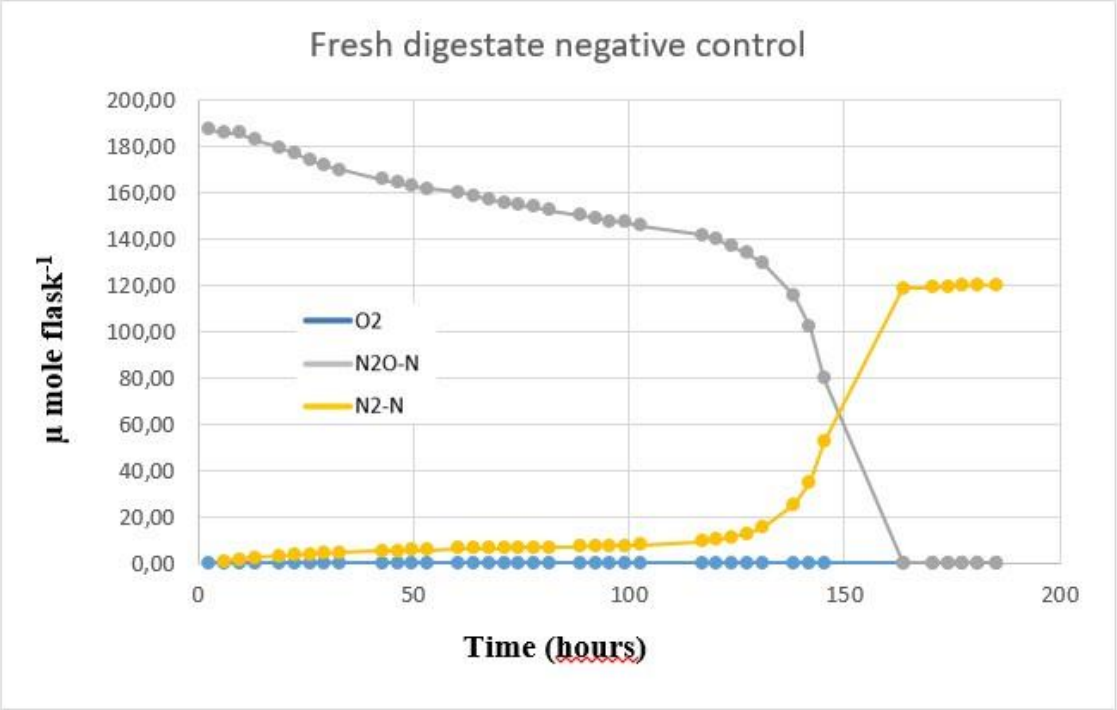


Figure 39 - The reduction rate of N_2O is very high in the fresh biorest. It looks like a population of indigenous bacteria that did not reduce N_2O in the beginning, but started to reduce nitrous oxide when they had to change their source of electron acceptor. This could be the reason why they suddenly start to reduce N_2O at such a high rate.

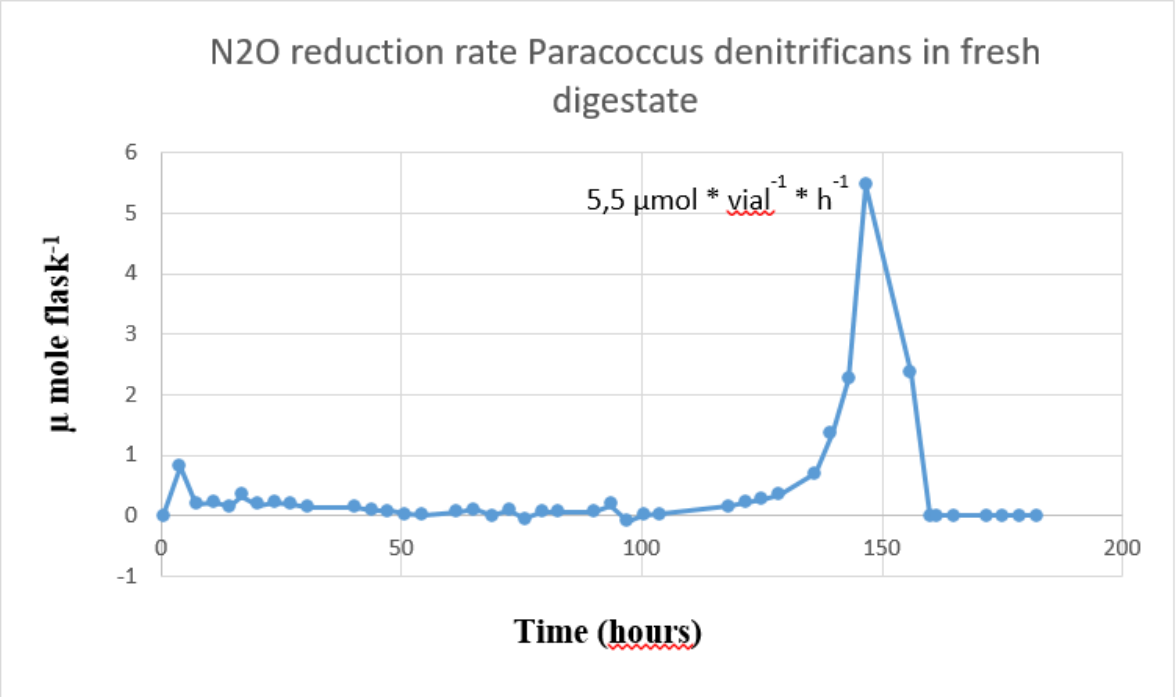


Figure 40 - We cannot say that the reduction rate is because of Paracoccus, because it started at the same time as the digestat with no Paracoccus. There is however a very slight increase in reduction rate, which may be because of slight activity from Paracoccus, but could also suggest that the Paracoccus cells became food or the indigenous bacterial community.

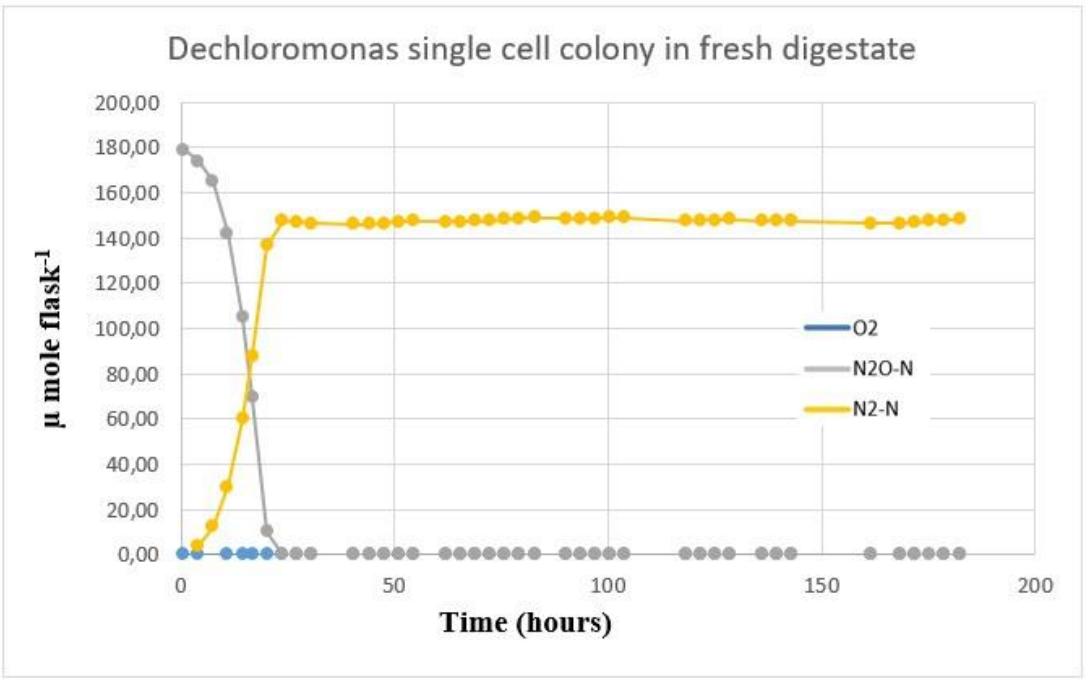


Figure 41 - Nitrous oxide reduction rate 8.4 µ mole * vial^{-1} * h^{-1}.

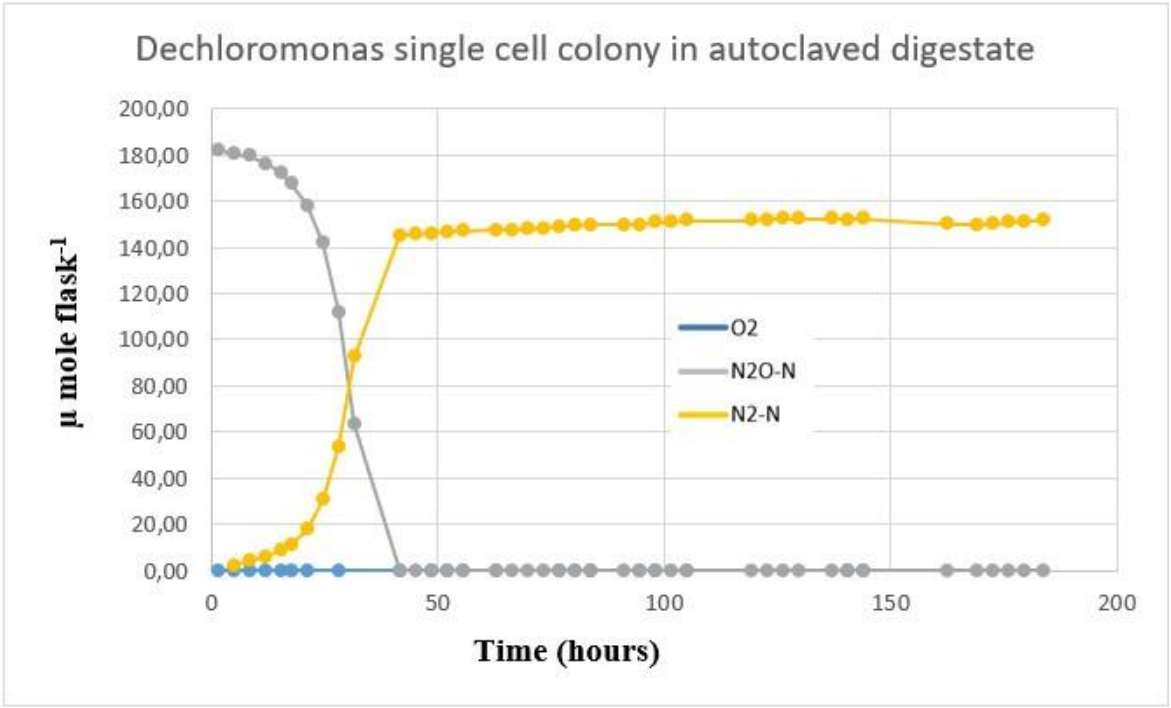


Figure 42 - Nitrous oxide reduction rate 6.9 µ mole * vial^{-1} * h^{-1}.

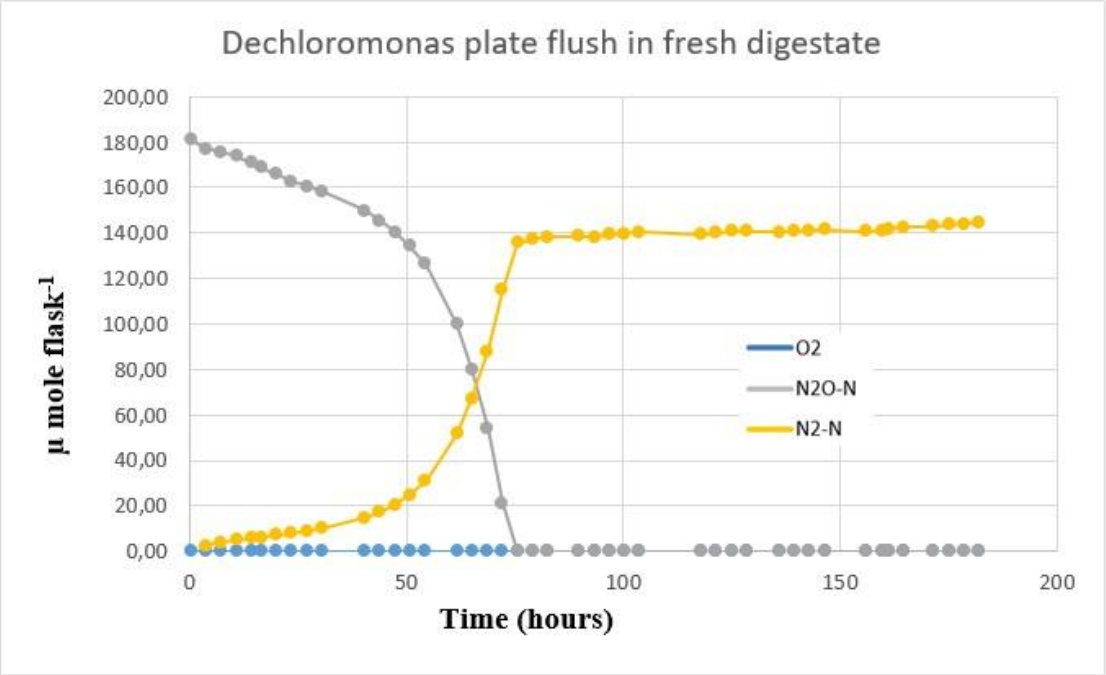


Figure 43 - Nitrous oxide reduction rate 4.6 µ mole * vial^{-1} * h^{-1}.

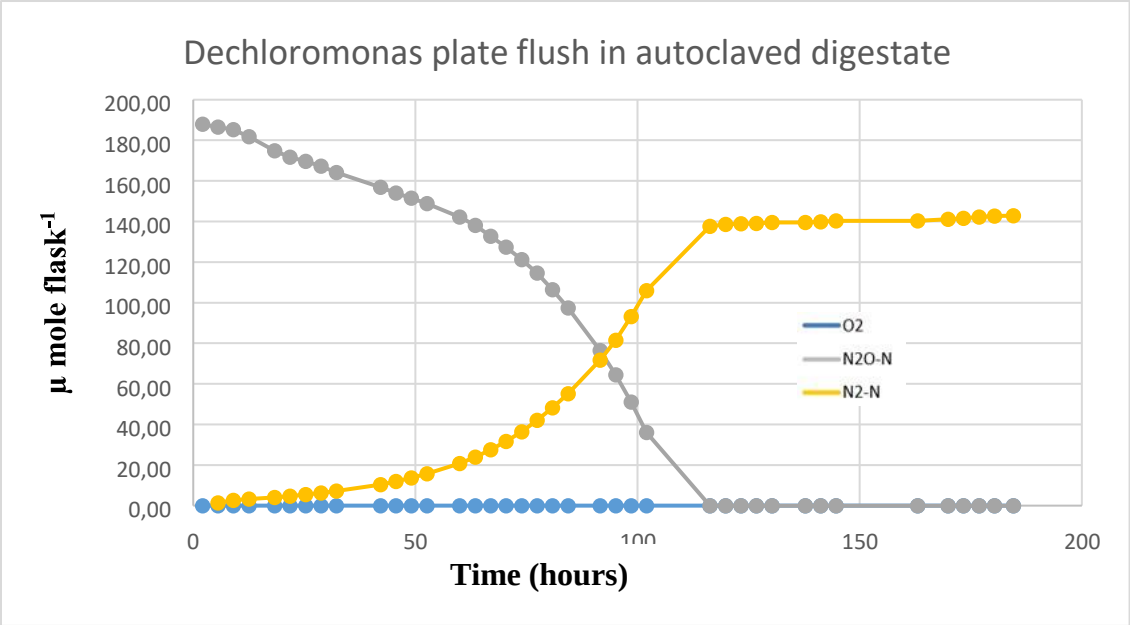


Figure 44 - Nitrous oxide reduction rate 2.1 µ mole * $vial^{-1}$ * h^{-1}.

It looks like the rate of nitrous oxide reduction was low in the beginning, which led to most of the reduction in N_2O coming from dilution through robot sampling in the beginning, because the robot took out more than the bacteria could reduce. This is also the reason why it looks like not all N_2O-N is being reduced to N_2-N, because the robot removed more than the bacteria did in the beginning of the experiment. Another explanation might be that the N_2O concentration measured is incorrectly measured to be too high, because nitrous oxide was added right before the robot started, so there might not yet have been equilibrium between headspace and liquid phase.

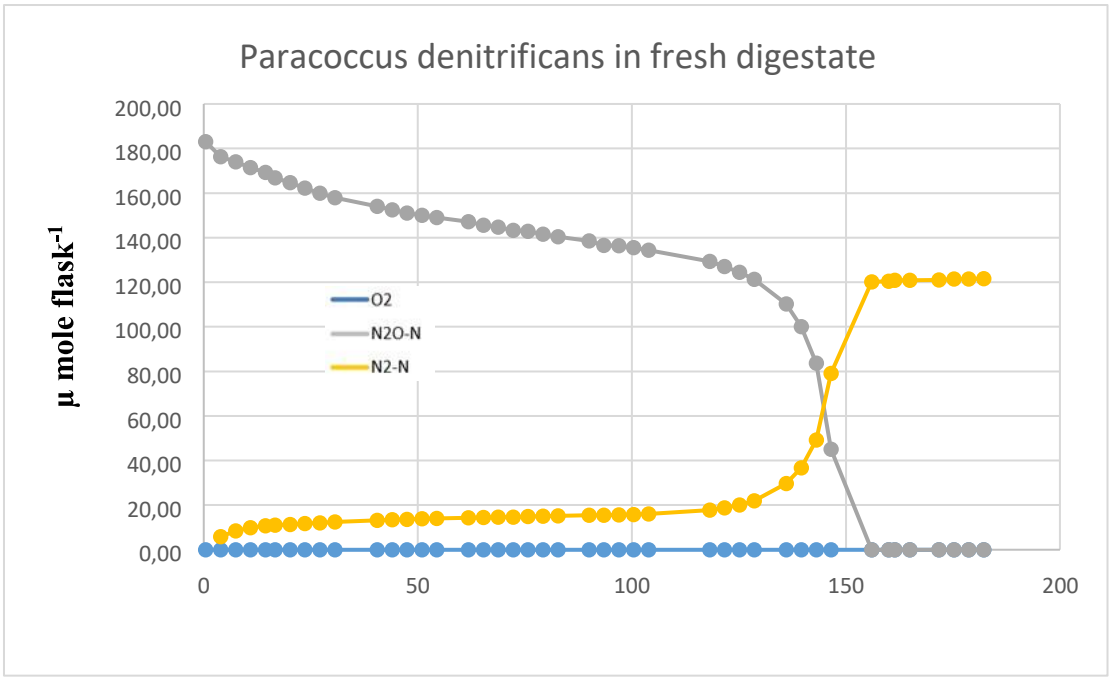


Figure 45

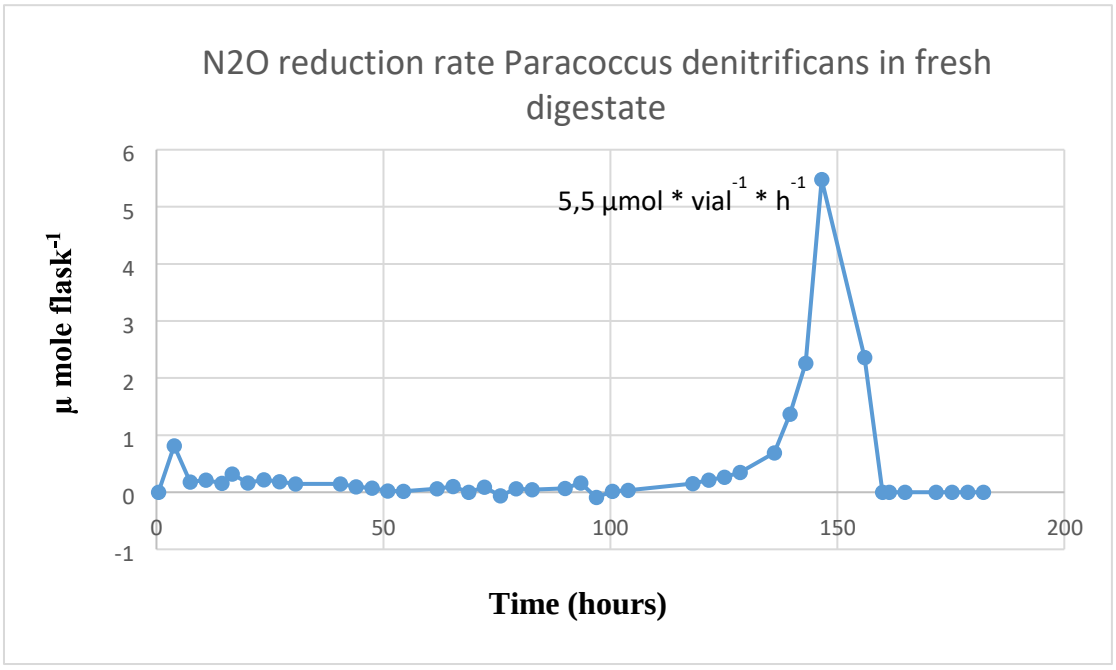


Figure 46

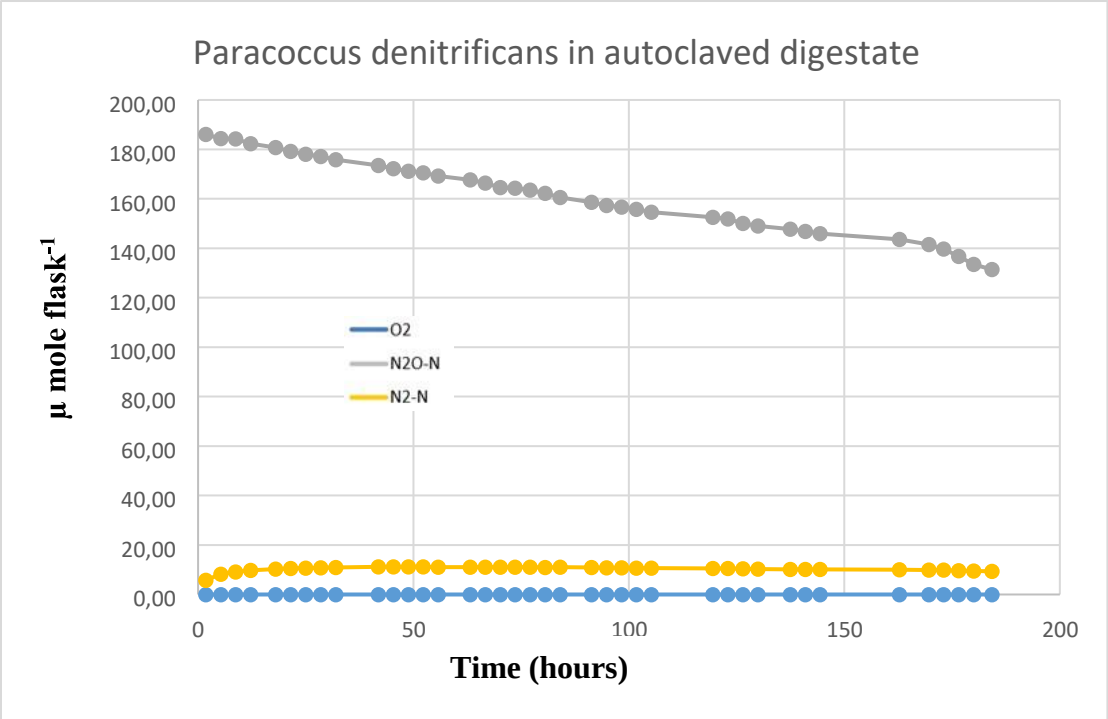


Figure 47 - The small increase in nitrogen gas at the beginning of the experiment is contributed to diffusion from the liquid phase, creating equilibrium.

Since there is no increase in nitrogen gas, we can assume that the decrease in nitrous oxide is dilution from robot sampling.

5. Discussion

5.1 pH regulation

We made the very unfortunate mistake of not thinking of the pH changing when doing these experiments. The high pH would have killed off bacteria that might otherwise have survived in the sludge. Out thought when we had negative results with no growth was that we might not have been able to make complete anaerobic conditions while growing the pure cultures to be inoculated into the robot, and that the reason why we did not have any growth could be explained by anoxic entrapment, and that the bacteria simply starved. Later I thought of the pH possibly changing, which we discovered very late. It is therefore only the last experiment that has been pH regulated back to a neutral pH of 6.95.

5.2 Oxygen consumption in digestate

The reason why the oxygen concentration is so low in all experiments containing digestate is because of the high reduction rate of oxygen in the digestate. This causes all the oxygen to be used almost immediately.

In biogas reactors CO2 is produced, and the liquid phase gets saturated with CO2. New CO2 is also continuously produced - replacing species that react with bases or other chemical species. CO2 can react with water in a series of reactions with several intermediates governed by the laws of thermodynamics and chemical equilibrium (bicarbonate, carbonic acid and carbonate). Dissolved CO2 that reacts with water, forming carbonic acid, acts as an acid. Adding a base will result in the formation of the salt forms of carbonic acid, and this creates the buffering effect.

The breakdown of proteins in anaerobic digestion generates ammonia as one of the end products, which is a base. Stripping off CO2 will result in the buffering capacity being close to nothing/dramatically lowered, and the concentration of ammonium will determine the pH in the sludge. The solubility of a gas in a liquid is proportional to the partial pressure of the gas in the atmosphere over the liquid, thus for example doubling the pressure will also double the partial pressure. The partial pressure of co2 is low in normal air. In addition the solubility is dependent on temperature - the higher the temperature, the lower the solubility of gases in liquids become. It is likely that a combination of high temperature, and low CO2 concentration in the autoclave have stripped CO2 out of the liquid phase.

High pH (low H+) increases the velocity of abiotic oxidation of reduced iron. The velocity will increase at high pH (dividing something with a number between close to zero and 1 gives a bigger number).

5.3 55 ºC heating

Sanitizing the digestate before freezing was done because we thought we could slow down the indigenous bacteria to create a window we could use to add bacteria and observe the added bacteria's growth before the indigenous bacteria started growing.

This has been proven through these experiments not to work at all, and it would probably have been best not to do this treatment.

We still used the digestate because we had a too limited amount of fresh frozen digestate to use in all experiments, and we had already started using this digestate for experiments.

5.4 **Dechloromonas**

Kjell Rune Jonassen made an enrichment culture with an indigenous strain of bacteria. Lars Bakken took this culture and plated it out on petri dished with Sistrom's media and with a digestate media. They were grown anaerobically. Lars picked one single cell culture from the petri dish with digestate media (made by Kjell), and flushed an entire petri dish with Sistrom's media. These were both used in my last experiment.

They are both Dechloromonas, but there might be different strains of Dechloromonas present. The reason I hypothesize this is because the reduction rate of nitrous oxide in the two are a little different.

The single cell colony picked, starts reducing nitrous oxide very early and does so very efficiently. The Dechloromonas flushed from the entire petri dish starts reducing nitrous oxide a bit later, and with a bit slower speed, though still very efficiently. This suggests to me that one of the strains either needed less time to divide and multiply, or it utilized another compound before starting to reduce nitrous oxide.

They are regardless both very strong denitrifiers, and seem to grow comfortably on all the digestate treatments.

When comparing the autoclaved digestate with added Dechloromonas, we can see that they are very comparable with the untreated digestate. This suggest that Dechloromonas might have a large role in denitrifying in untreated digestate.

Den ene startet tidligere enn den andre, og når det kommer til reduksjonshastigheten kan disse sammenliknes med ubehandlet slam.

5.5 Dilution from sampling

The results from the robot measurements are interpreted in an excel sheet that helps us regulate and interpret the data.

An example is that the excel sheet helps us calculate out the dilutions (except nitrous oxide) from the robot, making it easy to see if the changes in the gasses are microbial activity or dialution.

When the robot takes a sample from a flask, it removes some of the gas from the headspace. Each time the robot takes out a sample, there is a small leakage through the septum, either in or out of the flask depending on the gas concentrations. This is regulated for.

It also takes into account that every time it samples from the flask, the gas concentrations entering the robot needle are not the same at that excact point, as the remaining concentrations in the flask at the time of sampling.

The NO- analyser was not functional during my experiments, which is why I have not measured the NO concentrations in my experiments. The other gasses are however sufficient to get a good image of the microbial growth without.

There was some problems with the robot during the time of my thesis, which made it impossible for me to use results from 3 additional experiments. I did run a pure culture experiment with Bacillus vireti which had some severe integration problems. I also ran an experiment with a Bradyrhizobium and a strain of Pseudomonas stutzeri. They are not included because the experiment was done before we realized the pH changes, and so there was nothing happening in the flasks from these bacteria. There was also some problems with the needle on the robot, making the results hard to show.

We did also have an idea that we should try to count the amount of cells in the digestate using PCR. The PCR count would then be confirmed by physical counting in the microscope, and CFU on petri dishes. This was spent a significant amount of time on in the lab, but we did not get the PCR counting work out unfortunately, which is why it is not included here.

There was a lot of problems last minute with the conversion of this document from a doc to a PDF, which led to a lot of mess in the document. Time ran out, and there was no possibility of getting to fix it.

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