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Out of breath: entrapping the denitrifier *Paracoccus denitrificans* in anoxia and rescuing it by spikes of oxygen

Kjetil Johnsen Hauge
Master of Science, Biotechnology

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Faculty of Biotechnology, Chemistry and Food Science
Norwegian University of Life Sciences
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Sammendrag

N_2O er en dobbel fare, da det er en kraftig drivhusgass, og den største kjemiske svekkeren av ozonlaget som ikke er regulert av Montrèalprotokollen. Utslippene har økt på grunn av økt mikrobiell aktivitet som igjen skyldes økt menge reaktivt nitrogen i miljøet, som primært kommer fra menneskelig bruk av kunstgjødsel.

Heterotrofe denitrifiserende bakterier er muligens den største kilden til N_2O, men også ett mulig «sluk», siden de har det eneste enzymet som spesialiserer i å redusere N_2O. Forståelse av denitrifikasjonsenzymene på ett regulatorisk nivå vil hjelpe oss å lage modeller som kan hjelpe oss i å redusere N_2O utslipp i fremtiden.

Denne masteroppgaven utforsker hvordan modellbakterien *Paracoccus denitrificans* kan «fanges i anoksia»: i teorien er dette mulig om cellene opplever en plutselig fjerning av alt oksygen, fordi de da mangler energi til å syntetisere de enzymene som kan sette den i stand til å respirere anoksisk (denitrifisere). Eksperimentene ga oss økt regulatorisk innsikt. Masteroppgaven var delt i tre deler. Den første delen var å finne en effektiv metode for å fange *P. denitrificans*, dette lyktes med oksygenfjerningsmetoden med kallenavnet «GOX», som bruker glukose og enzymene glukose oksidase og katalase for å fjerne rester av O_2 i flasker. Min andre oppgave var å bruke GOX for å «fange» *P. denitrificans* i anoksi. Dette ble gjort via robotinkubasjon en stamme *P. denitrificans* celler med det genmodifiserte genet nirS:mCherry, som lager ett fluoriserende rødt protein når nirS (nitritt reduktase) blir uttrykt. Disse cellene ble også farget med «FITC» Fluorescencs Isitiocyanate, ett fluoriserende grønt fargestoff som binder seg på kovalent til proteiner på cellens overflate. Dette lyktes også, via målinger av gasskinetikk og fluorescens mikroskopi. Min tredje og siste oppgave var å demonstrere oppgave 2 med ett flow cytometer, da dette ga flere fordeler, spesielt tidsbesparelse, kontra fluorescens mikroskopi. Også dette lyktes. Oppgaven som helhet viste viktigheten av O_2 til å rekruttere denitrifikasjonsenzymer når anoksi nærmer seg; før alt oksygenet er brukt opp. Fenomenet ble observert i *P. denitrificans* , men man kan forvente at andre denitrifiserende bakterier har det samme behovet for å sysntetisere denitrifikasjonsenzymer i forkant av anoksia.

Abstract

N_2O, is a double danger, as it is a potent greenhouse gas, and the largest depleting substance of the ozone layer not regulated by the Montrèal protocol. The emissions have increased due to increased microbial from increased N inputs, mostly due to anthropogenic use of fertilizers.

Heterotrophic denitrifiers are possibly the largest source of N_2O, but also a potential sink, as they have the only enzyme that specialize in reducing N_2O. Understanding the regulation of denitrification pathways at regulatory level will help us in creating models to help mitigate N_2O emissions in the future.

This thesis explored how the model organism denitrifier *Paracoccus denitrificans* can become "entrapped in anoxia": in theory, this is possible if cells are exposed to a sudden removal of all oxygen, because they would then lack the energy to synthesize the enzymes needed to enzymes for anaerobic respiration (denitrification). The experiments provided us with an increased knowledge of the regulatory biology. The thesis was subdivided into three parts. The first part was to find an effective entrapment assay, that proved fruitful with the enzymatic O_2 scavenging method nick named "GOX", utilizing the enzymes glucose oxidase, catalase combined with glucose to scavenge trace amounts of O_2 in experimental bottles where anoxia is important. My second task was to entrap *P. denitrificans* in anoxia using GOX. This was achieved by robot incubation, using a genetically modified strain of P. denitrificans, that expressed a red fluorescent protein, mCherry, when the gene nirS (nitrite reductase) was expressed. Cells of this strain was also stained with FITC, "Fluorescence Isothiocyanate", a fluorescent green dye, binding covalently to proteins on the cell surface. This task was also successful, as measured by fluorescence microscopy and gas kinetics. My final task was to demonstrate the previous task using flow cytometry, a method that offered several advantages, chief among them time-saving, contra fluorescence microscopy. This task was also successful. This master thesis illuminates the crucial role of O_2 when recruiting enzymes for anoxic respiration. The phenomenon was observed in the model organism *P. denitrificans*, but is expected to be relevant also for other denitrifying organisms.

Abbreviations

ABS	Absorption
ECD	Electron capture device
FITC	Fluorescein isothiocyanate
GC	Gas chromatograph
GOX	Glucose oxidase and catalase.
OD_{660}	Optical density at 660 nm wavelength
TCD	Thermal conductivity detector

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1. Introduction

1.1 N_2O, the double danger: N_2O, nitrous oxide, commonly called "laughing gas", depletes ozone, and is a powerful climate gas. According to the Intergovernmental Panel on Climate Change it has a 298 times larger climate effect than CO_2 in a 100-year perspective (IPCC, 2007). In their summary, the panel also states that N_2O has a life-span of a 114 years, nearly 10 times that of CH_4, methane, the second largest anthropocene climate gas (ibid). Nitrous oxide is also the largest anthropogenic source of the depletion of the ozone layer (Ravishankara et al., 2009), and its emission is not regulated by the Montrèal protocol (ibid). Nitrous oxide therefore presents a double danger. And its concentration in the atmosphere has been escalating since 1850, due to anthropogenic activity. Initially this came from combustion engines, and rice and legume crops (Galloway et al., 2004). However, after the invention of the Haber-Bosch process, microbial use of fertilizer from agriculture has become the largest source. The N_2O levels in the atmosphere, as measured by ice core samples from Law Dome, Antarctica, varied between the year 1 to 1100 A.D., from ~263 to ~275 parts per billion (ppb) in the atmosphere (Macfarling Meure et al., 2006). As of November 2019, nitrous oxide levels are at 332.62 ppb in the atmosphere, as measured in situ at Mauna Loa, in Hawaii (Elkins et al.). But why are N_2O levels rising, and what are the sources of N_2O? To understand this, we need to understand the nitrogen-network, and how atmospheric N_2 is transformed into its chemically reactive, bioavailable forms.

1.2 Nitrogen and its network: "Nitrogen has arguably the most complex cycle of all the major elements" -Galloway et (al. 2004). I will not attempt to explain the full scope of the N-network/cycle, as that is far outside the reach of this master thesis. But a functional understanding is required. Most nitrogen is chemically inert, such as the 78% of our atmosphere that N_2-gas makes up. However, chemically reactive nitrogen is added to the environment, through both natural, particularly biological sources, and anthropogenic activity (Schlesinger, 2009). Lightening and combustion of fossil fuels create some reactive nitrogen in NO_x and NH_x forms. But the primary inputs of reactive nitrogen into the network are biological nitrogen fixation and industrial nitrogen fixation by the Haber Bosch process (ibid). After the industrial revolution and the invention of the Haber-Bosch process, the total amount reactive nitrogen has increased dramatically (Galloway et al. 2004). In 2009, Schlesinger estimated that the Haber-Bosch process adds 125 Tg of chemically reactive nitrogen annually. Fossil fuel combustion adds 25 Tg (ibid) and human cultivation of symbiotic plants that fixate nitrogen (e.g. legumes), adds another 20 Tg per year (ibid). This brings the total input of

anthropogenic reactive nitrogen up to 170 Tg per year. Natural sources of reactive nitrogen through biological N-fixation and lightening is estimated to 125 Tg per year (ibid). Making for a total of 295 Tg of chemically reactive nitrogen added to the atmosphere, hydrosphere and biosphere annually. For most of the history of life on the planet biological nitrogen fixation and lightening created most of the reactive N available. Anthropogenic activity has more than doubled the input of reactive N in the atmosphere, biosphere and hydrosphere.

1.3 Specific pathways:

Nitrogen fixation is performed by prokaryotes, particularly bacteria, usually in symbiosis with plants. The prokaryote fix atmospheric nitrogen (N_2) by reducing it to ammonia (NH_3) (Newton, 2007). This is a very energy-demanding process, but vital for all life on the planet, as all life require reactive N. Haber Bosch is an anthropogenic method for N-fixation, and is also energy demanding, using high pressure to combine N_2 and H_2 into NH_3. We would not be able to feed the current human population without Haber-Bosch or an equivalent N-fixing technique (Galloway et al., 2004). The fixed NH_3 can be protonated into ammonium (NH_4^+). Both NH_3 and NH_4^+ can be assimilated into organisms to build proteins, this is from a biological perspective their most important function. But both can also act as energy sources. Nitrifiers are autotrophic organisms utilizing NH_3 oxidation to nitrate (NO_3-) as an energy source (Spanning et al., 2007a). They oxidise NH_3 into nitrite (NO_2-) and NO_2- into NO_3-. This can be done stepwise by different organisms, or completely by one (commamox). This oxidation process has small "leaks" and release N_2O as a byproduct. The prokaryotes are autotrophic and require O_2 for the process. NH_4^+ can be oxidized anerobically with nitrite (NO_2-), a process called anammox (Op den Camp et al., 2007), which result in N_2-gas and water, and yield energy to the autotrophic anammox organisms. This is one of the major ways of removing reactive nitrogen, but it represents a smaller sink than denitrification. NO_3^- can be incorporated into organisms, and is also the initial substrate for the heterotrophic, anaerobic respiration process denitrification. Denitrification yields energy to the organism by stepwise reduction of NO_3- to NO_2- to nitric oxide (NO) to N_2O to N_2 (Hassan et al., 2016).

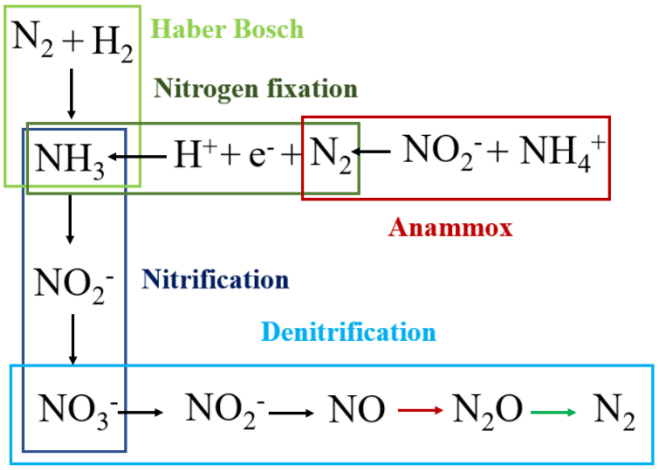

Figure 1: Some key processes in the nitrogen network. The red and green arrow in denitrification represent the source and sink, respectively, of N_2O within denitrification.

The last step, reducing N_2O to N_2, is performed by a reductase, coded by the gene *nos*Z. *nos*Z is the only known gene coding for an enzyme that specialize in reducing N_2O into N_2. The N cycle have several other pathways, but only two major ways of removing reactive nitrogen: Anammox and denitrification, and only denitrification has enzymes specialised for reducing N_2O.

1.4 Denitrification: Denitrification is the major pathway that remove reactive nitrogen from the biosphere to the atmosphere. In waste water treatment it is frequently combined with nitrification to remove NH_3 from waste water, releasing a combination of N_2O and N_2 into the atmosphere (Kampschreur et al., 2009). There are several variations of denitrification in nature. One is nitrifier-denitrification, where autotrophic, ammonia oxidising bacteria oxidize NH_3 to NO_2^-, followed by reduction of NO_2^- to N_2O, all happening in one organism. Another is heterotrophic denitrification, by prokaryotes or fungi, reducing NO_3^- to N_2 to sustain respiratory metabolism in anoxia. Both release N_2O, and they seem to be the primary sources of N_2O in the atmosphere (Bakken & Frostegård, 2017). There is some debate about the role of nitrifier-denitrification, both for the organisms, and whether it plays a significant role as a source of N_2O. Bakken & Frostegård argue in their 2017 editorial that investigations using isotope tracing has grossly exaggerated nitrifier denitrification. And they further argue that heterotrophic denitrification seems to be the primary source of N_2O. Regardless of whether N_2O is produced by heterotrophic denitrifiers or nitrifiers, only heterotrophic denitrifiers can reduce N_2O-emissions by reducing N_2O, to harmless N_2, since only heterotrophic denitrifiers have the gene for nitrous oxide reductase (N_2OR/*nos*Z). Research on heterotrophic denitrifiers gas emissions is well over a half a century old (Šimek & Cooper, 2002). An "ideal" or "platonic" heterotrophic denitrifier would release ½ mol of N_2 per one mol of NO_3^- and have a N_2O/N_2 ratio of 0/100, but such an

	NAR	NIR	NOR	N_2OR	pH 7.4 soil series A	pH 3.7 soil series B	pH 3.7 soil series C
Full-fledged	N_2				8	0	1
NIR, NOR, N_2OR		N_2			0	0	3
Only N_2OR				N_2	2	1	0
NAR*/NIR*, N_2OR	NO_3^- and NO_2^- reduced			N_2	0	0	2
NAR, NIR, NOR	N_2O				1	2	0
NIR, NOR		N_2O			1	4	4
NAR, NIR	NO				4	0	0
Only NIR		NO			0	2	0
Only NAR	NO_2^-				19	13	1
DNRA	NO_3^- and NO_2^- reduced to NH_4^+				1	1	0

Figure 2: Denitrifiers with full-fledged and truncated pathways. A full-fledged organism can perform the complete denitrification pathway because it has and is able to express the genes nar/nap, nir, nor and nosZ, coding for the enzymes NAR/NAP, NIR, NOR and N_2OR, which catalyze the four steps of denitrification NO_3^-→NO_2^-→NO→N_2O→N_2. Lycus (et al., 2017) isolated denitrifying organisms in soil, and found that the majority had truncated pathways, as illustrated.

ideal organism does not exist, since N_2O is a free intermediate (a minimum of N_2O is inevitably released). What gases, and particularly what N_2O/N_2 ratio heterotrophic denitrifiers release *in situ* depends on several factors (Lycus et al., 2017; Šimek & Cooper, 2002). The first major factor is truncation: Not all heterotrophic denitrification bacteria contain all genes needed for the complete denitrification pathway; some lack genes for some of the steps of denitrification. A denitrifier can have all the reductases, or lack 1-3 of the four enzymes required to reduce NO_3- all the way to N_2, see figure 2. Thus, denitrifiers exist, which produce either NO, N_2O or N_2 as their final product. An important distinction is between the denitrifiers possessing either nitric oxide reductase (NOR) or nitrous oxide reductase (N_2OR), or both the enzymes. Truncated denitrifiers with NOR but not N_2OR are net-producers of N_2O, as they can only produce N_2O and not reduce it. While truncated denitrifiers with N_2OR but not NOR can only reduce N_2O, not produce it. And denitrifiers with both NOR and N_2OR can both produce and reduce N_2O. Genes therefore directly affect the individual organism's ability to denitrify and its product, but also leads us to the second factor, community composition. The price of genome sequencing has decreased drastically after the the turn of the millennium and continue to decrease in price (van Nimwegen et al., 2016). This has made metagenomic analysis of microbial communities a viable method for researching gas emissions from soil. Several scientists have attempted to use such genomic information to predict the propensity of soils to emit N_2O. i.e that the N_2O emissions from soils correlate with their *nor/nosZ* gene abundance ratios. Some of the studies show that N_2O-reduction correlates negatively with genetic abundance of nosZ (or positively with *nor/nosZ* or *nir/nosZ* abundance), while other studies have not managed to replicate this (Liu et al., 2014; Lycus et al., 2017). It appears that the gene abundance ratios (*nir/nosZ* and *nor/nosZ*) is not the most important factor controlling the propensity of microbial community to produce N_2O.

The fact that gene abundance is a poor predictor of N_2O emission could reflect that only a fraction of the genes in a community is expressed. Thus, it could be more interesting to quantify what genes are actually expressed, as the genes expressed are obviously more important than having but not utilizing a gene. This is the third factor. Measuring gene expression, rather than gene abundance, as a measure of the communities' capacity to produce or consume N_2O. By measuring the transcript abundance of *nor* and *nos*Z, the *nos*Z/*nor* transcript abundance ratio would thus predict if a community is a net N_2O-sink or source. However, Bergaust and colleagues showed in their 2010 paper that even with high levels of N_2O-reductase transcription occurring, N_2O was not reduced when pH was low. pH is the

fourth factor affecting N_2O-emissions, and have been described as “the master variable” in soil (Šimek & Cooper, 2002). It is well known that soils capacity to reduce N_2O decrease with decreasing soil pH (Šimek & Cooper, 2002), and papers by Bergaust and Liu (Bergaust et al., 2010; Liu et al., 2014) showed that this was not due to lack of *nosZ* transcription at low pH. This could suggest that low pH inhibits N_2O reductase-enzyme, or that low pH hampers the synthesis of functional N_2O reductase. The latter was proven to be the case, both for the model bacterium *Paracoccus denitrificans* (Bergaust et al., 2010) and for soil bacteria (Liu et al., 2014). In both cases, N_2O reductase synthesized at pH 7 was functional at low pH. They concluded that the primary reason for low N_2O reduction capacity of acidic soil is that low pH impedes the synthesis of functional N_2O reductase.

1.5 Assembly of N_2Oreductase: Copper-atoms are inserted into the N_2O-reductase enzyme when the enzyme is in the periplasm of the cell. The periplasm, unlike the inside of the cell has a poorly regulated pH, greatly influenced by the pH of the external environment. The inhibition seems to stem from low pH effect on the CuA site in protein structure (Bergaust et al., 2010; Fujita & Dooley, 2007). This explains the more than 50 year old observation that the N_2O/N_2 product of denitrification, increase with decreasing pH in soil (Šimek & Cooper, 2002). The bacteria in the soil tries to utilize the N_2O for respiration by expressing *nos*Z but are unable to assemble functional N_2O reductase due to low pH. But the making of functional N_2O reductase is not completely inhibited in acidic soil, since soil is a complex medium with many microniches. Some of the bacteria may therefore assemble N_2O-reductase and reduce N_2O in soil that seems to be too acidic for this to happen, due to a neutral/alkaline pH microniche. This takes much longer time in acid than in neutral/alkaline soil, however, and as a result much of the N_2O escapes from anoxic microenvironments before functional N_2O reductase is in place.

1.6 Transcriptional regulation of denitrification: Denitrifying bacteria have two alternative methods for respiration, since the O_2-levels in the environments they live in fluctuate (Bergaust et al., 2010). Denitrifying bacteria prefer O_2 as an electron acceptor during respiratory metabolism for an obvious reason: The generation of proton motive force (PMF; charge separation across the membrane) is more efficient for oxic than for anoxic respiration (Spanning et al., 2007b). In response to O_2 depletion, however, the bacteria may express the genes for denitrification and start to respire by denitrification. The gene expression is regulated by the proteins sensing the concentrations of oxygen and nitrogen oxides (NO, NO_2^- and NO_3^-), and the regulatory network for denitrification genes has been characterized for a

number of model organisms (Spanning et al., 2007b), this is the fifth factor affecting N_2O-emissions.

1.7 Bet hedging: The classical major factors, described above, influencing N_2O-emissions in soil are genes and truncation, community composition, gene expression, soil pH and N_2O-reductase assembly. But recently another factor affecting N_2O-emissions have been discovered: Bet hedging. The phenomenon requires some explanation: Imagine a platonically perfect denitrification bacteria with perfect knowledge of the future, of fluctuating O_2-levels and the availability of substrates in the denitrification pathway. The organism would create the ideal amount of enzymes and adapt perfectly to future conditions. It would create the required enzymes for denitrification when the O_2 levels depletes. Such a bacterium would also be perfect to mitigate N_2O emissions, as it would know the future levels and scavenge all available N_2O. This bacterium does of course not exist. And all organisms adapt to a compromise of their current conditions, while preserving the ability to adapt to changed future conditions. The denitrifier *Paracoccus denitrificans*, a commonly used model organism in denitrification research, faces a dilemma when experiencing oxygen depletion:

> *P. denitrificans* has an obligate respiratory metabolism. So when facing anoxia it requires the energy from aerobic respiration to synthesize the denitrification enzymes(“recruiting to denitrification”).
>
> But *P. denitrificans* does not know the future as our "ideal" denitrifier does. *P. denitrificans* does not know the length of the anoxic spell. If it is brief, it may be energetically wasteful to create all the denitrification enzymes. If anoxia lasts long, however, it will be penalized by not expressing the denitrication preoteome, it will be outcompeted by other denitrifiers that recruited the full proteomes. So how does a population of denitrifiers behave?

Till recently it was commonly assumed that all cells within a species population would react similarly to environmental impacts. The classic example of this is the diauxic growth in *Escherichia coli*, when provided with a mix of glucose and lactose: it “prefers” glucose as its C-source, and when glucose is depleted it uses stored energy to fuel the synthesis of enzymes for lactose utilization. While the change is taking place, the population experiences a lag phase, with low respiration and little/no growth. Monod (1949) discovered the phenomenon by measuring respiration rates and named it *diauxic lag.* It was commonly assumed that all cells within the population would synthesize the new enzymes during such a diauxic lag.

However, more refined analyses of such phenomena have revealed cell differentiation during diauxic lag: some express the new enzymes, while others do not (Siegal, 2015). Several mechanisms can cause such cell differentiation: One is differentiated inheritance i.e. that when the cell is undergoing binary fission, all protein required for a specific phenotype emigrate to only one daughter cell. This creates two phenotypically distinct daughter cells from the same progeny, a bifurcation. If the bifurcation results in stable subpopulations, in the same environment, it is called bistability (Becskei et al., 2001). Another possible explanation for phenotypic differences arise from the stochastic initiation of gene expression. Since cells contain few copies of many regulatory molecules, these are more exposed for stochasticity, or "noise" (Elowitz et al., 2002). In the case of *P. denitrificans*, the *nir*S gene, once initiated, induces a positive feedback loop, via nitric oxide, NO (which enhance *nir*S transcription), described more below. The stability of the different phenotypes can be regulated by such feedback loops. There are several ways this happens; once a new subpopulation forms it may supress the remaining undifferentiated population from differentiating. It may also be time dependent, by internal or environmental factors. So if the a cell does not respond by a certain time, it is "locked" to a phenotype. But why would bacteria "choose" a strategy of not expressing a phenotype suited to their new environment? Because nature has selected for bacteria that differentiate their isogenic population for higher fitness over time. By splitting their population, the bacteria prepares for an uncertain future, one part of the population preparing for one outcome, and the rest another. This increases the fitness of the total isogenic population (Ackermann, 2013). The cell differentiation has been coined *bet hedging*, since this is essentially what the population does: hedging its bets on the future conditions.

1.8 Bet hedging in *Paracoccus denitrificans:* *Paracoccus denitrificans* is an alpha-proteobacterium, and a frequently used model organism in denitrification research. It has an obligate respiratory metabolism and is a full-fledged denitrifier. By modelling the kinetics of respiratory metabolism in *P. denitrificans,* Hassan et al. (2014 & 2016) were able to reproduce experimentally observed respiration kinetics during the transition from oxic to anoxic respiration in response to oxygen depletion. But only by assuming bet hedging: that only a fraction of the population expressed the genes for nitrite and nitric oxide reductase (*nir*S and *nor*). The modelling suggested a very low probability for the initiation of *nir*S transcription (~0.005 h^{-1}), which implied that a substantial fraction of the population would

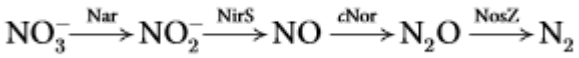

Figure 3. Substrates and genes used by Paracoccus denitrificans in denitrification. From Hassan et. al., 2016.

not initiate nirS transcription before complete exhaustion of oxygen. This part of the population would thus be entrapped in anoxia, unable to provide energy for synthesising any new proteins. The model thus includes two essential elements for the bistability of the population (stochastic product induces *nir*S transcription), a positive feedback loop and a negative feedback loop (entrapment in anoxia). Direct proof of the phenomenon was provided by (Lycus et al., 2018). Lycus et al. (2018) demonstrated bet hedging by using the *P. denitrificans* strain PD1222 mCherry:nirS. The strain was genetically modified and contained an inserted *mCherry* gene onto the *nir*S-gene. The *nir*S gene codes for the protein nitrite reductase, it reduces nitrite (NO_2) into nitric oxide (NO). By inserting the *mCherry*-gene, a fluorescent red signal was produced when expressing *nir*S. So, the part of the population expressing *nir*S would fluoresce red. This was combined with cell-staining, using the fluorescent green dye fluorescein isothiocyanate (FITC). The dye would dilute 50% every generation, giving a measure of growth. NirS-mCherry and FITC could be quantified using fluorescence microscopy. Lycus et al. (2018) performed the experiments in serum flasks where the headspace atmosphere was replaced with helium by "He-washing" (repeated evacuation and He-filling). Oxygen-levels could be regulated as needed, as far down as ~1 umol $bottle^{-1}$. The experiments showed that in response to oxygen depletion, an isogenic population divided into two distinct subpopulations. All cells expressed the gene *nos*Z (N_2O reductase), while a subpopulation expressed the genes *nir*S and *nor*, coding for NirS and NOR, which reduce nitrite to nitric oxide, and nitric oxide to nitrous oxide, respectively The authors suggested that this bet hedging would improve the fitness (Lycus et al., 2018). The majority would save energy by only expressing N_2O reductase and was ready for a reversal. They did not overcommit in case of oxygen return, but would have a minimum of respiration (by reducing N_2O) in anoxia, should it last. While the smaller subpopulation committed, investing their energy in synthesising NIR and NOR. If the anoxia turned out to be a brief spell, the majority would benefit, not wasting their energy. Should the anoxia last, the larger population would be penalized, though keeping a low anaerobic respiration by reducing trace N_2O provided by cells with all denitrification enzymes. While the smaller population would be able to grow rapidly. The implication of this research is that *P. denitrificans* will function as a N_2O-sink when O_2 becomes limiting.

1.9 Anoxia: Qu et al. (2016) suggest that O_2 repress *nir*S more effectively than *nos*Z. While Lycus et al. (2018) provided clear evidence for bet hedging in the sense that only a fraction of the population express *nir*S and grow fast in anoxia, they provided no direct

observation of entrapment in anoxia. In fact the only direct evidence of entrapment in anoxia was provided by Højberg et al. (1997), who transferred cell entrapped on filters from fully oxic to fully anoxic conditions, which arrested growth. Such immediate transfer to complete anoxia was not possible with batch cultivation technique used in our laboratory because He-washing inevitably left traces of O_2 in the vials (Bergaust et al., 2010). The "entrapment in anoxia" phenomenon is important because it sheds light on the perils of oxygen depletion for any denitrifying organism: if they fail to express NAR and/or NIR in due time, they will become entrapped in anoxia. Which thus explains why the oxygen-repression of denitrification gene expression must be relieved while oxygen concentrations are still sufficient to allow aerobic respiration to produce the energy to synthesize denitrification enzymes. The problem is that 1) we lack a direct demonstration of entrapment of *Paracoccus denitrificans* in anoxia and 2) we do not know how widespread this is among denitrifying bacteria. For this reason, the NMBU Nitrogen Group initiated a study of entrapment of denitrifying bacteria in anoxia, as a part of their general investigations of the regulatory biology of denitrification. If a method for removing trace amounts of O_2 from He-washed, vials was developed, it could answer a couple of questions unaddressed by Lycus et al. (2018):

1. Although the entrapment experiments conducted by Lycus et al. (2018) provided direct evidence that cells without NIRS would be permanently entrapped in anoxia if NO_2^- was their only available electron acceptor, they did not prove that subsequent addition of a small dose of oxygen would help them "over the edge".
2. The study by Qu et al. (2016) suggested that O_2 repress *nirS* more effectively than *nosZ.* In theory therefore, all cells carry a few NOS molecules. Thus, an aerobic *P. denitrificans* culture, experiencing a sudden anoxia, will, given enough time, recruit the full denitrification phenotype in anoxia if provided with N_2O.

My experimental work is an integrated part of this study, culminating in three tasks:

1.10 My task 1; developing an efficient entrapment assay for *Paracoccus denitrificans:* The task was to find a method to effectively transfer aerobically grown cells to complete anoxia. We could use the cell immobilization technique developed by Højberg et al. (1997) but it has two major drawbacks: it is complicated and it does not allow the measurement of gas kinetics after transfer to anoxia. The He-"washing" (repeated evacuation and He-filling), as described by Molstad et al. leaves 100-400 ppmv O_2 behind. This is

evidently enough to sustain the synthesis of denitrification enzymes in aerobically grown cells transferred to He-washed vials (Bergaust et al., 2010). Thus, I had to find a chemical procedure that 1) efficiently removes the residual O_2 after He-washing of the serum vials and 2) which has no inhibitory effects on the respiratory metabolism (and growth) of the organism.

1.11 My task 2; direct demonstration of entrapment in anoxia: Once the method was established, I wanted to test whether it could be used to demonstrate that aerobically grown cells of *P. denitrificans* would become efficiently entrapped if transferred to vials without oxygen, and whether the subsequent injection of a small dose of O_2 would help them to express denitrification enzymes. In this experiment, I used the *mCherry-NirS* construct (Lycus et al., 2018), which report the synthesis of nirS by red fluorescence (mCherry-NIRS), in combination with FITC staining of cells to track growth (dilution of FITC fluorescence by growth), as described in Lycus et al.. For these experiments, I used both fluorescence microscopy and flow cytometry (see My task 3).

1.12 My task 3; test if flow cytometry could replace fluorescence microscopy for detecting mCherry:NirS expression, and declining FITC fluorescence as a measure of growth of individual cells: Lycus et al. (2018) successfully detected mCherry:NIRS expression and FITC dilution (by growth) in *Paracoccus denitrificans*, using fluorescence microscopy. This is time consuming, however, and flow cytometry was an attractive alternative. First a flow cytometer with the ability to detect FITC dilution and mCherry-NIRS expression had to be tested. If such an instrument could replace fluorescence microscopy, it would be used to demonstrate entrapment using the O_2-scavenging technique.

2 Materials

2.1 Water: Distilled ultrapure water (Type 1) nick named “Milli-Q water” was made in house by “Synergy® Water Purification System” (Merck).

2.2 Contents of solutions

2.2.1 Trace elements solution

Amount	Component
100mL	Milli-Q water
1.765g	Ethylenediaminetetraacetic acid (EDTA) (triplex 3)
10.95g	$ZnSO_4 * 7H_2O$
5.0g	$FeSO_4 * 7H_2O$
1.54g	$MnSO_4 * H_2O$
0.392g	$CuSO_4 * 5H_2O$
0.248g	$CaCl_2 * 6H_2O$
0.114g	H_3BO_3

The solution was premade in house, and stored at 4°C

2.2.2 Vitamins solution

Amount	Component
100mL	Milli-Q water
1.0g	Nicotinic acid
0.5g	Thiamine HCl
0.010g	Biotin

The solution was premade in house, and stored at 4°C

2.3 Recipes

2.3.1 Stock 10X Sistrom's medium

At the onset of the experiments 2L of a 10X stock of Sistrom's medium was prepared. The stock was frozen in 150- and 200-mL batches. The 10x stock solution contained:

Amount	Component
1.0L	Milli-Q water
27.1g	KH2PO4
1.95g	NH4Cl
40.45g	Succinic acid
1.0g	L-glutamic acid
0.4g	L-aspartic acid
5.0g	NaCl
2.0g	Nitrilotriacetic acid
2.44g	$MgCl_2 * 6H_2O$
335.0mg	$CaCl_2 * 2H_2O$
20.0mg	$FeSO_4 * 7H_2O$
200.0ul	$(NH_4)_6MO_7O_{24}$ (1%-solution)
1.0mL	Trace elements solution
1.0mL	Vitamins solution

To make Sistrom's medium a frozen 10X stock portion was thawed, and diluted 1/10 in Milli-Q water. All the other ingredients were dissolved in the Milli-Q water. The thawed solution was diluted 1/10 in Milli-Q water. The pH of the medium was adjusted by adding KOH-pellets and fine-tuning by pipetting drops of 10M KOH until a pH of 7.00 was reached.

2.3.2 Cysteine HCl stock solution

Amount	Component
10.0mL	0.1M sodium sulphide (N_2-sparged)
10.0mL	0.14M Cysteine HCl (N_2-sparged)

The N_2-sparged components was combined in a N_2-sparged bottle. This had been done in advance of the experiments in house by a different group on NMBU. This scavenger was nick-named "Cysteine HCl".

2.3.3 GOX stock solution

Amount	Component	Supplier
10.0mL	Milli-Q water	
11.4mg	Glucose oxidase (100-250 units*mg^{-1})	Sigma
2.8mg	Catalase (2k-5k units*mg^{-1})	Sigma

Glucose oxidase and catalase was dissolved in milli-Q water. A stock solution then had 200 units*ml^{-1} glucose oxidase and 1000 units*ml^{-1} catalase.

2.3.4 Glucose stock solution

Amount	Component
10.0mL	Milli-Q water
1.43mg	Glucose

The solution then had a concentration of 800 mM.

2.4 Gas standards

The GC was calibrated by three gas standards:

Component	Low/air standard	High standard	NO standard
N_2	780 000	>800	999 975
O_2	210 000	>500	-
CO_2	361	10 000	-
N_2O	0.58	150	-
NO	-	-	25
Ch4	1.84	10 000	-
He	-	The rest	-
Other trace gases	The rest	-	-

All concentrations are given in ppmv (=uL L^{-1}). Supplier is AGA AS

2.5 Instruments and centrifuges

Instruments	Supplier
Mettler Delta 320 pH-meter	Mettler Toledo
Spectrophotometer Shimadzu UV-1280	Shimadzu Europe
CellStream^{Tm} flow cytometer	Merck
Robotized incubator	NMBU Nitrogen Group
PTR-25 Mini Rotator	Grant Instruments
Zen Hamamatsu microscope	Hamamatsu

Centrifuges	Supplier
3 mL Eppendorf "Minispin" centrifuge	Eppendorf
Beckman Avanti J-25	Beckman Coulter

2.6 Software

Software	Supplier
KINCALC	NMBU Nitrogen Group https://www.nmbu.no/en/research/groups/nitrogen/spreadsheets-
ImageJ	National Institutes of Health, USA https://imagej.nih.gov/ij/
MicrobeJ	Ducret et al. 2016 microbej.com
CellStream Analysis v. 1.21	Merck

2.7 Bottles: 120 ml serum bottles were used for incubation. A 3 cm Teflon covered magnetic stirring bar was added to the bottles, for efficient stirring to ensure fast transfer of gases between the culture medium and the headspace. Each serum bottle received 50 of Sistrom's medium. The bottle tops were then covered by aluminium foil to prevent contamination, and the bottles were autoclaved.

2.8 Sealing the bottles: To accurately monitor the gas composition, and therefore bacterial/enzymatic gas production and consumption, we first had to ensure neglible exchange of gases between headspace of the serum bottles and the atmosphere. This was done by sealing the serum bottles with a butyl rubber septum and crimp-sealing them, rotating approximately 60 degrees per crimping and crimping three times. The only gas exchange would then be through the butyl rubber septum, ensuring a miniscule, but calculable leakage of N_2 and O_2 into the bottles (Molstad et al., 2007). For bottles to be used for pure cultures, sterile techniques were used when replacing the aluminum foil with an autoclaved rubber septum. For gas-standard bottles this was done without sterile technique, in bottles without liquid medium.

2.9 Replacing the headspace, "He-washing": To allow the determination of N_2 production, the air in the headspace was replaced with He. This was done by placing the reference serum bottles on an automated gas-exchanging system, described by Molstad et al. (2007). The system consists of a helium-line and a vacuum line with valves controlled by a computer, programmed to run cycles of evacuation and He-filling. The gas manifold of the system has 15 outlets with manual valves, butyl rubber tubes with fitting for mounting sterile filter (0.45um) syringes which are used to connect the vials to the manifold, (piercing the septa). The system can be programmed for running cycles of evacuation and He-fillings to remove most of the atmospheric gases, primarily N_2 and O_2, from the vials.

For each experiment, bottles with standard gas mixtures were prepared for calibrating the gas chromatograph (GC). They were first evacuated (240 seconds), using the same automated gas exchange system, and then filled with standard gases from high pressure gas cylinders. The contents of the standards are described above. Calibrating using the gas standards is described under "The robot incubator" below.

To remove atmospheric gases from the medium bottles, a thorough method was required. The standard protocol used was to place the bottles on the automated gas-exchanging system, evacuating the bottles for 180 seconds, before refilling with helium for 30 seconds, waiting for another 30 seconds before repeating the three steps 5 times. They were continuously stirred during this process. The bottles were then referred to as "Helium-washed" and contained 200-400 ppmv of O_2 and 700-1000 ppmv N_2. In the hours and days after He-washing, trace amount of gases was released from the butyl rubber septum, the

Teflon covered magnet, and the inner surface of the glass bottle into the headspace and the medium of the bottles. After He-washing the medium bottles and filling the standard bottles, the pressure in the bottes was >1 atm. To equalize this to the atmospheric pressure, a syringe containing 3 mL of water would pierce the septum. The over-pressure from the bottle would bubble through the water in the syringe, but no atmospheric gases would enter the bottles. When it stopped bubbling the bottles would have ~1atm of pressure. Bottles destined for experiments where measurement of N_2 production was not needed, were "nitrogen-washed", rather than He-washed. By piercing the septum of a bottle with two needles. One providing a continuous flow of N_2-gas and the other releasing the overpressure out into the room for 90-180 seconds. The bottle was then referred to as "nitrogen-washed".

2.10 Bacterial stock: The model organism used throughout the experiments was a genetically modified strain of the obligate and full-fledged denitrifier, bet-hedging, alpha-proteobacterium called *Paracoccus denitrificans*. The genetically modified strain is called PD1222 mCherry:nirS, because it has the gene *mCherry* inserted onto *nir*S. *mCherry* codes for a fluorescent red protein, while *nir*S codes for nitrite reductase (NIR) (Lycus et al., 2018). Thus cells expressing NIR will fluoresce red. This could be measured with photometric methods such as fluorescence microscopy and flow cytometry. Bacterial stocks were produced by sampling from an exponentially growing culture and frozen in freeze tubes, cell disruption was prevented by adding at total of 15% by volume glycerol, and the cultures stored at -80 degrees Celsius. These frozen cultures were then used throughout the experiments.

3. Methods:

3.1 Incubation and tracking gas composition; The robot incubator: The robot incubator is a robotic system described in detail in two papers (Molstad et al., 2007; Molstad et al., 2016), see figure 4. For my experiments it functioned as an incubation system for monitoring gas composition in the headspace (frequent sampling) of bottles containing bacterial cultures or chemical solutions, which were stirred by magnets to secure near equilibrium between headspace and the liquid. It also had the capability to control the temperature of the growth medium. The sampling was done by a thin syringe coupled to a peristaltic pump, transporting the gas through the injection loops of the gas chromatograph, GC, ending at a T-piece with constant He-flow (Fig. 4). After sample injection the pump is run in reverse, replacing the sampled gas with He to sustain ~1 atm pressure in the vial. The frequency of sampling could be programmed *ad lib*, but the frequency was limited by the time taken to analyze each sample ~5 minutes. The system is equipped with a custom made python shell, which sorts and displays the gas concentrations in each bottle, while the system is running. It was of some importance to monitor the gas kinetics as the experiment progressed, both for guiding the sampling of the cultures for microscopy and flow cytometry, and for spotting and solving problems such as clogged sampling needle, severe gas leaks into the peristaltic pump, malfunctioning of the GC and NO analyser, as this would give incorrect results. If such problems occurred, the sampling could

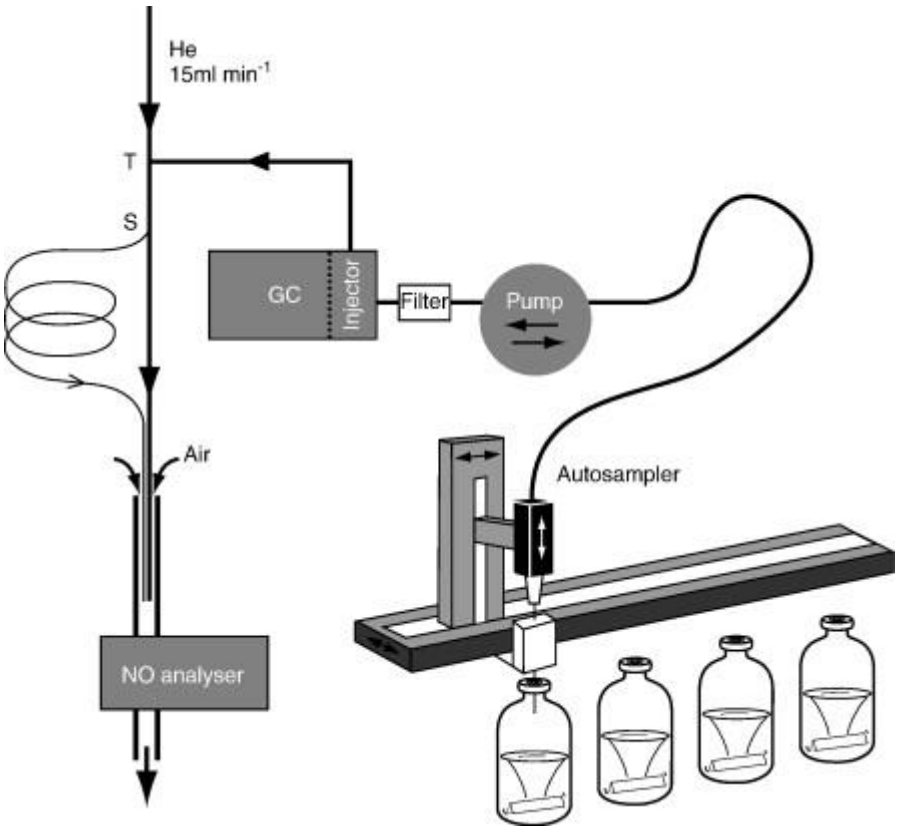

Figure 4: Schematic overview of robot sampler, from Molstad et. al (2007). Sample gas to the NO analyzer is the outlet from the injector of the microGC. In the new version of the robot (Molstad et al 2016, gas samples for the NO analyzer were taken from a third loop in the GC.

be stopped, while the incubation continued, until the error was corrected. Then the sampling continued.

3.2 KINCALC: After the experiment was done, the data could be exported to a specialized excel sheet, KINCALC, available from the NMBU nitrogen group home page, (Bakken, 2019). The spread sheet calculates the amount of each gas in headspace and medium, rates of scavenging and calculated respiratory electron flow. A prerequisite for obtaining correct estimates is that the spreadsheet is parametrized:

Response factors for each gas is determined by the peak areas for the various gases for the injections from the bottles with the standard gases. The low/air standard contained low levels (0.58 ppmv of N_2O) and was therefore used to calibrate the electron capture device, ECD, a device with a higher sensitivity, but more rapid "saturation" of signal than the thermal conductivity detector, TCD. To calibrate the higher signal strength of the TCD, the "high standard" (150 ppmv of N_2O) was used. TCD has a longer linear range, but a lower sensitivity than the ECD. "Low/air standard" was used to calibrate O_2 (210 000 ppmv) and N_2 (780 000 ppmv). High standard was used to calibrate CO_2 (10 000 ppmv).

The sample volume varied between experiments, depending on which robot is used, and the wear and tear of the peristaltic pump. The exact sampling volume must be known to calculate rates correctly, and this is estimated by the measured decline in N_2 and O_2 in the low/air standard.

Temperature is essential, since it determines the mol volume of the gases, and their solubility in the liquid. KINCALC calculates the solubility of the various gases, based on Wilhelm et al. (1977), but for CO_2, dissociation depending on pH must be taken into account. Thus, setting the right temperature and pH of the medium is essential for KINCALC to give us correct rates.

As for the sample volume, leakage of O_2 and N_2 varies between experiments, and this is estimated by the increasing O_2 and N_2 concentrations in the standard bottle with "high standard", which contains marginal amounts of O_2 and N_2 (600-800 ppmv).

Zero-offset for O_2: "Zero-offset" occurs due to small leaks into the system when sampling. So even in a completely anoxic bottle, the GC gives us a false positive of O_2 signal. To determine this, the O_2-peak areas in sampled bottles that can be

assumed to be O_2 -free are used; either bottles with chemicals with actively O_2-scavenging chemicals or, more commonly, respiring bacteria in which the O_2 peak areas reach similarly low plateaus. Judged by the variability of such low plateaus it is clear the GC cannot determine O_2 concentrations lower than ~20 ppmv accurately. We only know that it is lower than 20 ppmv (Bakken L. pers. comm.).

3.3 Sampling and fixation of cultures: Bacterial samples from the incubation robot were taken with needle and syringe by piercing the septum and extracting either 0.9 or 4.5 ml of sample and transferring to either a 3ml Eppendorff-tube or a 15 ml falcon tube, depending on the analyses to be done. In cases when it was essential to avoid that sampling caused O_2 contamination, the needle and syringe was "nitrogen-washed" first. After sampling another syringe and needle was "nitrogen washed" by pumping it 3-5 times into a "nitrogen-washed" bottle, and used to replace the sample volume with autoclaved Sistrom's medium. After sampling another syringe and needle was "nitrogen washed" and used to replace the sample volume in the sampled bottle with autoclaved Sistrom's medium. The sample was fixated by adding 30-38% formaldehyde (1:9 vol/vol formaldehyde and sample). The sample tubes were then vortexed and wrapped in aluminum foil to protect from light bleaching, and placed in a fridge at 4 degrees Celsius, before photometric tracking methods were used (fluorescence microscopy and flow cytometry).

3.4 Photometric tracking of growth by detecting NirS-mCherry and FITC-fluorescence, in single cells:

3.4.1 Optical density: To track growth of a culture, optical density (OD_{660}) measured spectrophotometrically, was used to estimate bacterial density. The spectrophotometer was calibrated at 660 nm so ABS=0, using Sistrom's medium or Milli-Q water, as they had identical OD_{660}. *P. denitrificans* had an OD_{660} of 1 at a cell density of $1{,}25*10^9$ cells*ml^{-1}.

3.4.2 FITC-staining: To differentiate growing and non-growing cells, "FITC-cell tracking" was used: cells were first stained with fluorescein isothiocyanate, FITC, a fluorescent green dye, that binds covalently to proteins at the surface of the cells. Free FITC was then removed by washing the cells, which were then used to inoculate vials. Growth results in dilution of the FITC, while non growing cells remain strongly fluorescent (Lycus et al., 2018).

The initial solution was made by dissolving fluorescein isothiocyanate (FITC) in dimethyl sulfoxide (1mg FITC mL^{-1}). This solution was then diluted, 1 ml solution per 4 ml

Sistrom's medium, making 5mL staining solution. The staining solution was filtered with a syringe and a 0.22um syringe-filter. Bacteria were stained by mixing 2.4 mL bacterial suspension (OD_{660}=0,9±0.1) with 0.6 mL filtered FITC staining solution in a 15 ml falcon tube. The falcon tube(s) was then placed in a rotator, rotating speed 5 min^{-1} in a dark chamber for 10 minutes.

The excess stain was removed by two different methods, depending on the size of the experiment. For smaller experiments, that was all but one experiment, the stained culture was transferred to several 3ml Eppendorf tubes and centrifuged at max speed (13.4*1000rpm) for 3 minutes in a "Mini-spin" centrifuge. In the large experiments the cultures were transferred to 50 ml Falcon tubes, centrifuged at 4ºC, 10 000G for 6 minutes in a "Beckman Avanti^{Tm} J-25" centrifuge with a J12 rotor. After centrifuging the supernatant was removed, and the bacterial culture resuspended with an equal amount by volume autoclaved Sistrom's medium. The centrifuging and resuspendation step were then repeated. The cultures were then thoroughly agitated in a 15 ml falcon tube, and could now be used for inoculation. The FITC binds covalently to proteins on the outside surface of the cell. The amount of FITC fluorescence declined somewhat during the first ~2 hours after inoculation, possibly due to protein-shedding (Bergaust & Bakken pers. com.), but then reached stable levels in non-growing cells, while growing cells lost FITC fluorescence in proportion to growth, i.e reducing the FITC fluorescence with 50% at each cell division. This was used to measure bacterial growth patterns using flow cytometry and fluorescence microscopy.

3.5 Fluorescence microscopy: Washing and mounting; the culture was fixed with a 10% by volume 30-38% formaldehyde solution. The first step was spinning down the culture, creating a bacterial pellet, removing supernatant, then replacing the lost volume with equal amount of a phosphate-buffer saline solution (PBS), and resuspending the bacterial pellet. Then the washing was repeated, the culture spun down, and most of the supernatant was removed, leaving of 1-5 ul of liquid to resuspend the bacterial pellet. 0.9 ul of the resuspended culture was then pipetted over to a glass slide and covered with a coverglass which was pressed hard down onto the glass slide. The low volume of the sample, relatively high spread and hard pressure applied to the cover sheet ensured a slide with immobilized cells. This was important as the fluorescence microscopy had an exposure time of approximately 1 second per image. Any cells moving would be blurry and give us poor information. The fluorescence microscope was a Zen Hamamatsu and offered us the use of several fluorescence channels. The ones used in my experiments was FITC fluorescence and *mCherry* in combination with

phase contrast. Fluorescence microscopy gave me unrivalled resolution of the cultures but were quite time-consuming.

3.6 Flow cytometry: Flow cytometry was performed by diluting the fixed culture down to 10^7 cells mL^{-1} (securing $\leq$10 000cells $*s^{-1}$) before loading into the flow cytometer. The flow cytometer was a CellStream (Merck) and was the only flow cytometer tested able to detect *mCherry* at low enough levels to be used in our experiments. CellStream had the ability to detect individual cells, their *mCherry*-signal strength and their FITC-fluorescence.

3.7 O_2 scavenging: Two different O_2 scavengers were tested for their ability to scavenge O_2, and if they had any inhibitory effect on *P. denitrificans*.

3.7.1 Cysteine HCl: "Cysteine HCl" is the nick name of an oxygen scavenging teqchnique (Patel et al., 1995). A bottle of 50 mL Sistrom's medium would receive 0.5 mL of Cysteine HCl stock solution via syringe injection.

3.7.2 GOX: GOX is the nick-name to an enzymatic O_2 scavenging method (Thorndycroft et al., 2006). A bottle with 50 Sistrom's medium would receive 1 mL glucose solution, followed by 1 mL GOX solution via syringe injections. When combined, one mol of glucose could theoretically remove 0.5 mol O_2. Catalase would remove H_2O_2 to avoid toxicity, see figure 5. It is possible that we would have a catalase peak shortly after GOX-injection. Both stocks were made fresh <30 minutes before GOX-inoculation for each experiment. When diluted in 50 mL medium the concentration was 4 units glucose oxidase$*ml^{-1}$, 20 units catalase$*ml^{-1}$ and 16mM glucose. This scavenger technique was nick-named "GOX".

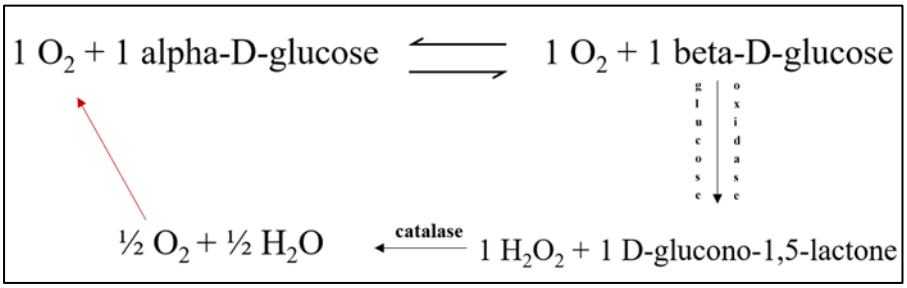

Figure 5: The *GOX reaction*: *Glucose oxidase catalyze the oxidation of glucose, producing gluconolactone + H_2O_2. Catalase cleaves H_2O_2, liberating ½ O_2 per H_2O_2, which is then removed by glucose oxidase. The net effect is that 1 mol glucose can remove 0.5 mol O_2*

3.8 Experimental designs:

3.8.1 Cysteine HCl-scavenging of O_2: The experiment consisted of 2 He-washed bottles, both containing 50 mL pH 7.0 Sistrom's medium. One bottle received Cysteine HCl-treatment. The other did not. Both bottles had a 3 cm Teflon-covered magnet, and the magnetic stirrer turned on to 600. The bottles were placed in the robot incubator at room temperature, over-pressure bubbled of, and headspace sampling set to every 15 minutes. The scavenging effect was so small that O_2 was increasing due to the frequency and leaks during headspace sampling. Therefore sampling was turned off for ~66 hours, to measure longer term scavenging. The frequency was then increased to every 15 minutes again. The incubation was run for ~71 hours.

3.8.2 Cysteine HCl toxicity: The experiment consisted of 4 He-washed bottles, all containing 50 ml pH 7.0 Sistrom's medium, and 2mM KNO_2. All bottles had a 3 cm Teflon covered magnet. 2 of the bottles received Cysteine HCl-treatment. One received it 1 week before incubation started, the other received it the day before. After placing the bottles in the incubation robot water-bath, 1mL of N_2O was added via syringe injection to the headspace. Over-pressure was then bubbled off, and magnetic stirring turned on to 600. All bottles were inoculated with 5mL of an anaerobic *P. denitrificans* PD1222 mCherry:nirS culture, that had grown over-night, OD_{660} ~0.1. The robot incubation was then started and ran for ~24 hours, and at room temperature. pH in the bottles were not measured after Cysteine HCl was added. So to control for possible inhibitory effects from change in pH when using Cysteine HCl, an uninoculated bottle was treated with Cysteine HCl, and pH was measured.

3.8.3 GOX-scavenging of O_2: The experiment consisted of 8 bottles containing 50 ml pH 7.00 Sistrom's medium. All bottles had a 3 cm Teflon-covered magnet, 6 bottles were He-washed. 4 of the He-washed bottles received atmospheric air, at two different target levels of O_2, 1% and 5% of headspace concentration. They received the atmospheric air by syringe injection. The final 2 bottles were not He-washed, and thus contained atmospheric levels of O_2. This gave 4 different initial O_2-concentrations, run in duplicate. Then, after transferring the 8 bottles to the robot incubator, the over-pressure was bubbled of, and the magnetic stirrer switched on to 600. The head-space sampling was started and ran for one headspace sampling cycle. After the first cycle the GOX- and glucose solution was added, and headspace sampling continued. Due to a miscalculation a double concentration dose of the GOX enzymes were added, but correct levels of glucose. The incubation was run for ~28 hours, and at room temperature.

3.8.4 GOX toxicity: The experiment consisted of 4 He-washed bottles with 50 mL pH 7.0 Sistrom's medium and 2mM KNO_2. All bottles had a 3 cm Teflon-covered magnet. 2 of the bottles received glucose- and GOX-solution the day before inoculation and robot incubation. This was to ensure that the O_2 had been scavenged before the experiment started. Due to following the calculations from the previous experiment, a double concentration of enzymes was added, but the correct concentration of glucose.

A *P. denitrificans* PD1222 mCherry:nirS culture was grown anaerobically in 50 mL pH 7.0 Sistrom's medium with 2mM KNO_2, over-night, reaching an OD_{660} ~0.1. After placing the bottles in the incubation robot water-bath, the over-pressure was bubbled off, then magnetic stirring was turned on to 600. Then all bottles were inoculated with 5 mL of the anaerobic over-night culture. The NO-analyzer did not work; therefore, no NO-standard was used. The experiment was run at room temperature for ~19h.

3.8.5 GOX entrapment: A total of 12 bottles were used for the experiment. All bottles had 50 mL of pH 7.0 Sistrom's medium, and a 3 cm Teflon-covered magnet. 8 bottles received 2mM KNO_2. All 12 bottles were then He-washed. After He-washing, to the 4 bottles that did not receive KNO_2 and to 4 of the bottles that did receive KNO_2, received 0.5mL of N_2O by syringe injection to the headspace.

This created three distinct series, containing different combinations of KNO_2 and N_2O. I then split the three different series in two parallels. One parallel received 1mL glucose- and 1mL GOX-solution, the other parallel received 2mL milli-Q water. This took approximately 10 minutes, then the over-pressure was bubbled off, magnetic stirring set to 600, then the incubation in the robot started. The headspace sampling started after 2 hours, due to a miscommunication.

I grew a *P. denitrificans* PD1222 mCherry:nirS culture aerobically over two nights in 50 mL pH 7.0 Sistrom's medium with 2mM KNO_2. Then I diluted the culture ¼ by volume in Sistrom's to measure ABS. The diluted culture had an OD_{660} ~0.33. I inoculated the bottles with 1 mL of the undiluted culture ~3.5 hours after the start of the robot incubation, 5.5 hours after the GOX treatment. The incubation was run at room temperature for ~48 hours. The NO-analyser did not work and NO was therefore not measured.

~23 hours after GOX treatment, 19.5 hours after inoculation, 900ul of sample was taken from one culture from each duplicate. The cultures was fixed with 100ul 38% formaldehyde. I photographed the cultures using fluorescence microscopy later.

3.8.6 Flow cytometer sensitivity test: To test the ability of various flow cytometers to detect *nirS-mCherry* expression and FITC staining, I raised *P. denitrificans* PD1222 mCherry:nirS (i.e the strain with the gene for mCherry coupled to the gene for nirS) by aerobic growth. The cells were FITC stained, and transferred to near anaerobic vials, and allowed to grow by anerobic respiration for 3 hours. This secured a mix of cells with and without NIRS-mCherry (thus with red fluorescence), all with FITC fluorescence. Cells raised aerobically without FITC were used as non-fluorescent controls. All cell suspensions were fixed by formaldehyde (as described above) and used to test the ability of the various flow cytometers. The actual testing was done by other members of the Nitrogen research group.

3.8.7 GOX entrapment measured with flow cytometry: Several aerobic cultures of *P. denitrificans* PD1222 mCherry:nirS were grown over-night and pooled together. This culture was split in two, half was to be FITC-stained, the other half was not. The culture to be FITC-stained was transferred to a cold room, stained and disperse through needle and syringe. The cultures was stained for 30 minutes. The experiment was run with 3 replicate bottles for each treatment (50 mL pH 7.0 Sistrom's medium with 0 or 2mM initial KNO_2, He-washed prior to the experiment). All bottles received KNO_2 as the experiment progressed, but some replicates did not receive KNO_2 before O_2 was depleted, after 5 hours of incubation. Two of the bottles for each treatment were sampled for flow cytometry throughout (see table 3 in appendix for exact sampling schedule), while the third was not, in order to secure observations of gas kinetics without the disturbance of sampling. In total there were 18 bottles, with the following treatments:

FITC: He-washed bottles (no GOX) inoculated with a FITC stained culture. Bottles 1-3, first 2mM KNO_2 was added after 5 hours. The results of bottle 2 and 3 are presented in this thesis.

Reference: He-washed bottles (no GOX) inoculated with an unstained culture. Bottles 4-6, first 2mM KNO_2 was added after 5 hours. The results are not presented in this thesis.

FITC+GOX: He-washed and GOX-treated bottles (1 day before) inoculated with a FITC stained culture. Bottles 7-9, first 2mM KNO_2 was added after 5 hours. The results of bottle 8 and 9 are presented in this thesis.

GOX: He-washed and GOX-treated bottles inoculated with an unstained culture. Bottles 10-12, first 2mM KNO_2 was added after 5 hours. The results are not presented in this thesis.

FITC+GOX+initial KNO_2: He-washed and GOX-treated bottles (1 day before) inoculated with a FITC stained culture. Bottles 13-15, first 2mM KNO_2 was added at 0 hours. The results are not presented in this thesis.

GOX+initial KNO_2: He-washed and GOX-treated bottles (1 day before) inoculated with an unstained culture. Bottles 16-18, first 2mM KNO_2 was added at 0 hours. The results are not presented in this thesis.

KNO_2 was added at points when headspace concentrations of N-gases (N_2+N_2O+NO) indicated NO_2 was about to be depleted. See table 3 in appendix for an overview. After 69 hours of incubation 350 uL O_2 was injected to one of the sampled bottles in each triplicate, in order to check the ability of entrapped cells to switch to denitrification. The experiment ran for ~162 hours at 17 C. But only the initial ~140 hours are presented, due to O_2 injections and N_2O injections after 140 hours confounding the results.

4. Results

4.1 Cysteine HCl scavenging of O_2: The results of the attempts to determine O_2 scavenging by Cysteine HCl are shown in figure 6. Figure 7 shows cumulative N_2, visualizing increasing N_2 levels from leaks and septum diffusion. The two different time frames of the experiment show a marginal capacity of Cysteine HCl to scavenge O_2: $\leq$ 0.1 umol O_2 bottle^{-1} h^{-1} during the first 1.5 hours after injecting Cysteine HCl and ~ 0.03 umol O_2 bottle^{-1} h^{-1} for the subsequent 65 hours.

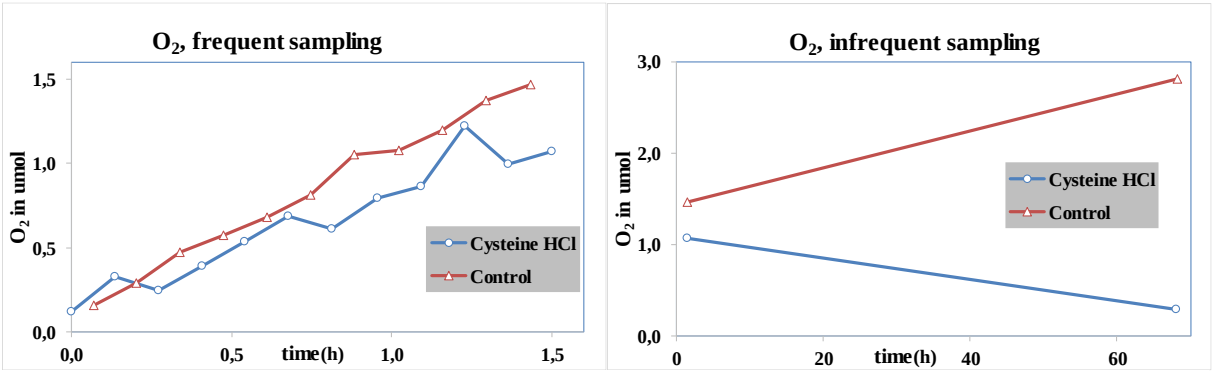

Figure 6. O_2 scavenging in Cysteine HCl: Measured O_2 (umol bottle^{-1}) in bottles with and without Cysteine HCl, at different sampling rates. The intial frequent sampling through the intial 1.5 hours (left panel), the measurement of the infrequent sampling in the subsequent 65 hours (right panel). The increasing O_2 during frequent sampling is due to a relatively high leakage of O_2: ~140 nmol O_2 per sampling (in comparison Molstad et al (2007) found O_2 leakage through sampling to vary between 20 and 50 nmol per sampling). The rate of O_2 -scavenging by Cysteine HCl when sampling frequently was ~0.1umol bottle^{-1} h^{-1}. (estimated by the diference in the rate of O_2 accumulation with and without Cystein HCl). Converted to rate per L liquid, this is 2 umol O_2 L^{-1} h^{-1}. In the vials which were unsampled for 65 hours, O_2 in the control vial increased by 1.2umol, while in the Cysteine HCl bottle it declined by 0.8 umol. The calculated rate of O_2 scavenging by Cysteine HCl in this time interval was thus ~0.03 umol O_2 h^{-1} =0.6umol O_2 L^{-1} h^{-1}.

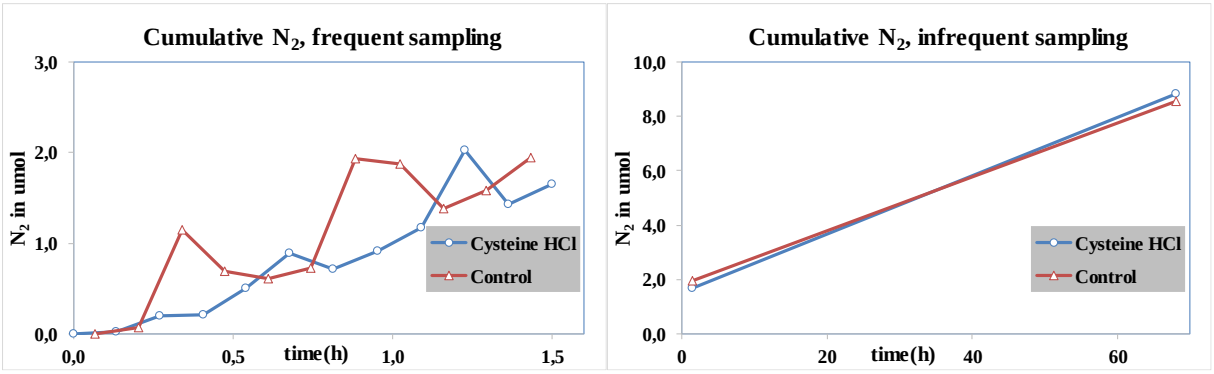

Figure 7. Cumulative N_2 in Cysteine HCl and control bottles: Cumulative levels N_2-N (umol bottle^{-1}) in bottles with and without Cysteine HCl, at different sampling rates. The intial frequent sampling through the intial 1.5 hours (left panel), the measurement of the infrequent sampling in the subsequent 65 hours (right panel). This plots show similar gas kinetics as the O_2 plots (fig. 6), with the exception of N_2 increasing in both bottles at infrequent sampling. While O_2 decreases in one the Cystein HCl treated bottle, and increases in the other, demonstrating an greater O_2 scavenging ability than the rate of diffusion through the butyle rubber septum.

4.2 Cysteine HCl toxicity: The effect of Cysteine HCl on the respiratory metabolism of *P. denitrificans* was tested by transferring anerobically raised cells to He-washed bottles with N_2O in the headspace, 2mM NO_2^- in the medium. Two replicate bottles for each treatment (with and without Cysteine HCl), and the two vials with Cysteine differed, as one received Cysteine HCl one week before inoculation, and the other the day before. Results are shown in fig. 8.

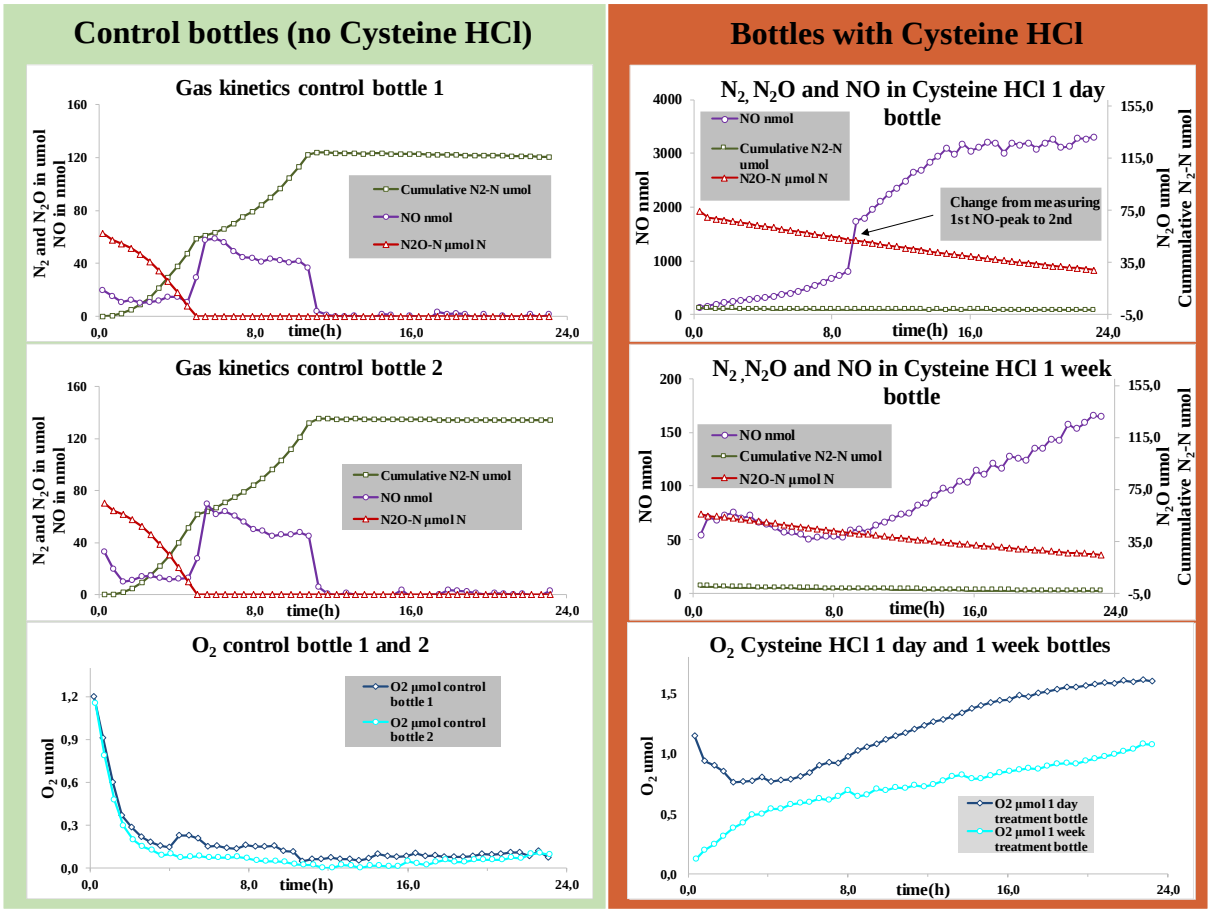

Figure 8. Toxicity of Cysteine HCl: The left panels show the kinetics in the bottles with He and N_2O in the headspace and 50 mL Sistrom's medium with 2mM NO_2^-, inoculated with *P. denitrificans* cells that were raised by anaerobic growth. The right panels show the same treatment, but with Cystein HCl added. One of the bottles in the right panel received Cysteine HCl one week prior to inoculation, the other the day before. Cumulative levels N_2-N (umol bottle^{-1}) , measured O_2 (umol bottle^{-1}), measured N_2O-N (umol bottle^{-1}) and measured NO (nmol bottle^{-1}). Notice that cumulative N_2 goes negative in the Cysteine HCl treated bottles, due to no denitrification, and problems calibrating KINCALC.

There was a no net production of N_2 in the Cysteine HCl bottles, and slowly increasing O_2 levels. N_2O decreased continuously in both bottles. NO increased in both bottles and reached several hundred nmol in the 1 week treated Cysteine HCl bottle, and several thousand nmol NO in the 1-day treatment bottle. pH was measured in a Cysteine HCl treated bottle to 7.06. In the control bottles N_2O was taken down stoichiometrically to N_2 during the first 4 hours. Thereafter N_2 continued to increase at a lower rate until reaching a plateau that accounts for the initial N_2O plus NO_2^- in the medium.

4.3 GOX scavenging: The results of the attempts to determine O_2 scavenging by GOX at different initial levels of O_2 are shown in figures 9-13 and table 1. All bottles had 50 mL Sistrom's medium, and no culture. The "0%" bottles were not completely anoxic, but He-washed before the experiment. The 1 and 5% bottles were also He-washed and received O_2 via syringe injection to reach their calculated target. The headspace of the samples was sampled for one cycle, then GOX was added (black line on plots).

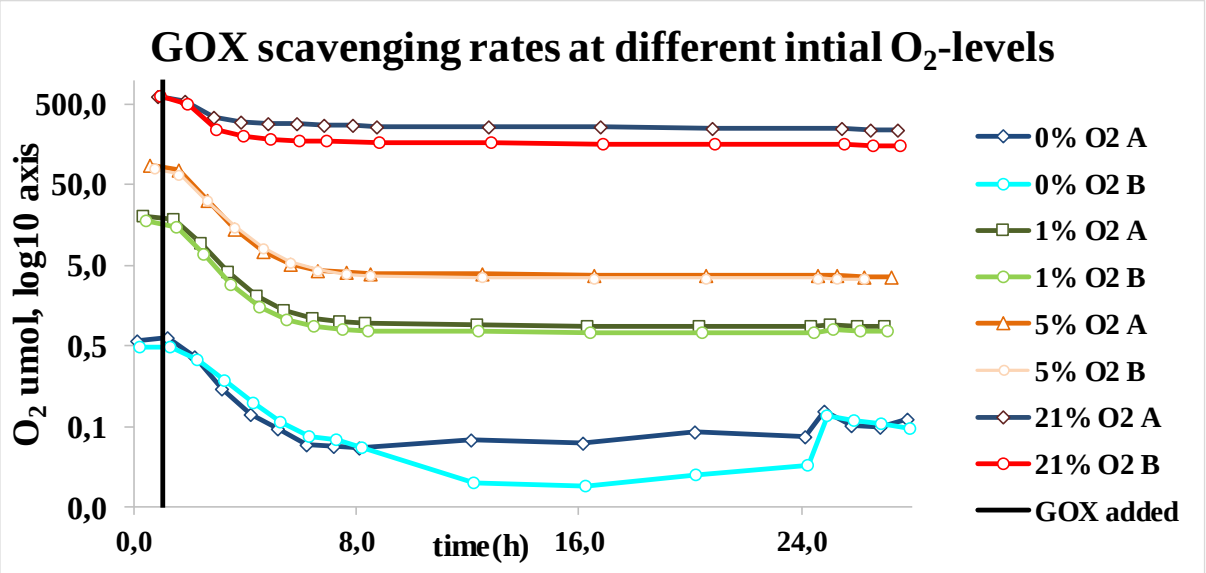

Figure 9. GOX scavengling of O_2. The panel show log_{10} (O_2, umol bottle^{-1}) dot plot with different initial oxygen concentraations in the headspace, plotted against time. The black line is the time when GOX and glucose was added to the bottles. Bottles named after target O_2 level, duplicate runs marked as A and B.

Table 1. Relationship between initial oxygen cconcentrations and maximum rate of O_2 scavenging by GOX. The table show the initial O_2 concentrations in the headspace (ppmv), the calculated O_2 concentration in the liquid (assuming equilibrium between headspace and liquid), and the maximum rate of O_2 scavenging. Regression analysis of rate (R, umol O_2 L^{-1} H^{-1}) against concentration ([O_2], uM O_2 in the liquid) showed that the rate is largely proportional with concentrations over the entire range. $V=[O_2]*15.6$; $r^2=0.98$

Measured initial O_2 (ppmv)	O_2 concentration in the liquid (uM)	Peak O_2 scavenging rate umol O_2 L^{-1} h^{-1}
196	0.27	6,78
165	0.22	4,83
7040	9.57	174
5911	8.04	160
29 529	40.1	858
26 608	36.2	686
210 050	286	4006
209 928	287	4863

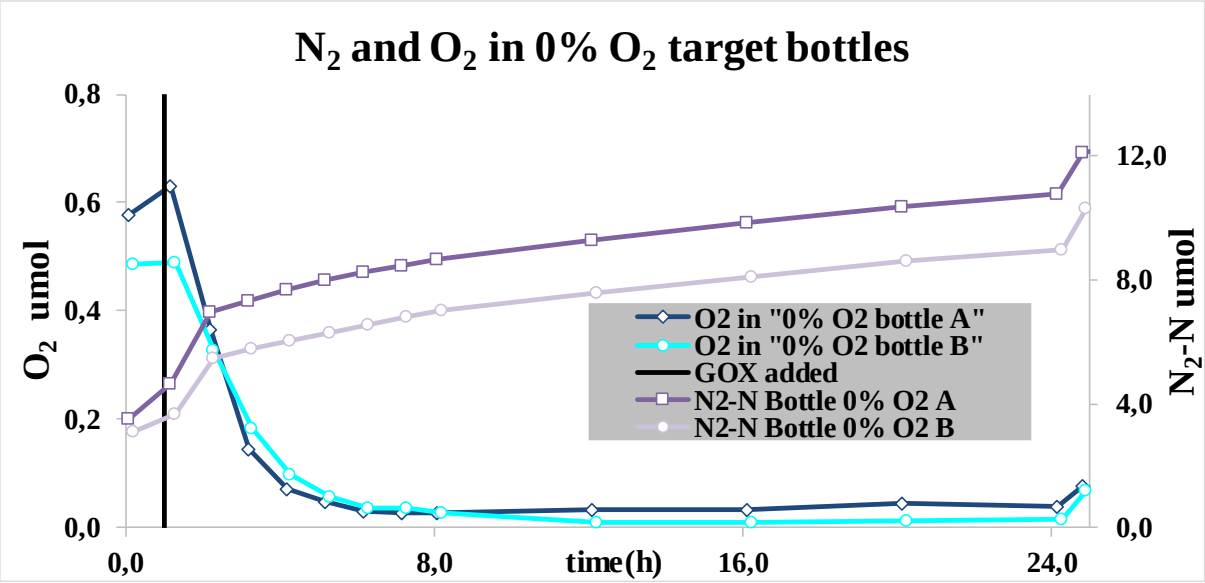

Figure 10. N_2 and O_2 dot plot in "0% O_2 target bottles". Measured O_2 and N_2 in (umol bottle^{-1}) for both replicates of 0% O_2 target bottles. O_2 on the primary Y-axis, N_2 on the secondary Y-axis. The black line is when GOX and glucose was added to the bottles. Duplicate runs on the sample plots, marked as A and B. O_2 decreases in both bottles, while N_2 increases from sampling and diffusion through the polymer septum

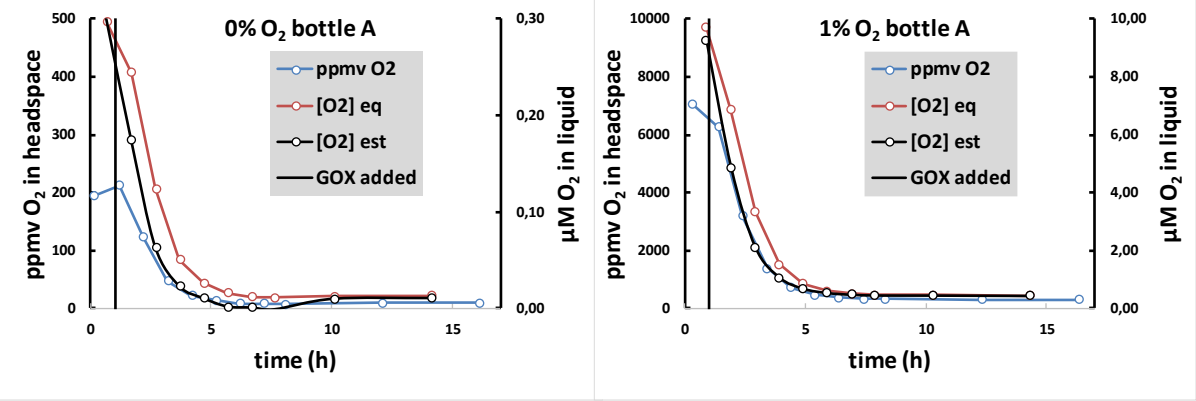

Figure 11. Dot plot of measured O_2 and calculated O_2 in liquid in 0% and 1% O_2 target bottles. Black vertical line is when GOX was injected. The panels shows the measured O_2 in headspace (ppmv = ul L^{-1}), and the calculated concentration of O_2 in the liquid (uM) for each time increment between the measurements: $[O_2]$eq is the concentration if we had equilibrium between the headspace and the liquid, and $[O_2]$est is the concentration estimated by takin transport limitation into account. This is estimated by the equation for O_2-transport from headspace to liquid $V=k_t(k_h*P_{O2}-[O_2])$ (Molstad et al. 2007), where V is the transport rate, which is estimated by the decline in O_2 in the headspace, k_t is the transport coefficient (1.2*10^{-3} L s^{-1}), k_h is the solubility of O_2 in water at the given temperature (=0.00147 mol L^{-1} atm^{-1}). P_{O2} is the partial pressure of O_2 in the headspace, and $[O_2]$ is the concentration of O_2 in the liquid (=$[O_2]$est). The left panel show the result for a bottle that was He washed without any O_2 injection (0% O_2 bottle), the right panel shows the result for the bottle with a inital O_2 target concentration of 1%.

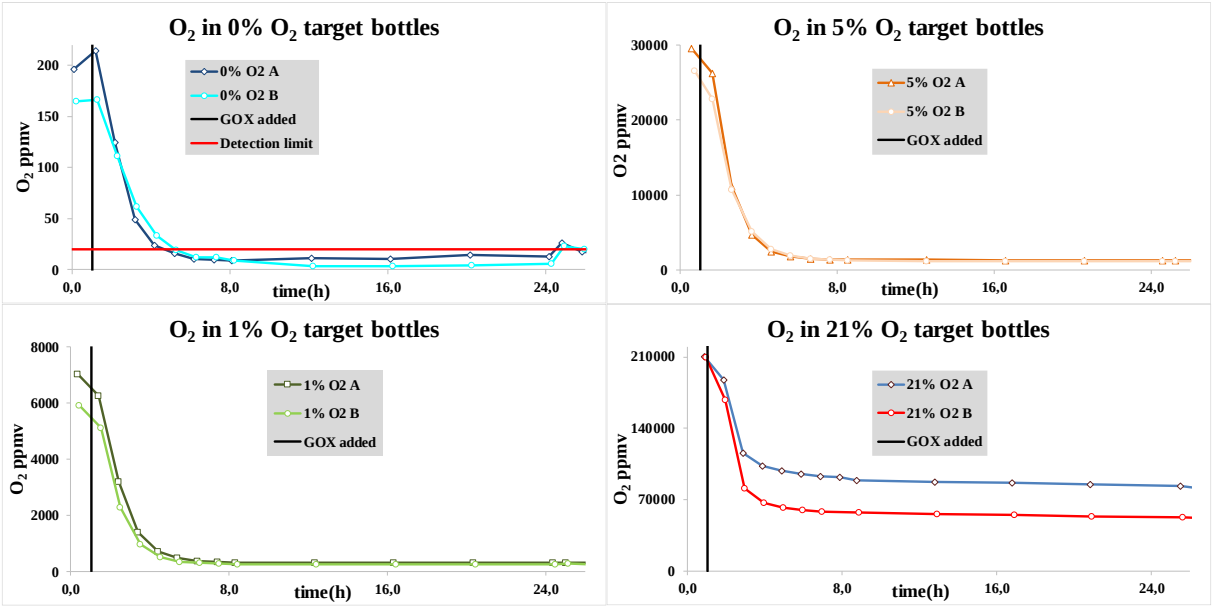

Figure 12. Detailed dot plots for GOX's O_2 scavenging for bottles with different initial O_2-levels. The black line is when GOX and glucose was added to the bottles. Each panel shows the measured concentration of O_2 in the headspace (ppmv) for a different initial O_2 level. Duplicate bottles for each treatment. The horizontal red line is the limit for quantifying O_2, which is around 20ppmv. This high limit of quantification is due to the "zero offset" for O_2: when sampling, leakage of O_2 will occur, resulting in a significant O_2 peak equivalent to 10-20 ppmv even if there is no O_2 in the bottle.

The bottles with the highest level of initial O_2 stopped scavenging O_2 the earliest and scavenged the largest total amount of O_2. The bottles with 1% initial O_2-levels stopped after 15-25 umol of O_2 was removed, while the bottles with 5% initial O_2, scavenge 70-80umol. In the bottles with 0% initial O_2-levels, GOX removed 2 umol of O_2, and the reaction takes O_2 below the detection limit (~20ppmv). All bottles, except 0% stopped scavenging after 5-10 hours. The higher the initial O_2 level was, the higher the peak scavenging rate became. The peculiar kinetics of O_2 scavenging suggested a first order reaction, but a gradual decay of the glucose oxidase enzyme. To inspect this in more detail, I calculated the turnover rate of oxygen in the liquid for each time increment: $r = V_{O2}/[O_2]$, where V_{O2} is the rate of O_2-scavenging (mol O_2 L^{-1} liquid min^{-1}), $[O_2]$ is the concentration of O_2 in the liquid (mol O_2 L^{-1}), hence the unit for r is min^{-1}. Fig. 13 shows r plotted against time for three treatments: 1, 5 and 21 % initial O_2. The figure strongly suggests a first order decay rate

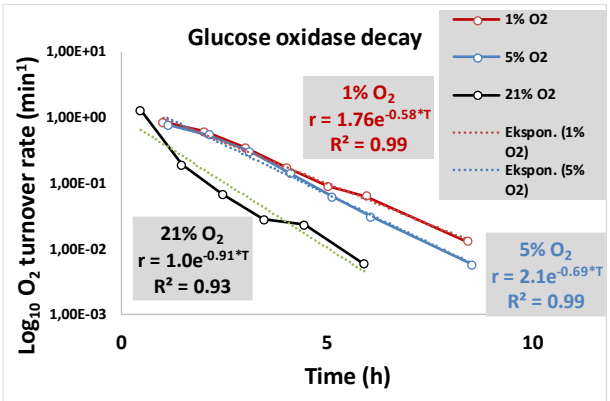

Figure 13. Dot plot of decay of glucose oxidase measured by the declining rate of O_2 scavenging. The panel shows the estimated turnover rate (min^{-1}) of oxygen plotted against time. Average values of two replicate bottles for each treatment (initial target O_2 = 1, 5 and 21%) are plotted against time after nijection of glucose oxidase, and an exponential function was fitted for the data (regression functions shown in panel).

of glucose oxidase, since the rates, when plotted on a log scale, showed a linear decline with time. By fitting exponential functions (nonlinear regression) to these data, we find apparent first order decay rates for glucose oxidase to be 0.58, 0.69 and 0.91 h^{-1} for the treatments with initial O_2 concentration = 1, 5 and 21%, respectively

4.4 GOX toxicity: The effect of GOX on the respiratory metabolism of *P. denitrificans* was tested by transferring anaerobically raised cells to He-washed bottles with 2mM NO_2^- in the medium. Two replicate bottles for each treatment (with and without GOX), the GOX bottles were treated with GOX the day before the experiment. Results are shown in fig. 14.

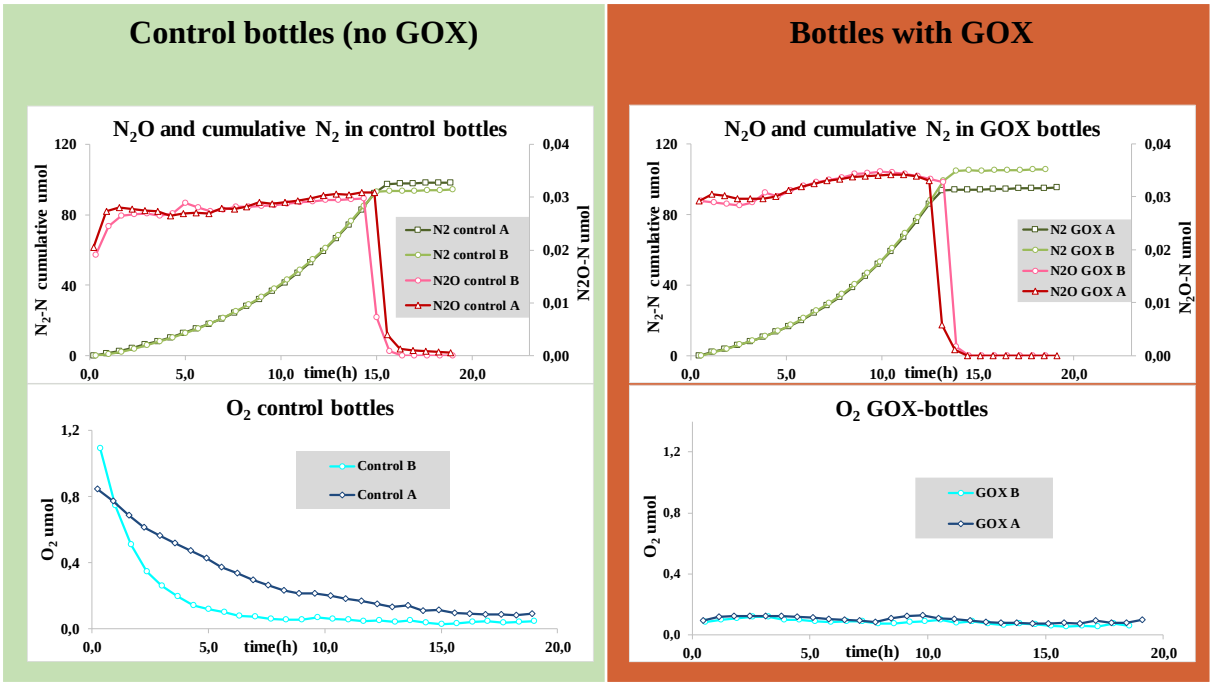

Figure 14. Dot plots of gas kinetics, testing the toxicity of GOX: The left panels show the kinetics of He-washed bottles with and 50 mL Sistrom's medium with 2mM NO_2^-. Inoculated with *P. denitrificans* cells that were raised by anaerobic growth. The right panels show the same treatment, but with GOX treatment the day before inoculation. Cumulative levels N_2-N (umol bottle^{-1}) , measured O_2 (umol bottle^{-1}) and measured N_2O-N (umol bottle^{-1}).

We see that all bottles start with low O_2, but the GOX-treated start with only 10-15% of the oxygen present in the He-washed control bottles. The O_2 in the control bottles is scavenged as the incubation progressed. We see a similar rate in denitrification in all bottles. All bottles reach similar peaks in speed, but the control bottles are slightly delayed in reaching it.

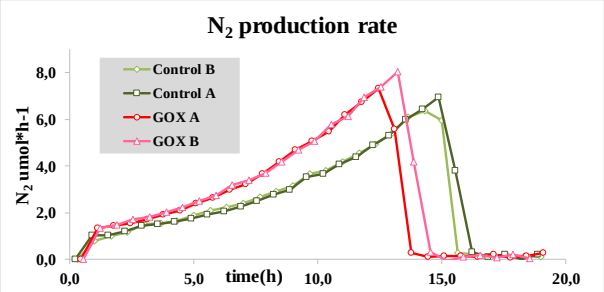

Figure 15: Dot plot of N_2 production rate in control and GOX bottles. umol N_2 produced per hour. The bottles all reach similar maximum N2- production rates, and stop when NO_2^- runs out. The control bottles however, are marginally lower throughout the incubation, and the GOX bottles increase their N_2 production the fastest, i.e they start denitrifying faster than the control bottles.

4.5 Entrapment in anoxia using GOX, measured with fluorescence microscopy and gas kinetics. In contrast with the previous experiments the entrapment assay bottles were inoculated with aerobically raised *P. denitrificans* PD1222 mCherry:nirS. All entrapment assay bottles were He-washed, contained 50 mL Sistrom's medium, and received either N_2O, NO_2^- or both. Half the bottles were GOX-treated the day before, the other half received Milli-Q water, and all combinations were run in duplicate. After incubation for ~24 hours one bottle from each replicate was sampled, and the cultures photographed in a fluorescence microscope.

4.5.1 Bottles with added $N_2O+NO_2^-$

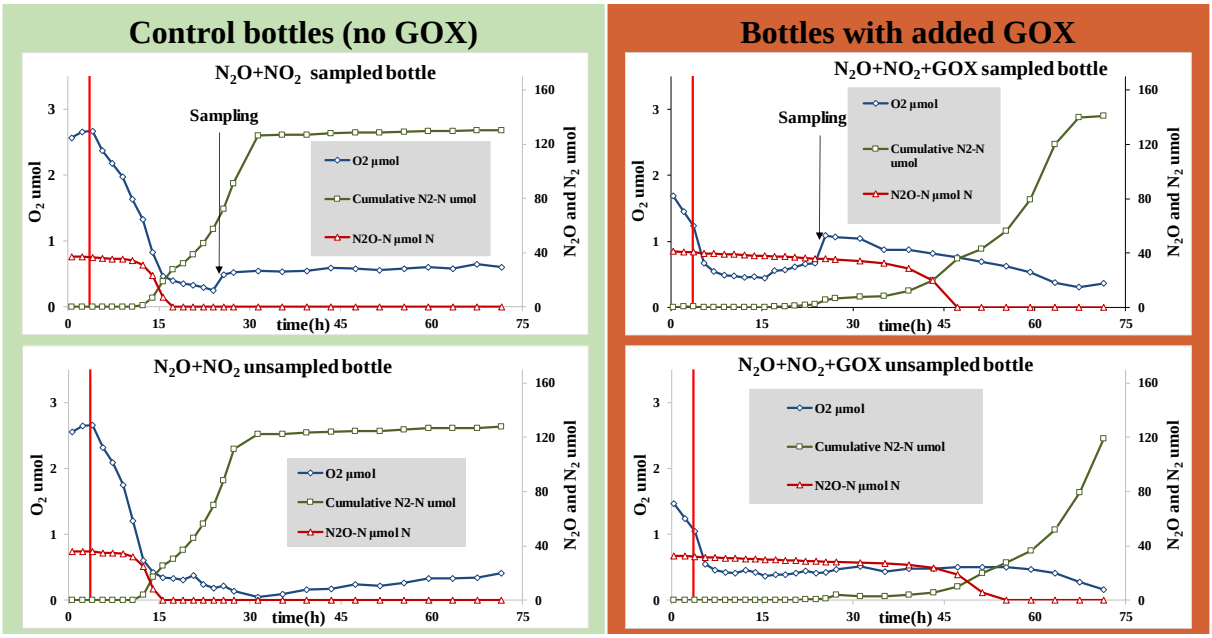

Figure 16: Dot pltos of gas kinetics in bottles with added N_2O and NO_2^-. The left panels show Helium-washed bottles with added N_2O and 50 mL Sistrom's medium with 2mM NO_2^- . The right panels had identical treatment, but also received GOX. The red line is inoculation with an aerobic *P. denitrificans* culture. Culture sampling marked marked with black arrow. Cumulative levels N_2-N (umol bottle^{-1}) , measured O_2 (umol bottle^{-1}), measured N_2O-N (umol bottle^{-1}). Bottles with GOX lags behind in N_2-production, but sampled GOX bottle is faster than unsampled bottle.

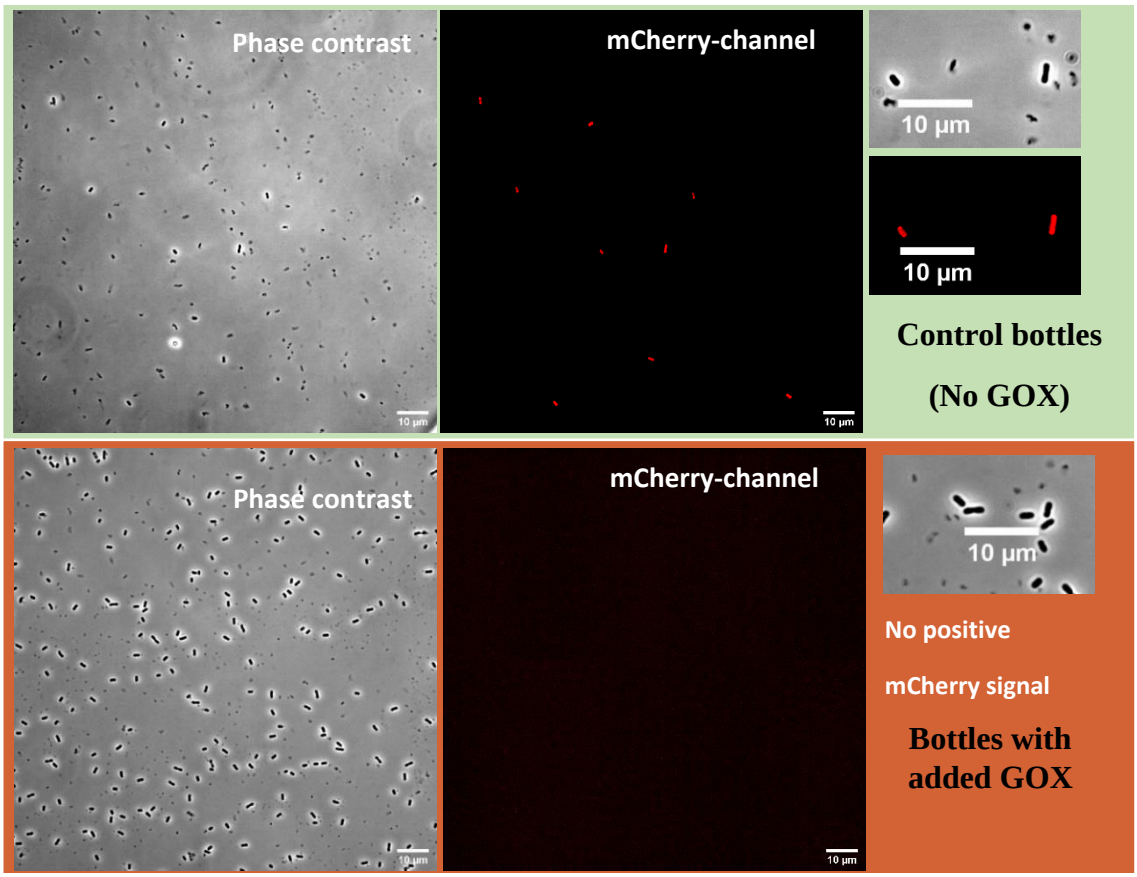

Figure 17: Phase contrast pictures in black and white on the left, mCherry fluorescence pictures in black and red in the middle, enlarged pictures on the right. Contrast and brightness adjusted. Bottles with added GOX has no positive cells with fluorescent mCherry signal, thus no NIRS positive cells. The control bottle has several cells with fluorescent mCherry, thus expressing NIRS, while having fewer cells overall.

4.5.2 Bottles with added NO_2-

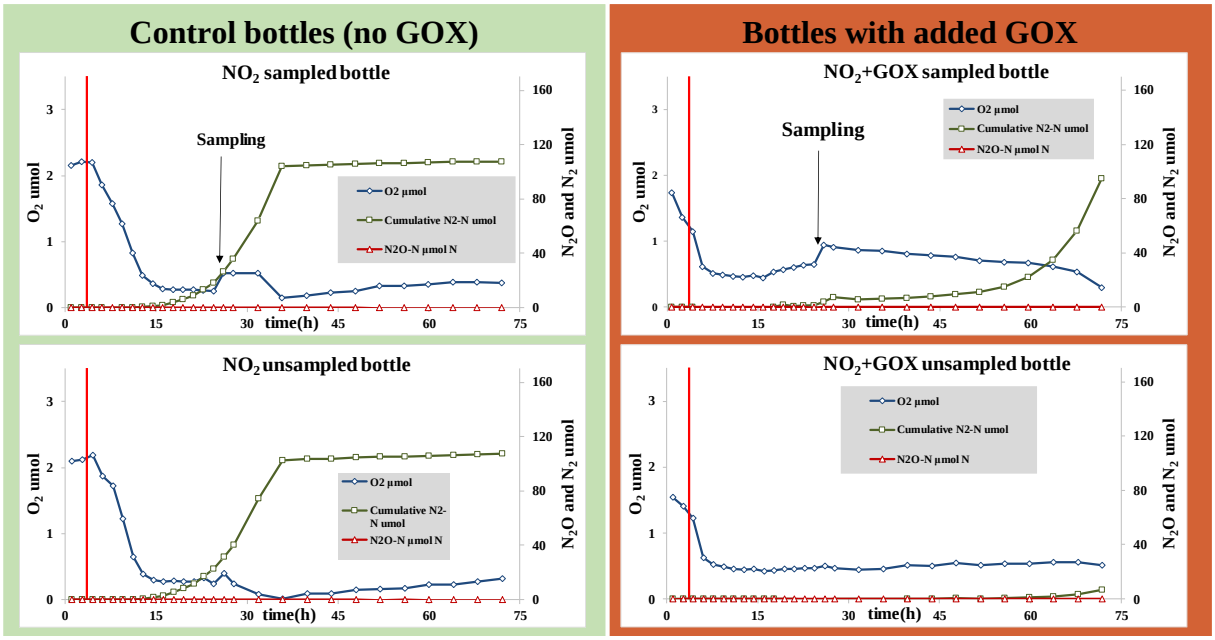

Figure 18: Dot plots of gas kinetics in bottles with added NO_2^-. The left panels show Helium-washed bottles with 50 mL sistrom's medium with 2mM NO_2^- . The right panels had identical treatment, but also received GOX. The red line is inoculation with an aerobic *P. denitrificans* culture. Culture sampling marked marked with black arrow. Cumulative levels N_2-N (umol bottle^{-1}), measured O_2 (umol bottle^{-1}), measured N_2O-N (umol bottle^{-1}). Bottles with GOX lags behind in N_2-production, but sampled GOX bottle is faster than unsampled bottle. N2-production is slower with only added NO_2^- than in bottles with both N_2O and NO_2^- added.

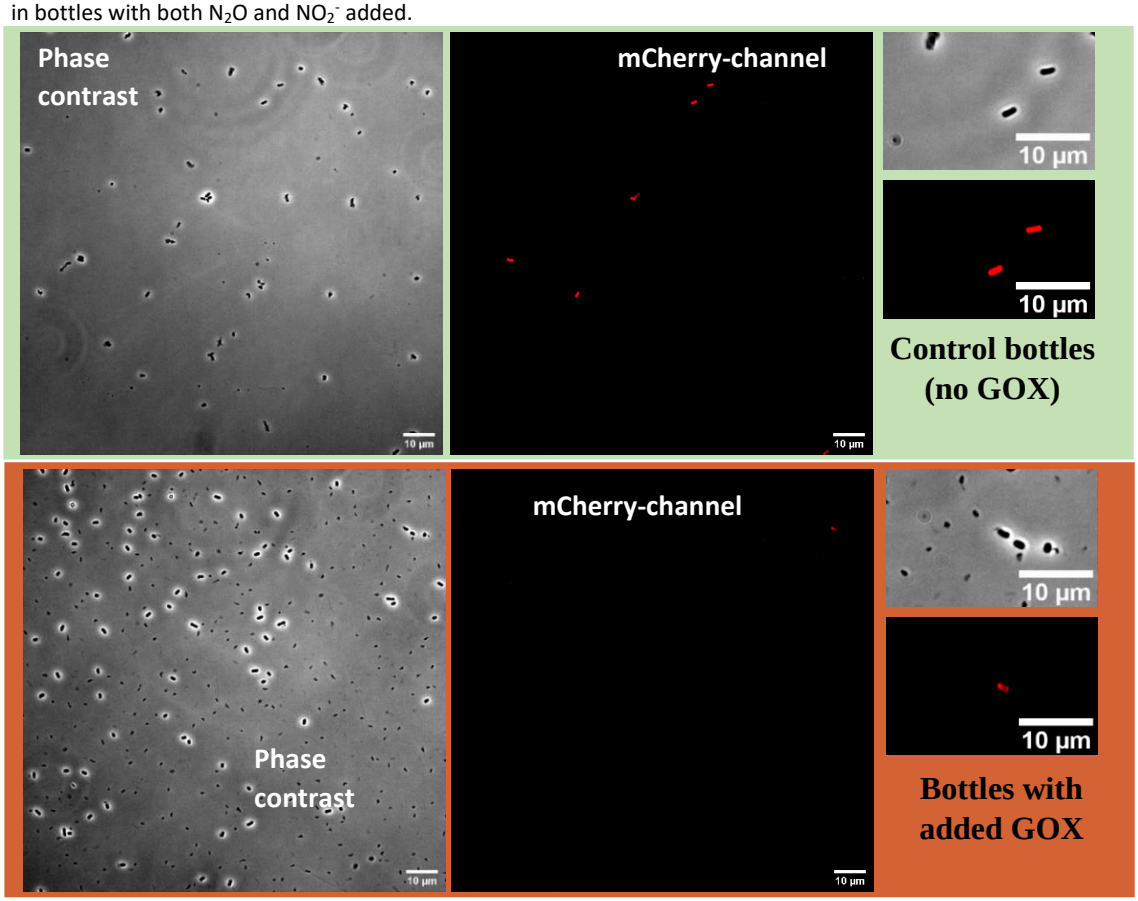

Figure 19: Phase contrast pictures in black and white on the left, mCherry fluorescence pictures in black and red in the middle, enlarged pictures on the right. Contrast and brightness adjusted. The bottles with added GOX has one cell with fluorescent mCherry signal, and more cells in total. The control bottle has several cells with fluorescent mCherry, thus expressing NIRS, while having fewer cells overall.

4.5.1 Bottles with added N_2O

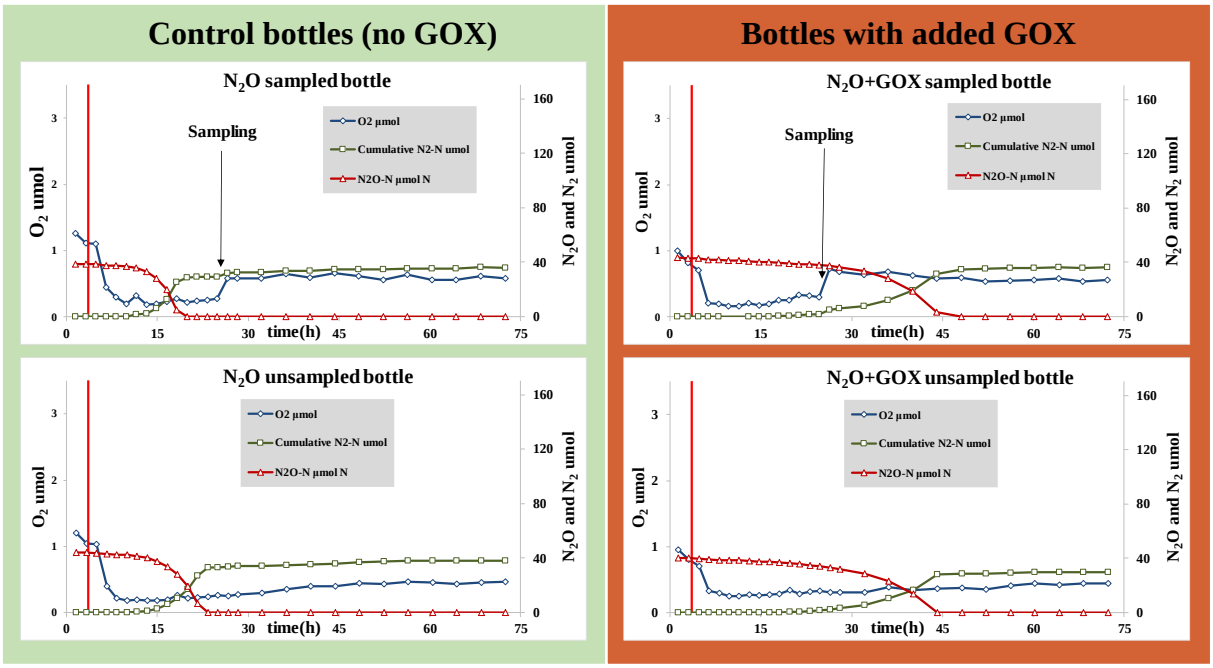

Figure 20: Dot plots of gas kinetics in bottles with added N_2O. The left panels show Helium-washed bottles with added N_2O and 50 mL Sistrom's medium. The right panels had identical treatment, but also received GOX. The red line is inoculation with an aerobic *P. denitrificans* culture. Culture sampling marked marked with black arrow. Cumulative levels N_2-N (umol bottle^{-1}), measured O_2 (umol bottle^{-1}), measured N_2O-N (umol bottle^{-1}). Bottles with GOX lags behind in N_2-production.

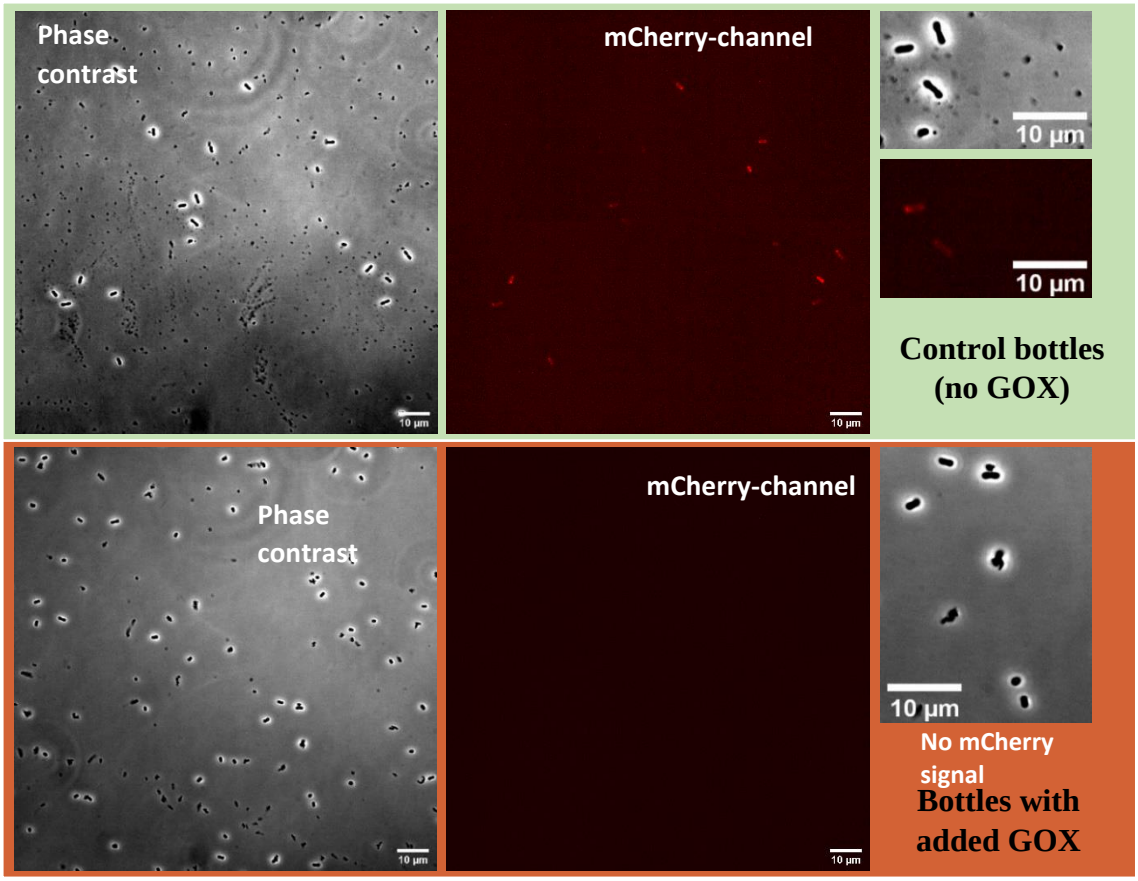

Figure 21: Phase contrast pictures in black and white on the left, mCherry fluorescence pictures in black and red in the middle, enlarged pictures on the right. Contrast and brightness adjusted. The bottles with added GOX has no cells with fluorescent mCherry signal, thus no NIRS positive cells. The control bottle has several cells with a weak fluorescent mCherry, thus expressing NIRS despite lacking NO_2^-.

We see in these bottles that the onset of N_2O-reduction and denitrification (reduction of NO_2^-) was delayed by scavenging of O_2 by GOX before inoculation of the bottles with aerobically grown cells. This is plausibly due to entrapment in anoxia, since the earlier experiments showed no toxicity of GOX when tested on anaerobically grown cells. This interpretation is strengthened by fact that few/no NirS-mCherry positive were observed in the samples from the GOX treatments, while the frequency was higher in the control vials without GOX.

Another striking result is that in the vials with N_2O only, the depletion of N_2O was delayed by ~25 hours by GOX, and the O_2 leak when sampling seemed to have little to no effect.

4.6 Flow cytometer sensitivity test

Paracoccus denitrificans PD1222 mCherry:nirS cells were raised by anaerobic growth and stained with FITC for testing the ability of a range of flow cytometers for their capacity to detect/enumerate NIRS-mCherry positive and FITC stained cells. The cells were mixed with aerobically grown cells (hence NIRS-mCherry negative) without FITC, and fixated with formaldehyde, providing a standardized mixture of positive and negative cells. The results are shown in table 2.

Table 2. Comparison of flow cytometers ability to detect FITC and NirS-mCherry.

Instrument	Detects FITC	Detects mcherry
CytoFLEX (Beckman)	**Yes**	**No**
MaxQuant HYB (Miltenyi)	**Yes**	**No**
Guawa Flow (Merck)	**Yes**	**No**
Novocyte Flow Cytometer (Acea Bioscience Inc)	**No**	**No**
Attune NxT Flow Cytometer (Thermo Fischer)	**Yes**	**No**
CellStream (Merck)	**Yes**	**Yes**

Only Cellstream from Merck was able to detect both FITC and mcherry. A unit was acquired and used in further entrapment tests.

4.7 FLOW cytometer studies of entrapment in anoxia

This experiment consisted of many smaller sub experiments in different bottles, a selection of the most relevant bottles is presented. All the bottles were He-washed and contained 50 mL Sistrom's medium. Half the bottles were GOX-treated, and all bottles received NO_2^- throughout the experiment, ensuring that the denitrifying cultures never ran out of NO_2^-, see table 3 in appendix. The experiment was run with three replicate bottles, every third bottle received O_2 after 69 hours to investigate escape from entrapment in anoxia. The bottles were inoculated with aerobically grown *P. denitrificans* PD1222 mCherry:nirS. For each sub experiment presented, the gas kinetics, using a large plot with the same scaling is shown for all bottles is shown first and at the top of the page. Under this plot are two smaller plot in the same figure, with the gas kinetics for the specific sub experiment with a result appropriate scaling. Finally, the flow cytometry for that specific bottle is presented.

Flow cytometry allows for a quantification of growing cells, as well as the number of cells that are entrapped: By plotting the distribution of cells in an logarithmic XY plot with FITC intensity along the X-axis, and nirS-mCherry along the Y-axis (fig. 23, 25, 27 & 29), the entrapped cells remain in the lower right corner of the plots (i.e cells without NirS-mCherry, and with constant high FITC because they do not grow), while cells that express mCherry are lifted up and subsequently move towards left if growing (hence dilute FITC). In the plots three populations are tentatively gated as illustrated:

- mCherry positive and FITC negative are presented as red circles, in the upper left corner.
- FITC positive and mCherry negative are presented as green circles, in the lower right corner
- The FITC positive and mCherry positive are presented as black crosses, due to problems with visualizing them with circles, stemming from their relatively low population. They are in the upper right corner to the middle of the plot.
- Other events are plotted with green dots, small events may be other things than cells, such as organelles and air bubbles. Some may be cells without mCherry and FITC. These are not gated.

The cultures were frequently sampled, only samples presented in flow cytometry fluorescence plots are marked with black arrows on the gas kinetics plots.

4.7.1 No GOX

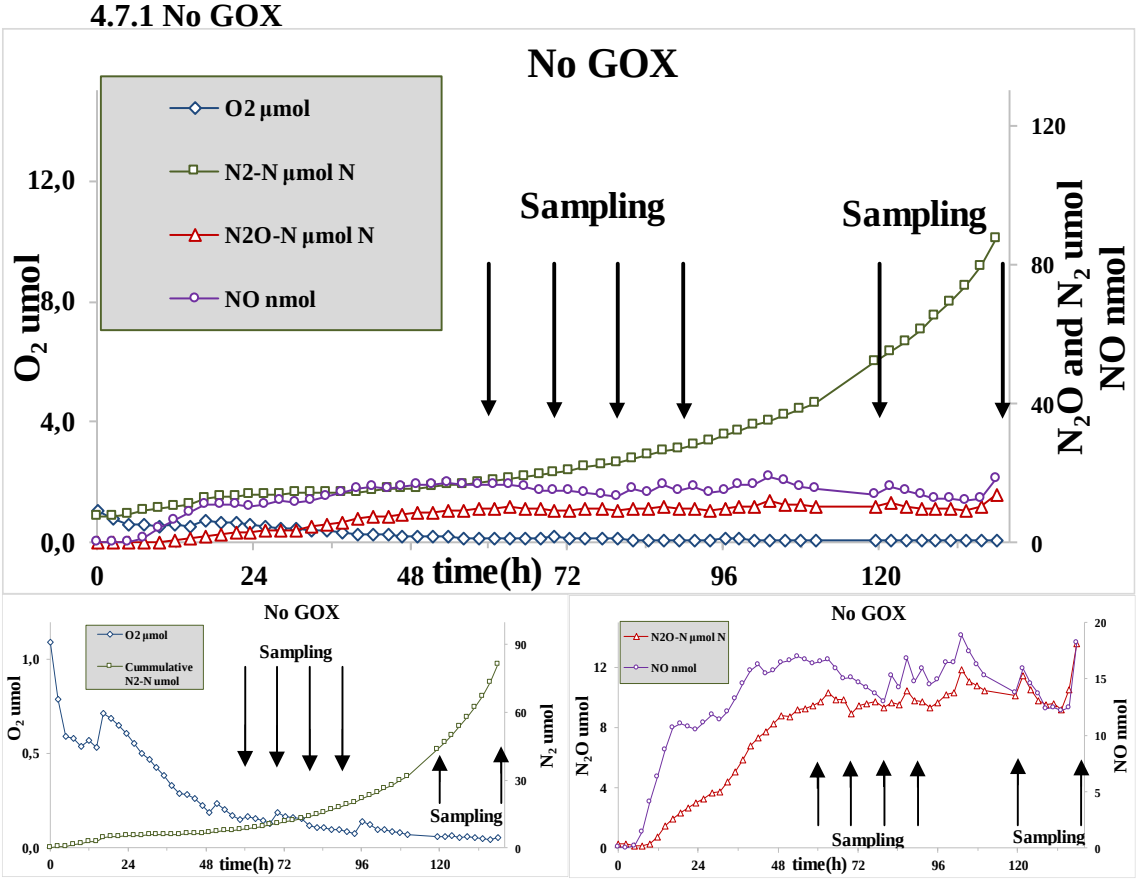

Figure 22. Gas kinetics, treatment "He-washed" Aerobically grown cells were transferred to He-washed bottles, and samples of the culture taken every 5-10 hours, and NO_2^- added at several points, see table 3 in appendix for an overview. The presented flow cytometer figure (fig. 23) were taken from the samples marked with arrows. Large plot with the same scaling used for other bottles. N_2-N (umol bottle^{-1}), measured O_2 (umol bottle^{-1}), measured N_2O-N (umol bottle^{-1}) and measured NO (nmol bottle^{-1}). Measured N_2 in large plot, cumulative in small plot. Denitrification, as measured by cumulative N_2 increases troughout the experiment and increases in speed throghout the incubation.

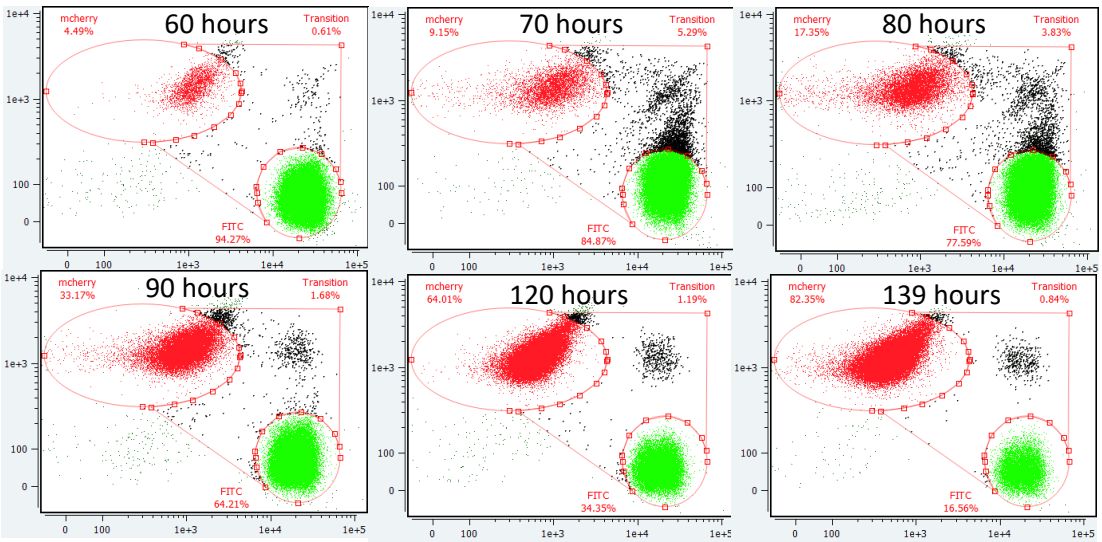

Figure 23. Flow cytometry plot for sampled cultures (black arrows in fig. 22) for treatment "He-washed" . Log X-axis for FITC-signal strength, log Y-axis for mCherry signal strength. The green circles in the lower right hand corner are entrapped *P. denitrificans*, and do not express nirS-mCherry. The black crosses in the upper right are *P. denitrificans* escaping entrapment, and the red circles are *P. denitrificans* expressing mCherry and denitrifying. The red population grows over time, becoming a larger part of the total population. The population is not entrapped

4.7.2 No GOX, spiked with O_2 after 69 hours

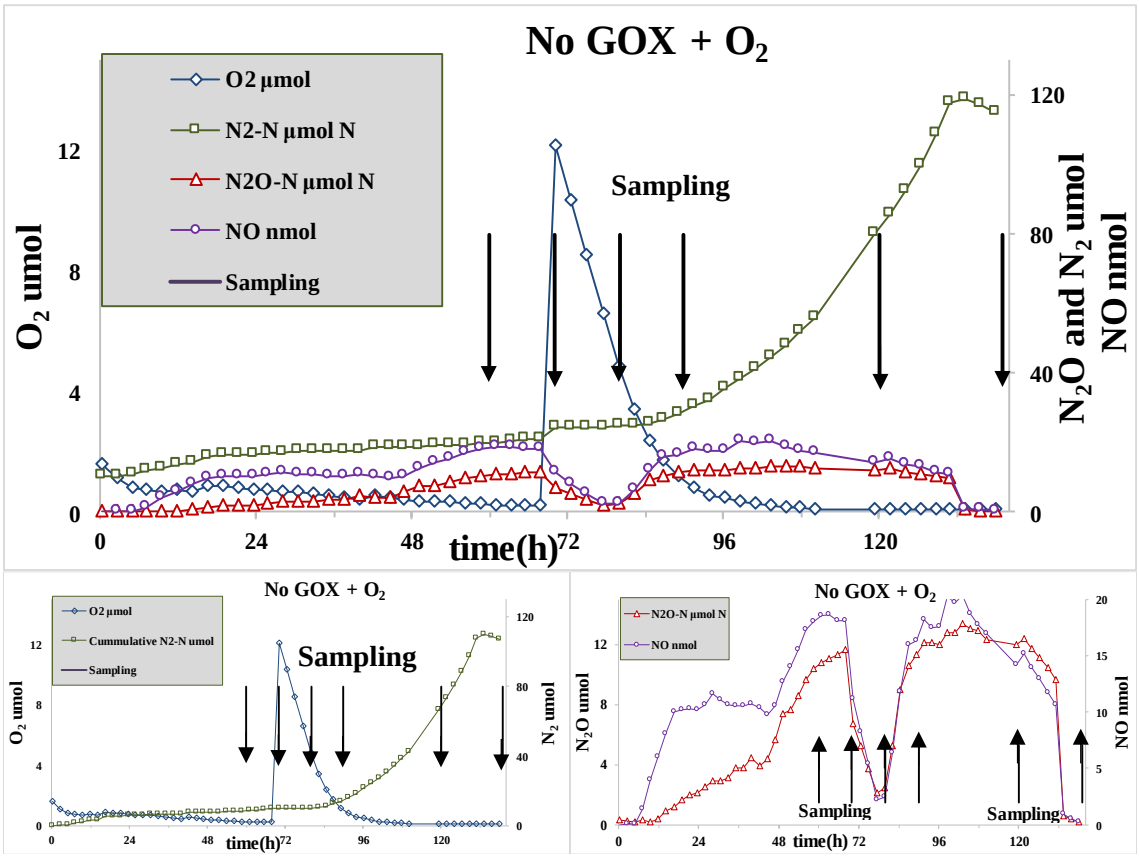

Figure 24. Gas kinetics, treatment "He-washed + O_2" Aerobically grown cells were transferred to He-washed bottles, and a sample of the culture taken every 5-10 hours, and NO_2^- was added at several points, see table 3 in appendix for an overview. O_2 was injected at 69 hours. The presented flow cytometer figure (fig. 25) were taken from the samples marked with arrows. Large plot with the same scaling used for other bottles. N_2-N (umol bottle^{-1}), measured O_2 (umol bottle^{-1}), measured N_2O-N (umol bottle^{-1}) and measured NO (nmol bottle^{-1}). Measured N_2 in large plot, cumulative in small plot. Denitrification, as measured by cumulative N_2 increases until O_2 injection, then stagnate before it increases faster than before the injection.

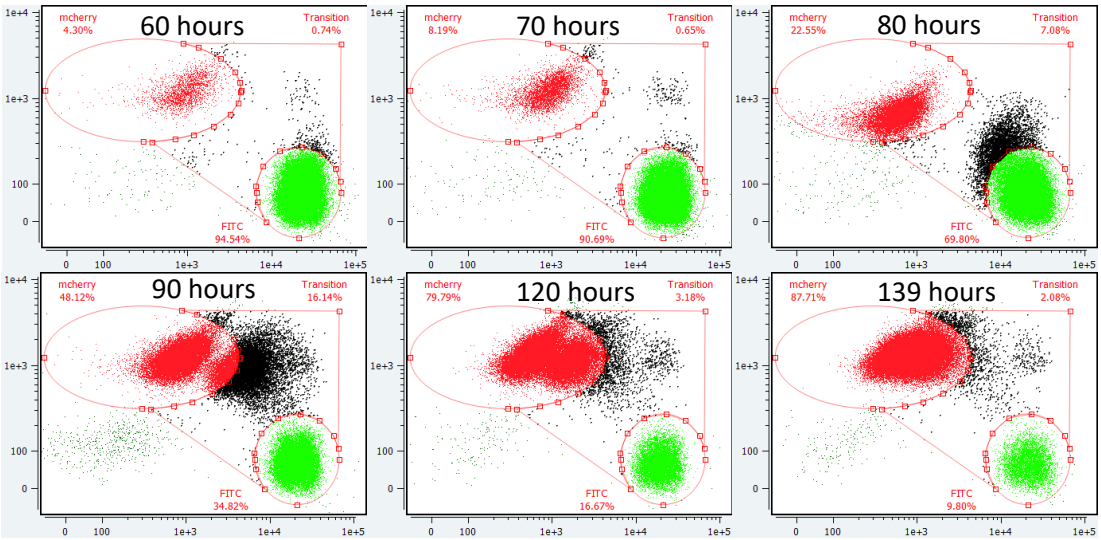

Figure 25. Flow cytometry plot for sampled cultures (black arrows in fig. 24) for treatment "He-washed" . Log X-axis for FITC-signal strength, log Y-axis for mCherry signal strength. The green circles in the lower right hand corner are entrapped *P. denitrificans*, and do not express nirS-mCherry. The black crosses in the upper right are *P. denitrificans* escaping entrapment, and the red circles are *P. denitrificans* expressing mCherry and denitrifying. The red population grows over time, becoming a larger part of the total population. The population is not entrapped, and recruit denitrifiers around 80 h due to the O_2 injection.

4.7.3 GOX

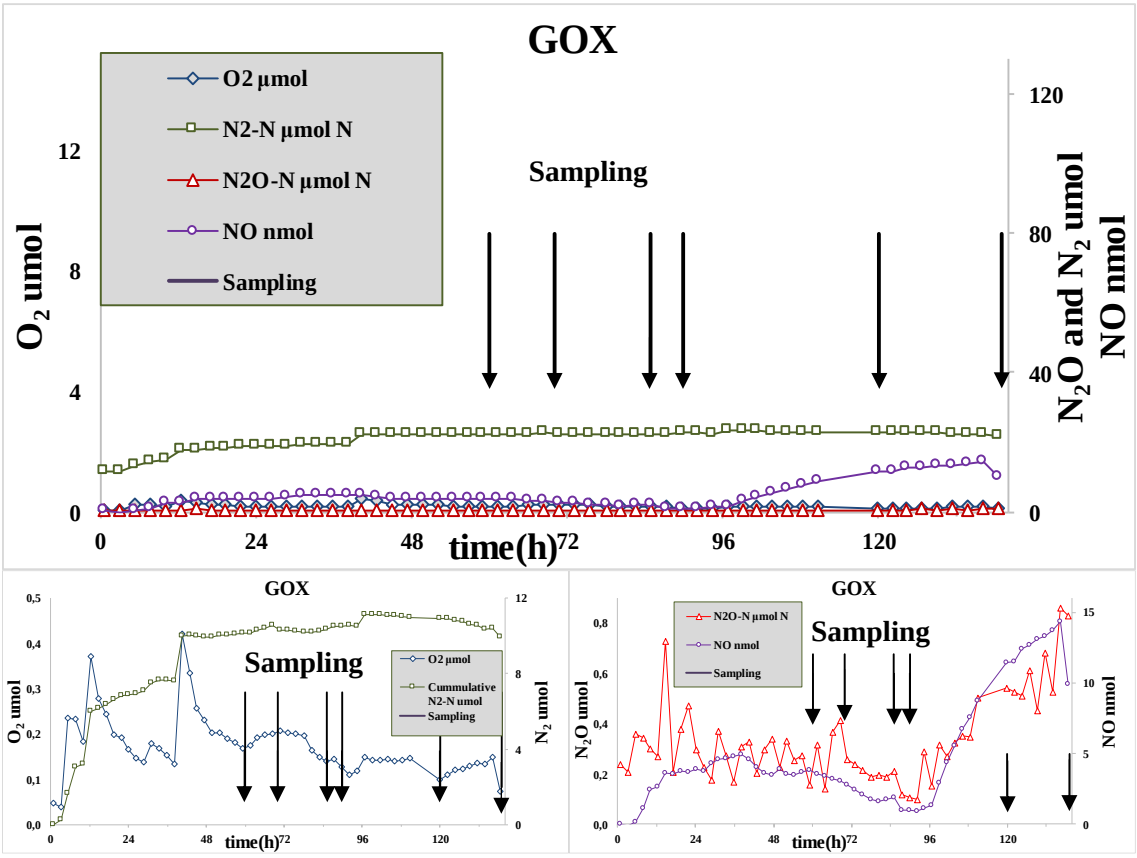

Figure 26. Gas kinetics, treatment "He-washed + GOX" Aerobically grown cells were transferred to GOX-treated and He-washed bottles, and a sample of the culture taken every 5-10 hours, and NO_2^- was added at several points, see table 3 in appendix for an overview. The presented flow cytometer figure (fig. 27) were taken from the samples marked with arrows. Large plot with the same scaling used for other bottles. N_2-N (umol bottle^{-1}), measured O_2 (umol bottle^{-1}), measured N_2O-N (umol bottle^{-1}) and measured NO (nmol bottle^{-1}). Measured N_2 in large plot, cumulative in small plot. Denitrification, as measured by cumulative N_2 is stagnant, and **the population is entrapped**.

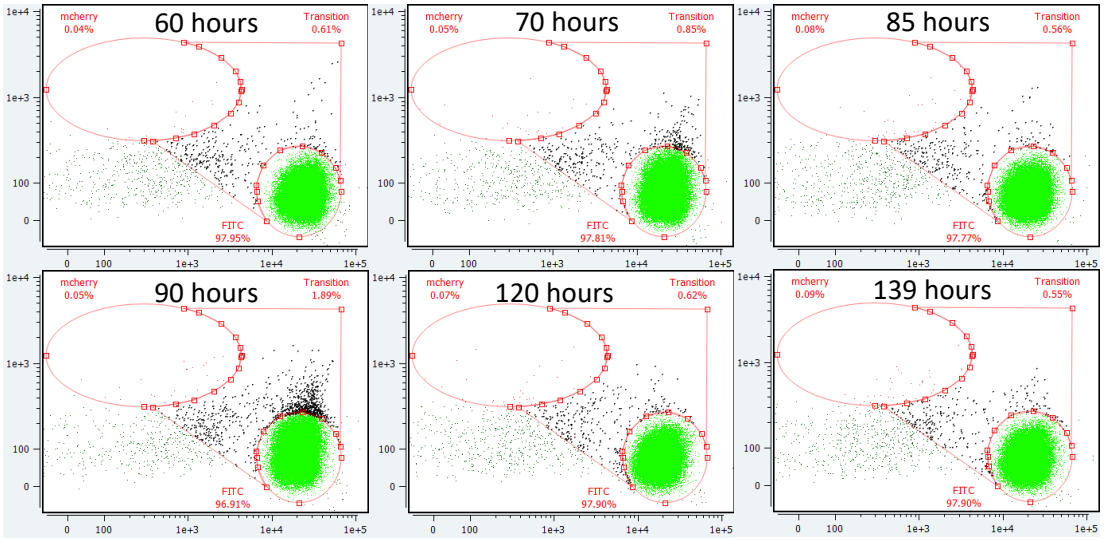

Figure 27. Flow cytometry plot for sampled cultures (black arrows in fig. 26) for treatment "He-washed + GOX" . Log X-axis for FITC-signal strength, log Y-axis for mCherry signal strength. The green circles in the lower right hand corner are entrapped *P. denitrificans*, and do not express nirS-mCherry. The black crosses in the upper right are *P. denitrificans* escaping entrapment, and the red circles are *P. denitrificans* expressing mCherry and denitrifying. There is no red population, and the green population stays at the same relative size. **The culture is entrapped**.

4.7.4 GOX, spiked with O_2 after 69 hours.

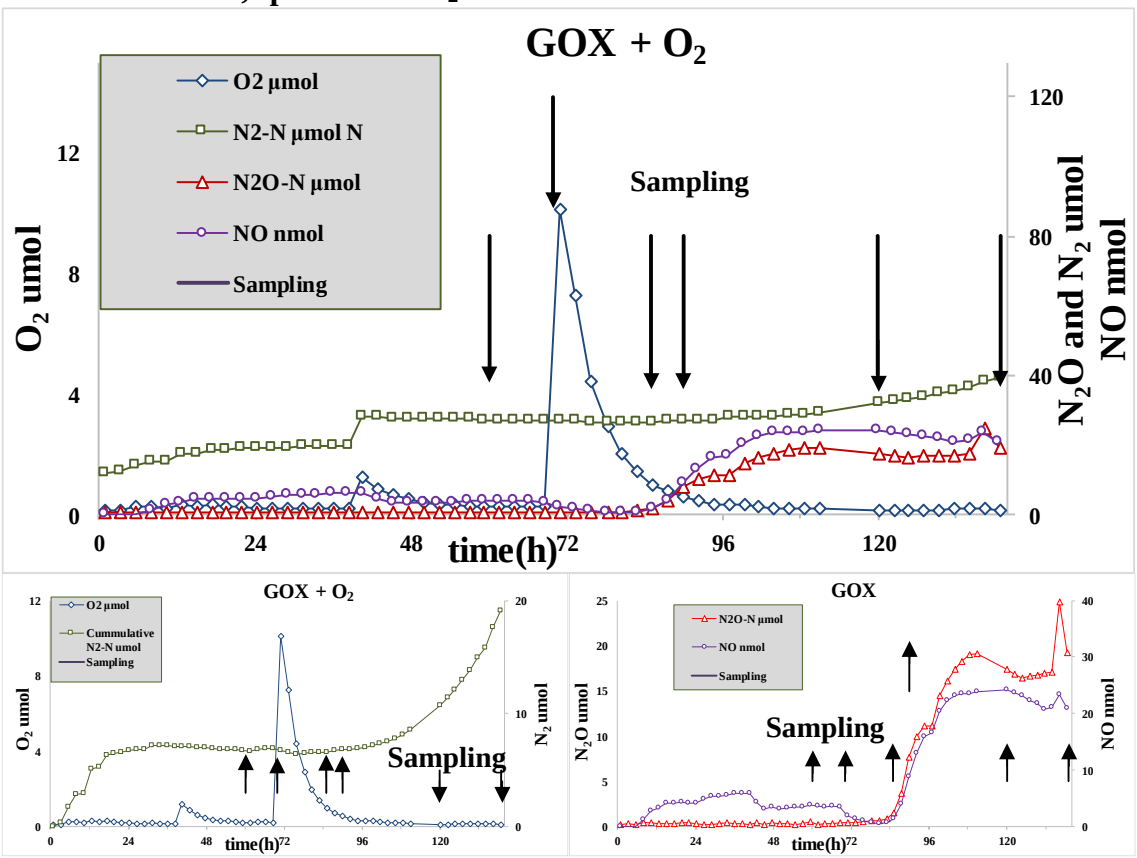

Figure 28. Gas kinetics, treatment "He-washed + GOX + O_2". Aerobically grown cells were transferred to GOX-treated and He-washed bottles, and a sample of the culture taken every 5-10 hours, and NO_2^- was added at several points, see table 3 in appendix for an overview. O_2 was injected at 69 hours. The presented flow cytometer figure (fig. 29) were taken from the samples marked with arrows. Large plot with the same scaling used for other bottles. N_2-N (umol bottle^{-1}), measured O_2 (umol bottle^{-1}), measured N_2O-N (umol bottle^{-1}) and measured NO (nmol bottle^{-1}). Measured N_2 in large plot, cumulative in small plot. There is not denitrification, as measured by cumulative N_2 , and the population entrapped, until the O_2 gives them energy to escape entrapment.

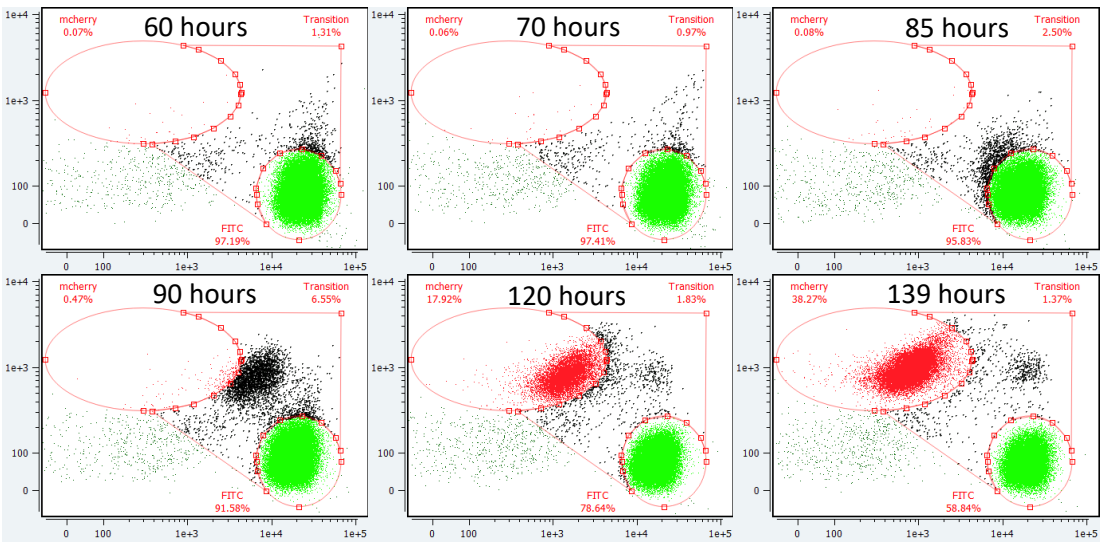

Figure 29. Flow cytometry plot for sampled cultures (black arrows in fig. 28) for treatment "He-washed + GOX + O_2" . Log X-axis for FITC-signal strength, log Y-axis for mCherry signal strength. The green circles in the lower right hand corner are entrapped *P. denitrificans*, and do not express nirS-mCherry. The black crosses in the upper right are *P. denitrificans* escaping entrapment, and the red circles are *P. denitrificans* expressing mCherry and denitrifying. There is no red population, and the green population stays at the same relative size. **The culture is entrapped, but the O_2 injection helps them escape.**

5. Discussion:

5.1 Tasks: The work presented in this master thesis was divided into three tasks: 1) Developing an efficient entrapment assay for *P. denitrificans.* 2) Direct demonstration of entrapment in anoxia for *P. denitrificans.* 3) Test if flow cytometry could replace fluorescence microscopy.

5.2 Entrapment assay: My first task was to develop an efficient entrapment assay for *P. denitrificans,* and I tested two different methods; Cysteine HCl and GOX, first I tested their O_2 scavenging ability, and then if they had any inhibitory effect on *P. denitrificans*.

5.2.1 Cysteine HCl: Cysteine HCl showed marginal O_2- scavenging ability; the estimated rate was ≤ 0.1 µmol O_2 bottle^{-1} h^{-1} (Fig 6). With frequent sampling both O_2 and N_2 levels increased in both the control bottle and the Cysteine HCl treated bottle (fig. 6 & 7). The scavenging was not effective enough to compensate for leaks of O_2 when sampling the headspace. When sampling frequency was decreased, by turning off sampling for ~66 hours, Cysteine HCl scavenged O_2 faster than the diffusion rate through the polymer (fig. 6, right plot).

I also tested if Cysteine HCl had any inhibitory effect on *P. denitrificans* by comparing the rate of denitrification in bottles without Cysteine HCl (the control bottles), the cells quickly depleted the residual O_2 and the N_2O in the headspace, and then started to respire NO_2^-. Seen as an increase in NO and the production of N_2. The cells exposed to Cysteine HCl were unable to reduce O_2 (the concentrations increased gradually, due to leakage when sampling). They did not reduce the N_2O in the headspace, and were unable to perform a balanced denitrification of NO_2^-. NO increased gradually, while the N_2 production was insignificant. It appears clear that *P. denitrificans* was strongly inhibited by Cysteine HCl.

Since Cysteine HCl failed to scavenge O_2 efficiently and inhibited *P. denitrificans*, it was discarded as a method to entrap the cells in anoxia.

5.2.2 GOX: In the bottles with lowest amount if initial O_2, the concentration of O_2 decreased, while the concentration of N_2 increased (fig. 10). At all levels of initial O_2, O_2 decreased (fig. 9-12). GOX clearly scavenged O_2 faster than sampling leaks and diffusion through the polymer septum. The O_2 scavenging rate of the GOX solution is dependent on the initial O_2 levels of the bottle (table 1). The initial rate was largely proportional to the concentration of O_2 (table 1), suggesting a first order kinetics. By calculating the actual concentration of O_2 in the liquid for each time increment (fig. 11), we could estimate the turnover rate as it evolved throughout the incubation. Plotting the turnover rate against time suggested a first order decay of glucose oxidase (fig. 13). This corroborates an earlier observation (Valdes & Moussy, 2000), that glucose oxidase decays rather rapidly in vitro if provided with glucose and oxygen. In the bottles with 21% O_2, depletion of glucose could have contributed to the declining rates of oxygen scavenging after ~4 hours, the glucose concentration was 16 mM, thus there was 800 µmol glucose in the bottle, which means that it takes 400 µmol O_2 bottle^{-1} to oxidize all the glucose (since the oxidation of 1 mol glucose consumes only ½ mol O_2, Figure 5). This is only 2/3 of the initial amounts of O_2 in the vials with 21% O_2. In fact, one of the two replicate vials with initially 21% O_2 lost 2/3 of the O_2 (fig.12).

The fact that GOX degraded fast, and was unable to deplete the oxygen completely in all other treatments than in the He-washed vials has practical implications for its use as an experimental tool: Removal of most oxygen (by He-washing) prior to GOX addition is a prerequisite, and if O_2 leaks into the bottle at a late stage of an experiment, GOX is probably unable to remove it.

As GOX had proven itself as an efficient O_2 scavenger, I had to test if it had an inhibitory effect on *P. denitrificans*. The organisms in the control bottles (i.e without GOX) started denitrifying, as it scavenged the low remaining O_2 levels (fig. 14, left plots). The organisms in the GOX bottles showed essentially identical N-gas kinetics (Fig. 14, upper right plot) as those in the bottles without GOX, although they were slightly faster. The GOX bottles was also slightly faster (~3h) to finish denitrification, as evidenced by the cumulative N_2 plateau and the depletion of N_2O. Thus, if GOX had any effect on the anaerobic respiration of *P. denitrificans,* it would be a slight enhancement, rather than inhibition. The most plausible explanation to this would be that GOC efficiently removed O_2: The traces of O_2 remaining after He-washing could possibly retard the rate of denitrification during the first 5-10 hours, thus explaining the marginally lower rates in the control bottles compared to GOX bottles (fig. 15). GOX itself however, seems non-inhibitory and non-toxic, and I have therefore successfully developed an entrapment assay for *P. denitrificans*.

5.3 Direct demonstration of entrapment in anoxia: My next task was to use my entrapment assay, GOX, to entrap aerobically grown *P. denitrificans* culture. I compared He-washed control bottles with He-washed + GOX treated bottles, in conjunction with different combinations of added KNO_2 and N_2O. I then used fluorescence microscopy to look at growth patterns.

5.3.1 N_2O+NO_2^- bottles: N_2O was taken down first in all bottles (fig. 16), as is expected if the expression of NOS is less suppressed by O_2 than NIR (Qu et al., 2016). The rise in cumulative N_2 lags slightly after N_2O is depleted, possibly due to more *P. denitrificans* cells having NOS than NIR+NOR. The GOX treated bottles are far slower at denitrifying than the control bottles, using ~3 times longer to scavenge N_2O. In the unsampled bottles the GOX treated one does not finish denitrification, while the control bottle finish denitrification after ~30 hours. In the sampled GOX bottle there is a small gas leak due to sampling the culture, and we see that cumulative N_2 start increasing shortly afterwards. It seems that this ~0.3umol O_2 leak into the bottle was enough to help the some of the cells to escape anoxia, by synthesizing NIR, as the sampled bottle finish denitrifying after ~70h. It therefore seems that the small amount of initial O_2 present in the control bottles when inoculating helps cells to synthesize enough NIR to engage in denitrification. This was confirmed by fluorescence microscopy of the cells: a substantial fraction of the cells in the control bottle were NIRS-mCherry positive (fig 17, upper pictures). In contrast, the GOX treated bottles had a very low fraction of NIRS-mCherry positive cells. None were detected, (fig 17. lower pictures).

5.3.2 NO_2^- bottles: Denitrification takes longer time to complete in bottles with only added NO_2^- (fig. 18) than in the bottles with both NO_2^- and N_2O added (fig 16). If more cells are able to synthesize NOS than NIR, before the onset of anoxia (as suggested by Lycus et al. 2018), a supply of N_2O would help cells with NOS to express *nirS* after oxygen depletion. Since their N_2O reduction would provide the necessary energy. This would explain why the bottles with only NO_2^- added are slower than those with both NO_2^- and N_2O (compare fig. 16

and 18). We also see that the unsampled NO_2^- bottle with GOX denitrify slower than the unsampled control bottle, and the reason to this appears to be the marginal leak of O_2 due to sampling: it appears that this induced an upshift in N_2-production. This phenomenon also occurred in the N_2O+NO_2^- bottles. Thus traces of O_2 seems to aid in recruiting the denitrification chain, also in the absence of N_2O.

The fluorescence microscopy confirm that GOX treatment reduce the number of cells recruiting to denitrification. The GOX bottle sample picture has more cells (fig 19, lower phase contrast picture) than the control bottle (fig. 19, upper phase contrast picture). And ~50% of the cells in the control bottle were NIRS-mCherry positive (fig. 19 upper mCherry pictures). While there was 1 positive in the GOX vial (fig, 19, lower mCherry picture) This is the same pattern we saw in the N_2O+NO_2^- bottles; a majority of the cells are entrapped in anoxia as a result of oxygen scavenging by GOX.

5.3.3: N_2O bottles: These are the bottles that finish denitrification the fastest, and the only bottles where both GOX and control bottles finish denitrifying (fig. 20). Again, the GOX treated bottles use ~3 times longer to finish denitrification, compared to the control bottles. The small oxygen leak when sampling, do not seem to accelerate denitrification in the sampled bottle, and all bottles manage to finish denitrification after ~45h. In the GOX treated vials, there is no NIRS-mCherry signal (fig 21, lower mCherry picture), while in the control bottles a weak NIRS-mCherry signal is expressed in some cells (fig. 21 upper mCherry plot). The signal is so dilute that the contrast to the background is slightly blurry, but it is there. NIRS-mCherry is expressed, despite there being no NO_2^- present. The absence of added nitrogen oxyanions (NO_2^- and NO_3^-) is no guarantee for their complete absence, however: many medium components contain traces of NO_3^- (Xu et al., 2000), and this is probably the case in Sistrom's medium as well. In fact, previous experiments with Sistrom's medium without nitrogen oxyanions added invariably show a detectable transient NO-peak as the cultures deplete the oxygen (Lars Bakken, pers comm). This strongly suggest that the medium contains traces of nitrogen oxyanions (presumably NO_3^-), although the concentrations are too low to result in detestable N_2 production. This could explain the observed expression of *NirS*, which is induced by NO via the NO sensor protein NNR (Spanning et al., 2007).

Nitrous oxide seems to help in recruiting enzymes upstream in the denitrification pathway.

5.4 Test if flow cytometry could replace fluorescence microscopy: First I had to find a flow cytometer sensitive enough to measure depletion of FITC, and detect nirS-mcherry production. With this flow cytometer I could the test if fluorescence microscopy could be replaced.

5.4.1 Flow cytometer sensitivity: Most flow cytometers were able to detect FITC in our samples, but unable to detect mCherry, except for CellStream^{Tm} (table 2). This was despite the suppliers claiming that the instruments could detect mCherry. The instruments may have been unable to detect mCherry in our samples due to a anthropogenic bias in the testing of the instruments. Most flow cytometers specialize in detecting eukaryotic, particularly human, cells. These are larger than the *P. denitrificans* cells used in our experiments, and the levels of mCherry present in our samples was most likely below the detection limit. But we managed to find a flow cytometer sensitive to detect NIRS-mCherry in *P. denitrificans*.

5.4.2: Entrapment measured with flow cytometry: My final task was to use my entrapment assay, GOX, to entrap aerobically grown *P. denitrificans* culture, and measure this entrapment using flow cytometry. I compared He-washed control bottles with He-washed and GOX treated bottles, grown with NO_2^-. I also injected one of each replicate bottle with O_2, to see if this recruited denitrification enzymes, and therefore helped parts of the entrapped population escape entrapment in anoxia.

5.4.3 He-washed (no GOX) treatment: Judged by their gas kinetics curves, the cultures in the bottles with FITC stained cells in He-washed vials (no GOX) produced some N_2 from the very beginning, and the rate of N_2 production increased with time (Fig 22), thus the culture was expected to contain cells that do respire and grow by reducing NO_2^- . The flow cytometry investigations confirm this (Fig 23), after 60 hours of incubation, 4.5 % of all cells had expressed *nirS-mCherry* (lifted up in the panel) and had already grown (shifted to the left, diluting FITC by growth). Throughout the rest of the incubation, the fraction of anaerobically respiring cells increases and reached 82 % after 139 hours. Cells that are entrapped in anoxia are found in the lower right corner of the panels: these cells have not expressed *nirS-mCherry* and retain strong FITC fluorescence throughout the entire incubation. In addition, we can identify a third population, "Transition" in the plots, cells that have expressed NIRS-mCherry, but not yet diluted their FITC (hence have not grown). These "transition cells" account for miniscule fraction of the total in the sample taken after 60 hours but reach higher in the samples after 70 and 80 hours, suggesting that some of the entrapped cells are able to initiate NIRS-mCherry synthesis at this stage of the experiment. In theory, this could have been induced by the small spikes of O_2 from the sampling (Fig 22, lower left plot) or scavenging N_2O by NOS-positive cells from other cells with the full proteome. The escalating fraction of cells with NIRS-mCherry and low FITC comply reasonably well with the expectation, assuming a specific growth rate of 0.07 h^{-1}, which is the anaerobic growth rate of *P. denitrificans* at 17 degrees Celsius (Bergaust pers comm). By assuming insignificant recruitment to denitrification after 60 hours, the fraction of actively growing cells can be estimated for each subsequent sampling (assuming that the active fraction has a growth rate of 0.07 h^{-1}). Figure 30 shows the relationship between predicted and measured fraction of active cells throughout.

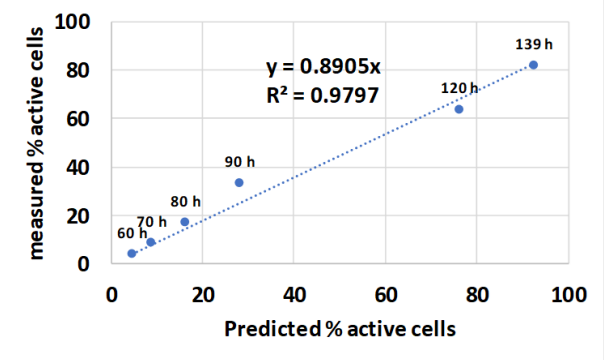

Figure 30. Comparison of measured and predicted percentage of actively growing cells (i.e. with mCherry and lowered FITC) for the experiment with He-washed bottles (no GOX), see Figure 22&23 for details. The predicted percentage is calculated assuming that the active population at time 60 h is growing by 0.07 h^{-1}, and that no subsequent recruitment takes place. The time of sampling for each point is given in the panel.

5.4.4 He-washed (no GOX) treatment, spiked with oxygen after 69 hours: This experiment was similar to the foregoing until 69 hours, when 12 µmol O_2 was injected to investigate if this could induce *nirS-mCherry* expression in the otherwise entrapped population (figure 24 and 25). The effect was strikingly clear: in response to the oxygen, approximately 7 % of the cells appear to be actively synthesizing NIRS-mCherry in the sample taken 11 hours after injecting the oxygen (time 80 h). This population increase their NIRS-mCherry content and grow, thus decreasing their FITC content, during the next 10

hours. Subsequently growing further, with a constant mCherry content, throughout the rest of the incubation, seen both as a dilution of the FITC signal and increasing numbers.

In theory (Hassan et al, 2016), the rate of recruitment, i.e. synthesis of NIRS, in hypoxia should be around 0.5% h^{-1}. Assuming that recruitment was ongoing for 11 hours before the sample was taken (i.e. 80 h), we should expect approximately 5% recruitment ($100*(1-e^{0.005*11})$), or somewhat higher because the cells recruiting early would have time to grow. The number of apparently recruiting cells in the "Transition"-gating at time=80 hours amounted to 7% of the total (upper right panel of fig. 25), but 9 % of the sum of entrapped cells and cells in "transition".

5.4.5 He-washed and GOX treatment: In this treatment, there was essentially no denitrification taking place (figure 26). There is a slight increase in N_2, this can be ascribed to release of N_2 from the Teflon coated magnet and the rubber septum, as well as small leaks when sampling the medium, as some increases in N_2 coincide with O_2 peaks. N_2O and NO remained low throughout the experiment, corroborating the fact that there was essentially no denitrification. We would therefore expect essentially no NIRS-mCherry positive cells at any time. This is confirmed by the flow cytometer XY-plot (fig. 27). Where the population in the corner of the plots stays the same size, in the same place, at all times, in the lower right hand of the plots. Not growing, entrapped in anoxia.

5.4.6 He-washed and GOX treatment, spiked with oxygen after 69 hours: This bottle is similar to the previous treatment, showing no denitrification (fig. 28). That is until 12 umol O_2 is injected after 69 hours of incubation. First O_2 is scavenged, and we see N_2 increase, at an increasing speed, and NO and N_2O also starts increasing after O_2 injection. In the flow cytometry XY-plot (fig. 29) we then see that a subpopulation starts expressing NIRS-mCherry, increasing their mCherry fluorescence, lifting the population up in the plot, while diluting FITC. Thus this subpopulation "travels" from the lower right corner, to the upper left corner of the plot from 85 hours-139 hours. The subpopulation had successfully used the injected O_2 to recruit the enzymes required for denitrification and was no longer entrapped in anoxia.

5.5 Summary: GOX was an effective and non-inhibitory method for scavenging oxygen, and lack of oxygen for an aerobic P. denitrificans culture seems to entrap them in anoxia. In addition, the flow cytometry investigations nicely corroborate the theory:

- recruitment to denitrification is dependent on oxic respiration
- Sudden disappearance of oxygen results in entrapment of the absolute majority of aerobically grown cells
- Later provision of oxygen (the O_2 spiking) induce synthesis of NIRS (hence recruitment to denitrification), but only in a fraction of the entrapped cells.
- The probability of recruitment to denitrification is low, approximately as estimated by Hassan et al (2016), i.e. 0.5% h^{-1}.

But the flow cytometry investigations also provided new information: the previous experiments (Hassan et al 2016, Lycus et al 2018) were unable to assess if cells entrapped in anoxia could possibly recruit gradually to denitrification. The results suggest that this does not happen (figure 26 and 27). Another striking phenomenon worth mentioning is the effect of N_2O on the ability to synthesize NIRS: if we compare the gas kinetics with GOX, with and

without N_2O (figure 16 and 18 respectively), we see that cells which have access to ample amounts of N_2O (fig. 16 lower right panel) recruit more efficiently to denitrification than those without N_2O (Figure 18 lower right panel): the N_2 production catch up much earlier!

This can be taken as a confirmation of the current understanding of the entrapment phenomenon: it is indeed due to lack of energy, since those cells within the culture which were able to respire N_2O (thus producing protonmotive force) could indeed synthesize nirS, and escape the entrapment.

Thus the regulation of enzyme recruitment when transitioning to anoxia is key for *Paracoccus denitrificans*, and potentially all denitrifiers. If they do not, they risk "running out of breath", facing entrapment in anoxia.

6 Appendix

6.1 Sampling of medium and addition of KNO_2 and O_2

Table 3, Overview of the flow cytometer sampling, O_2 and KNO_2 addition. Bottles 1-3 were He-washed and inoculated with an aerobic, FITC stained culture. Bottles 4-6 were He-washed and inoculated with an aerobic unstained culture. Bottles 7-9 were He-washed and GOX-treated and inoculated with an aerobic FITC stained culture. Bottles 10-12 were He-washed and GOX-treated, and inoculated with an aerobic, unstained culture. Bottles 13-15 had 2mM of initial NO_2^-, they were He-washed and GOX-treated, and injected with an aerobic FITC stained culture. Bottles 16-18 had 2mM of initial NO_2^-, they were He-washed and GOX-treated, and injected with an aerobic unstained culture. The gas kinetics and select flow cytometer fluorescence results for bottle 2 are shown in the "No GOX" on page 39 and for bottle 3 "No GOX+O_2" on page 40. The gas kinetics and select flow cytometer fluorescence results for bottle 8 are shown in the " GOX" on page 41 and for bottle 9 "GOX+O_2" on page 42. Bottles with less growth as measured by OD_{660} and gas kinetics were sampled less

Time / nick-name	Bottles sampled	Other
0h / inoculation	**No sampling, bottles inoculated.**	**Bottles 13-18 received 2mM KNO_2**
5h / I	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	**Bottles 1-12 received 2mM KNO_2**
10h / II	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
15h / III	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
20h / IV	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
30h / V	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
40h / VI	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
45h / VII	**5 & 6**	
50h / VIII	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
55h / IX	**5 & 6**	
60h / X	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
65h / XI	**5 & 6**	**Bottle 6 received 2mM KNO_2**
69h / No sample	**No sampling**	**350 uL O_2 added to every third bottle (3, 6, 9, etc)**
70h / XII	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
75h / XIII	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
80h / XIV	**2, 3, 5 & 6**	
85h / XV	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	**Bottle 5 received 2mM KNO_2**
90h / XVI	**2, 3, 5 & 6**	
95h / XVII	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
102h / XVIII	**2, 3, 5 & 6**	
120h / XIX	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	
139h / XX	**2, 3, 5, 6, 8, 9, 11, 12, 14, 15 & 18**	

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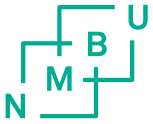

Norges miljø- og biovitenskapelige universitet
Noregs miljø- og biovitskapelege universitet
Norwegian University of Life Sciences

Postboks 5003
NO-1432 Ås
Norway