Acknowledgment

Abstract

Denitrification is a taxonomically widespread process used by more than 60 different genera of bacteria including archaea and some fungi, where nitrate is reduced to dinitrogen gas (N_2) by four enzymes; nitrate reductase (NAR & NAP), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (N_2OR). The end product of denitrification depends on the different bacteria species ability to synthesize the required enzymes involved in the denitrification process, some bacteria drive this process all the way to dinitrogen gas because they retain the genes necessary for the synthesis of all the four enzymes involved, while some only to N_2O and others emit a mixture of both N_2O and N_2 to the atmosphere. The release of the intermediates NO and N_2O are frequently observed in the denitrification process, when excess reactive nitrogen causes an overload of the enzymatic system, a phenomena known as "hole in the pipe". N_2O is a very potent greenhouse gas (300 times more than CO_2) with a residence time of up to 120 to 150 years in the atmosphere. Both NO and N_2O destroys the ozone layer, which protects us by absorbing and reflecting unwanted radiation and ultraviolet light. Several of the greenhouse gases have increased greatly since the preindustrial era up to today, and a large proportion of this increase is due to human activities.

The studies of a few model organisms have given insight in some of the phenotypic and genotypic regulation of denitrification, but more research is need to get a better understanding of why different bacteria that share similar genotypic traits displays a large variety of different phenotypic characteristics. Little is known about the phenotypic characteristics of *Thauera* during denitrification, mainly because this is not a model organism. The genera *Thauera* are found within the β-subdivision of Proteobacteria, capable of switching to denitrification, and use nitrate, nitrite or NO as electron acceptor when oxygen in the environment is scarce, while emitting either only N_2 or a combination of the gases NO, N_2O and N_2 depended on which reductase the bacteria is able to express. Two different methodological approaches were utilized in hope of getting a better understanding of *Thauera linaloolentis* phenotypic characteristics during denitrification.

The two gas kinetic experiments performed on the robotized incubation system, showed that *Thauera linaloolentis* performed a balanced transition from oxic to anoxic respiration at

neutral pH, with very low concentrations of NO (1-15 nM) and moderate concentrations of N_2O (40-31700 nmol). N_2O however displayed a tendency to increase in concentration with increased initial oxygen. The results obtained from electron flux showed that with higher initial oxygen, lower were the number of cells that managed the transition from oxic to anoxic respiration, but the fraction that managed the transition was still high even at 7% initial oxygen.

To verify the results obtained on the robotized incubation system, another experiment was performed where nalidixic acid was used for the quantification of growing cells by microscopic count. The result from this experiment revealed that the fraction of T. linaloolentis that managed the transition was indeed high (84, 77 and 73%), but decreased with increased initial oxygen concentrations. The study has provided new insight in Thauera linaloolentis phenotypic characteristics during denitrification, which was only speculated upon earlier.

Sammendrag

Denitrifikasjon er en taksonomisk utbredt prosessen brukt av mer enn 60 forskjellige arter av bakterier, inkludert Archaea og noen sopper, hvor nitrat blir redusert til dinitrogen gass (N2) av fire enzymer; nitrat reduktase (NAR & NAP), nitritt reduktase (NIR), NO reduktase (NOR) og N_2O reduktase (N2OR). Sluttproduktet av denitrifikasjon for de ulike bakterier artene avhenger av evnen til å syntetisere de nødvendige enzymene involvert i denitrifikasjon prosessen, noen bakterier drive denne prosessen helt til dinitrogen gass fordi de har genene som kreves for å syntetisere alle fire involverte enzymene, mens noen bare til N_2O og andre avgir en blanding av både N_2O og N_2 til atmosfæren. Utslipp av intermediatene NO og N_2O er ofte observert i denitrifikasjon prosessen, skjes når store mengder reaktivt nitrogen overbelaster det enzymatiske systemet, et fenomen kjent som "hull i røret". N_2O er en svært potent drivhusgass (300 ganger mer enn CO_2) med en oppholdstid på opp til 120 til 150 år i atmosfæren. Både NO og N_2O ødelegger ozonlaget, som beskytter oss ved å absorbere og reflektere uønsket stråling og ultrafiolett lys. Flere av klimagasser har økt kraftig siden den preindustrielle epoken og en stor andel av denne økningen skyldes menneskelig aktivitet.

Studier av noen få modellorganismer har gitt innblikk i noen av de fenotypiske og genotypiske reguleringene av denitrifikasjon, men mer forskning er nødvendig å få en bedre forståelse av hvorfor ulike bakterier som deler lignende genotypiske trekk viser et stort utvalg av forskjellige fenotypiske egenskaper. Lite er kjent om de fenotypiske egenskapene til *Thauera* under denitrifikasjon, hovedsakelig fordi dette ikke er en modell organisme. *Thauera* slekten finnes innenfor β-subdivision av Proteobacteria, i stand til å bytte til denitrifikasjon, og bruke nitrat, nitritt eller NO som elektron akseptorer når det er veldig lite oksygen igjen i miljøet, med utslipp av enten bare N_2 eller en kombinasjon av gassene NO, N_2O og N_2 avhengig av hvilke reduktase bakteriene er i stand til å uttrykke. To forskjellige metodiske tilnærminger ble brukt i håp om å få en bedre forståelse av *Thauera linaloolentis* fenotypiske egenskaper under denitrifikasjon.

To gass kinetikk eksperimenter ble utført på et robotisert inkubasjonssystem, som viste at *Thauera linaloolentis* utførte en balansert overgang fra oksisk til anoksisk respirasjon ved nøytral pH, med svært lave konsentrasjoner av NO (1-15 nM) og moderate konsentrasjoner av N_2O (40-31700 nmol). N_2O imidlertid vist en tendens til å øke i konsentrasjon ved økt

innledende oksygen. Resultatene hentet fra elektron fluks viste at ved høyere innledende oksygen, førte til at et lavere antall celler klarte overgangen fra oksisk til anoksisk respirasjon, men den fraksjonen som klarte overgangen var fortsatt svært høy selv ved 7% innledende oksygen.

For å bekrefte resultatene innhentet på det robotiserte inkubasjonssystemet, ble et annet eksperiment utført der nalidixic acid ble brukt til kvantifisering av voksende celler som ble senere telt i et mikroskop. Resultatet fra dette eksperimentet viste at andelen av *T. linaloolentis* som klarte overgangen var virkelig høy (84, 77 og 73%), men reduseres ved økt innledende oksygen konsentrasjon. Studien har gitt ny innsikt i *Thauera linaloolentis* fenotypiske egenskaper under denitrifikasjon, som tidligere var bare spekulert på.

List of contents

The aim of this thesis

The bacteria strains used in these experiments belong to the genera *Thauera*, found within the β-subdivision of Proteobacteria, capable of switching to denitrification, and use nitrate, nitrite or NO as electron acceptor when oxygen in the environment is scarce, while emitting either only N_2 or a combination of the gases NO, N_2O and N_2.

The main purpose of this thesis was to determine how big a fraction of a population of the denitrifying bacterium, *Thauera linaloolentis* that managed the transition from oxic to anoxic respiration, when incubated under defined conditions where an initial amount of oxygen was depleted. This was achieved through two different methodological approaches; estimation of electron transport rates based on gas kinetic measurements on a robotized incubation system, and the use of nalidixic acid for quantification of growing cells by microscopic count.

1. Introduction

Earth's atmosphere is mainly made up of nitrogen 78% and oxygen 21% (Fenchel et al. 1998; Richardson et al. 2009), but contains also various trace gases including greenhouse gases (CH_4, N_2O and CO_2) contributed from natural sources and human activities since the start of the industrial era. Atmospheric emission of greenhouse gases is known to influence and affect the global climate by enhancing the atmospheric greenhouse effect (Kasting & Siefert 2002), and the industrialized countries were to begin with quite ignorant and indifferent towards industrial and agricultural emissions of these gases before the global consequences were known. Since the industrial era, the amount of greenhouse gases emitted to the atmosphere have steadily risen to a level where it is today causing environmental problems like global warming, which have adverse effect on plants and animal life (Kasting & Siefert 2002). This also affects the microbial diversity and the individual microbial species found in different ecosystems.

Microorganisms are one of the many reasons why there exists life on earth, and they are found in all parts of the biosphere, where liquid water can be found. The largest microbial ecosystem which we know the least about is the Earth's crust (Lowe et al. 1993; Schlesinger 2006; Summit & Baross 2001) mainly because over ⅔ of Earth is covered with salt-water oceans (Madsen 2008; Russell et al. 2009) and at great depths many places. Bacterial diversity in terrestrial and aquatic ecosystems have been studied for several decades, but less than 1% of the bacterial and archaea species from natural habitats have been successfully cultivated on standard media with plating techniques (Ovreas & Torsvik 1998). Therefore have the development of new molecular techniques during the recent decade largely contributed to a better understanding of prokaryotic diversity in different habitats and of the complex interactions within and between ecosystems without the need of a cultivation step. Reassociation kinetics (Torsvik et al. 1990) is one of many molecular technique used to investigate prokaryotic diversity, some of the other molecular techniques used are nucleic acids measurements based on GC/ AT content (Holben et al. 1988; Liesack & Stackebrandt 1992), sequencing (Amann et al. 1995), amplified ribosomal DNA restriction analysis (ARDRA) (Kita-Tsukamoto et al. 2006; Sklarz et al. 2009) and Denaturing gradient gel electrophoresis (DGGE) (Ferris et al. 1996; Muyzer et al. 1993). By using different molecular techniques researchers have estimated that 1 gram of soil or 160 ml ocean water may contain

from 4000 (Torsvik et al. 2002) up to 40000 (Curtis et al. 2002) different genomes, depending on the techniques used but also on the habitat. In general terms, the bacterial diversity appears to be lower in most aquatic habitats compared to terrestrial habitats.

1.1. Greenhouse gases

CO_2 and methane will only be reviewed in this part of the introduction even though these gases are important greenhouse gases in the global gas budget and climate. The main focus of this thesis is on the denitrification process and its intermediates in the nitrogen cycle. Earth's anthropogenic CO_2 levels have increased considerably since the pre-industrial times contributing to the greenhouse effect, both well document and unequivocal evidence of human alteration. A large proportion of this CO_2 increase so far has fortunately been absorbed by the earth terrestrial primary production systems, mitigating the problem (Gruber & Galloway 2008; Vitousek et al. 1997), which at current state are not saturated by today's CO_2 levels, making terrestrial plants and forests a possible future sink for anthropogenic carbon dioxide (Falkowski et al. 2000). But an increased CO_2 concentration will also affect microorganisms found in terrestrial ecosystems, most likely would it cause a change in the species composition, diversity and dynamics since microorganisms reacts differently to increased CO_2 (Vitousek et al. 1997), mainly caused by an indirect effect of increased C input from root exudates.

It is hard to see a change in this trend of increasing CO_2 emission in the nearby future, Figure 1. At this moment it is more likely that the emission of CO_2 and other greenhouse gases will increase. As the human population increases so will the effects of human activities on the terrestrial and aquatic ecosystems, from increased use and conversion of land for agriculture use to the application of fertilizers alongside with fossil fuel consumption and industrial production (Gruber & Galloway 2008; Schlesinger 2006). Today land transformation contributes to about 20 percent (Vitousek et al. 1997) of the total anthropogenic CO_2 emission which will only increase with increasing human population, as will the emission of both methane and nitrous oxide.
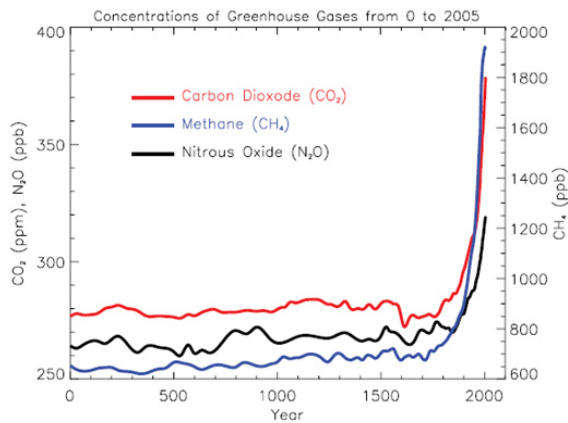
Figure 1: Shows historical and up to 2005, concentrations of the different greenhouse gases (CO_2, CH_4 and N_2O) in the atmosphere (Forster et al. 2007).

This has in many aspects altered and affected the global climate and the atmospheric composition especially in respect to the greenhouse gases (Vitousek et al. 1997). Carbon dioxide plays a central role in controlling the climate and one of the consequences of increased atmospheric CO_2 emissions, which has increased with between 30% (Vitousek et al. 1997) to 34% (Gruber & Galloway 2008) since the pre-industrial era, while others predict that the CO_2 has increase as much as 100% in this period (Falkowski et al. 2000), is a global warming of the planet due to the enhancing of the greenhouse effect (Gruber & Galloway 2008).

CO_2 is not the only gas that has increased substantially but also methane has more than doubled since the industrial era, due to increased agriculture activities and fertilizer use. But have received less attention than the other greenhouse gases because of a very low emission rate and also a very short residence time compared to the other gases, only 8-10 years, due to the reaction with the hydroxyl radical, OH (Houlton & Bai 2009; Kasting & Siefert 2002; Rowland 2006).

The greenhouse gas of concern is N_2O which has increased with over 14 % (Philippot et al. 2007) within this period, with a additional annually increase of 0.3%, Figure 2 (Schlesinger 2009). Although the concentration of nitrous oxide (0.3 ppmv) is low compared to that of CO_2 (387 ppmv) in the atmosphere, N_2O has a comparatively large impact on the global climate since it is 300 times more potent than CO_2 and has a residence time of up to 120 (Fields 2004) to 150 years in the atmosphere (Philippot et al. 2007; Richardson et al. 2009).
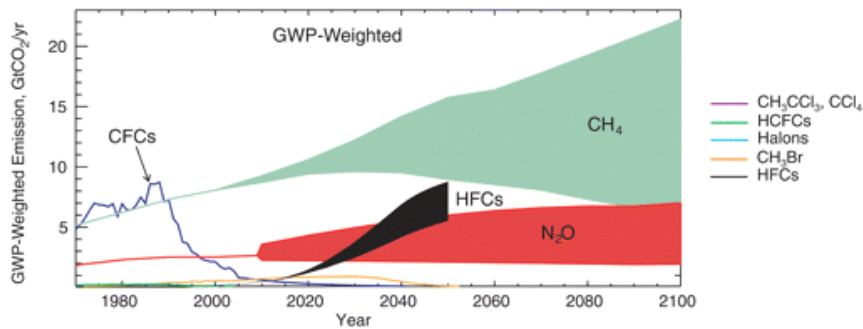
Figure 2: Shows the historical and projected GWP- (global warming potential) weighted emissions of the different greenhouse gases (Ravishankara et al. 2009).

There is consensus among many researchers that a large change in the atmospheric composition will have dramatic consequences for plant- and animal life, as well for humans (Schlesinger 2006; Vitousek et al. 1997). The consequences of this alteration have been and are still intensively debated, but no one knows for a fact what the outcome would be, only speculations on the basis of climate model simulations (Meehl et al. 2005). Some speculate that an increase in emission of various greenhouse gases would lead to a cascade effect, where global warming due to the greenhouse effect would cause the polar ice to melt, dumping large amount of freshwater into the ocean, which in turn could slow or at the worst case halt the Atlantic thermohaline circulation (Vellinga & Wood 2002), originated from the Gulf Stream completely (Curry & Mauritzen 2005; Kleiven et al. 2008). This is predicted to cause a annual colder climate of 2 °C (Bryden et al. 2005) to 5 °C (Vellinga & Wood 2008) in the northern hemisphere, offsetting some of the temperature increase caused by the greenhouse effect (Wood et al. 2003). Increased atmospheric and surface temperature in general would cause the sea level to rise due to added freshwater from the polar ice caps and also to thermal expansion (Meehl et al. 2005). Human activities over the past 100 years have so far caused an increase in the average global temperature of about 0.6 degrees, Figure 3 (Conrad 1996; Philippot et al. 2007; Tortora et al. 2001; Willey et al. 2008).
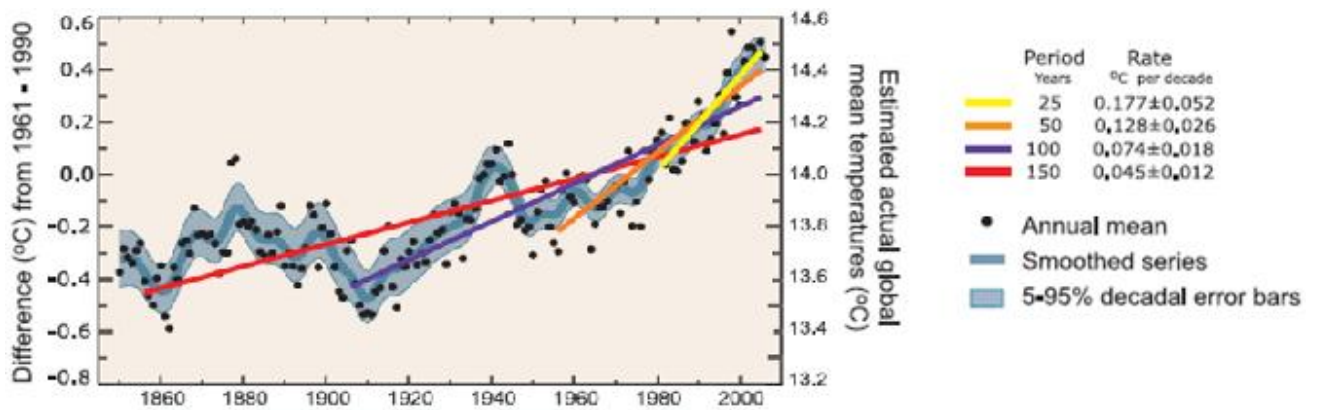
Figure 3: An overview of the annual global temperature increase from 1860 to 2005, with time periods ranging from 25 to 150 years (Trenberth et al. 2007)

1.2. Ozone depleting gases related to the nitrogen cycle (N_2O and NO)

The atmosphere is a gaseous layer around Earth which can be divided into 5 different layers, from the troposphere to the outmost layer, the exosphere. The ozone layer is a very important part of the stratosphere, mainly because the ozone layer protects us by absorbing and reflecting unwanted radiation and ultraviolet light. Especially UV light of 260 nm which is very mutagenic and causes the formation of thymine dimers in DNA (Conrad 1996).

Nitrous oxide, also known as laughing gas, is a very stable and inert greenhouse gas in the troposphere where it is mainly emitted, but when the gas reaches the stratosphere it is broken down to NO by ultraviolet light (Conrad 1996; Philippot et al. 2007). Nitrous oxide in the stratosphere is well known to catalyze the destruction of the ozone layer, but this gas has also another less know property and that is to moderate the destructive effect atmospheric chlorine has on the ozone, by binding some of it into a harmless form as $CIONO_2$. Ravishankara et al. 2009 expect the atmospheric chlorine concentration to reach preindustrial levels in the future (Figure 4) which would increase N_2O's destructiveness with about 50%, and lead to a further depletion of the ozone layer.

NO, on the other hand, is a highly reactive radical that interacts with oxygen species forming among other tropospheric ozone and smog, which exacerbate pulmonary disease in humans (Fields 2004) One of the many consequences of human activities, as the making of certain

chemicals and the release of ozone gases especially CFC gases in the beginning of the 20th century, are the depletion of the stratospheric ozone layer which is of major concern for the environment and has been a major issue of the 20th century (Ravishankara et al. 2009). It is also been shown that the effects of increased harmful ultraviolet light (UVB), due to a weakened ozone layer, greatly interferes with the nitrogen cycling in soil which lasts for several years, up to a decade (Zepp et al. 2003).

The two important actions that can be taken to hasten the recovery and mend some of the damages done to the ozone layer are to restrict and control future emission of different ozone depleting gases that destroy this protective shield around earth. Especially the emission of N_2O due to its great destructive potential (300 times greater than CO_2), this will also have beneficial effect on the global climate (Ravishankara et al. 2009).
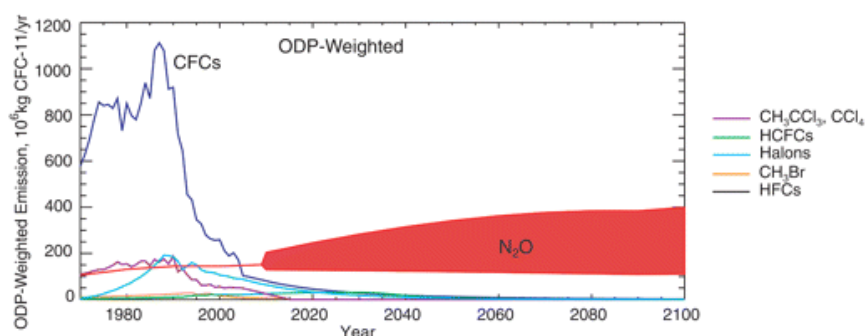
Figure 4: Historical and projected ODP- (ozone depleting potential) weighted emissions of ozone depleting gases (Ravishankara et al. 2009).

1.3. Greenhouse gases related to the nitrogen cycle (N_2O) and the impact of human activities

The atmosphere is mainly composed of nitrogen gas and oxygen (99%) and a small percentage, less than 1% of trace gases. Even with the small percentage, trace gases are very important in light of the global climate, concerning the greenhouse effect and the increase in global temperature (Kasting & Siefert 2002). Several of the greenhouse gases have increased greatly since the preindustrial era up to today, and a large proportion of this increase is due to human activities (Hungate et al. 2003) as fossil fuel combustion, conversion of land for

agriculture use, the use of fertilizers and several industrial processes (Fields 2004; Vitousek et al. 1997).

The total annually emission of N_2O alone is estimated to be about 10 million metric tons (10 Tg year^{-1}) and of this around 6.3 Tg year^{-1} originates from agricultural soils and other human activities (Philippot et al. 2007; Ravishankara et al. 2009). It is therefore of great interest to reduce the nonindustrial emission of N_2O. Humans as the dominant species on earth has and is changing the atmospheric composition in a record pace in relation to the level that was before the industrial era, and that is just one of many changes caused by human enterprise (Vitousek et al. 1997).

One of the human inventions that are of great threat to the global environment is the combustion engine with the burning of fossil fuel. It has contributed greatly to emissions of trace gases especially greenhouse gases to the atmosphere. And the simplest way to reduce this threat is to reduce the usage of fossil fuel and replace it with a cleaner alternative which is renewable energy (Gruber & Galloway 2008).

1.4. Regular anoxic spells and bacterial survival mechanisms

Bacteria that live in environments that experience regular anoxic spells need several different strategies for survival, and must therefore be able to respond to different environmental changes by altering their metabolic pattern, while others have through evolution evolved to live in symbiosis with plants and plant roots in the rhizosphere, which has its advantages (Conrad 1996; Philippot et al. 2007).

Most prokaryotes are able to perform both aerobic and anaerobic respiration and only a few bacterial species can grow under either of the respiratory systems alone. Microbes that do not manage the transition from an oxic to anoxic environment and vice versa would most likely perish or at least become dormant (Conrad 1996; Madsen 2005). The type of respiration prokaryotes perform is mainly dependent on the oxygen availability in soil, as well as in other ecosystems. Oxic respiration provides normally a energy yield about 32 (Fenchel et al. 1998) to 38 (Madsen 2008) ATP per glucose molecule and is therefore more favorable than anoxic

respiration due to the higher energy yield. In the absence of oxygen microbes utilize anaerobic respiration to gain energy, but the yield is not as great as for oxic respiration. Both denitrification and nitrate ammonification which are two forms of anoxic respiration provides a energy yield respectively 7 and 35% lower than oxic respiration (Strohm et al. 2007), while fermentation only yield 2 – 4 ATP (Fenchel et al. 1998)

Oxygen varies greatly in soil, which can be divided into several categories depending on the soil texture and of its characteristics, from sandy soil to clay. This has an effect on the trace gas consumption, production and transport since different soils would have different properties and also variable microbial composition. The different layers in soil are dominated by different prokaryotes and microorganisms in their microhabitats depending on the redox potential, pH, nutrient composition, concentration and availability in the soil environment which are some of many factors that determine the microbial diversity alongside with the availability of appropriate electron acceptors and donors (Conrad 1996; Madsen 2005; Torsvik et al. 2002). Nutrients in soil and many other ecosystems are almost always in short supply especially carbon and nitrogen (Madsen 2005), which means harsh competition between microorganisms to grow and gain an advantage over other species in the same habitat. But nitrogen is not only used by prokaryotes and other microorganism but also by plants for growth (Willey et al. 2008).

The exchange of gases between soil and the atmosphere is due to the activities of bacteria and other organisms found in the biosphere and also of chemical processes, but the interaction of the gas flux are complex and poorly understood. It is believed and showed through several studies that the gas flux of N_2O are influenced by certain types of environmental changes and soil conditions, which may either cause an increase or decrease in the emission rate.

Emissions of NO and N_2O in wetland soil are e.g. quite small due to the fact that this type of soil is mostly water saturated and therefore devoid of oxygen at a depth of just two to three millimeter. Another important contributing factor that also decides the depth of the oxic zone in soils is the rate between influx from above and oxygen consumption by the microflora (Conrad 1996).Continually waterlogged systems which do not experience any oxic spells are not dominated by the denitrification process, mainly because this system lacks the appropriate electron acceptor nitrate, which is only produced under oxic conditions (Zumft 1997). It is rather a environment dominated by fermentation and methanogenesis since it mostly contains

the electron acceptors CO_2 and H^+ (Conrad 1996). Soils on the other hand which are in general oxic, but experience regular anoxic spells emit more of these gases then wetland soils.

Also seasonal and natural events like freezing – thawing, drying – wetting, floods and droughts influence the composition and condition of the soil which in turn would affect the composition of the microbial communities by affecting their enzyme activity, synthesis or proliferation. Overall, all these events would affect both the emission rate and the composition of trace gases emitted from soil to the atmosphere. A large accumulation of these gases, especially N_2O, in the atmosphere will have profound impact on the climate and the atmospheric ozone balance (Conrad 1996; Philippot et al. 2007)

1.5. Nitrogen cycle

Nitrogen (N) is one of the chemical compounds essential for life to exist on Earth, needed by all organisms. On average up to 6.25% of an organism's dry mass consist of nitrogen and this compound is vital in the synthesis of nitrogen rich molecules as amino acid, purines, pyrimidines, cofactors and other cell substance (Fields 2004; Richardson et al. 2009).

Nitrogen is cycled between the atmosphere, the geosphere and the different ecosystems (biosphere), and as mention earlier our atmosphere consists of about 78% nitrogen gas (N_2). Therefore, one would intuitively think that there is an unlimited supply of nitrogen for all microorganisms and plants, but in reality this is not the case. Instead, nitrogen availability is quite limited in both terrestrial and marine ecosystems (Richardson et al. 2009) since N_2 is a very stable and inert gas, due to the triple bond between the two nitrogen atoms, (Fields 2004) that cannot be directly assimilated by most organisms. Only a limited number of prokaryote organisms can perform nitrogen fixation, Figure 5 (Gruber & Galloway 2008; Vitousek et al. 1997), converting atmospheric N_2 into ammonia, which is then used for biosynthesis of major building blocks of life such as nucleic acids and amino acids. The nitrogen that has been fixed into biomolecules will, after mineralization, become available to other organisms. Nitrogen limitation is thus a controlling factor that is reflected in the terrestrial ecosystems net primary production, and studies have demonstrated that addition of nitrogen to an ecosystem gives on average a growth response of about 29% (Elser et al. 2009; LeBauer & Treseder 2008)
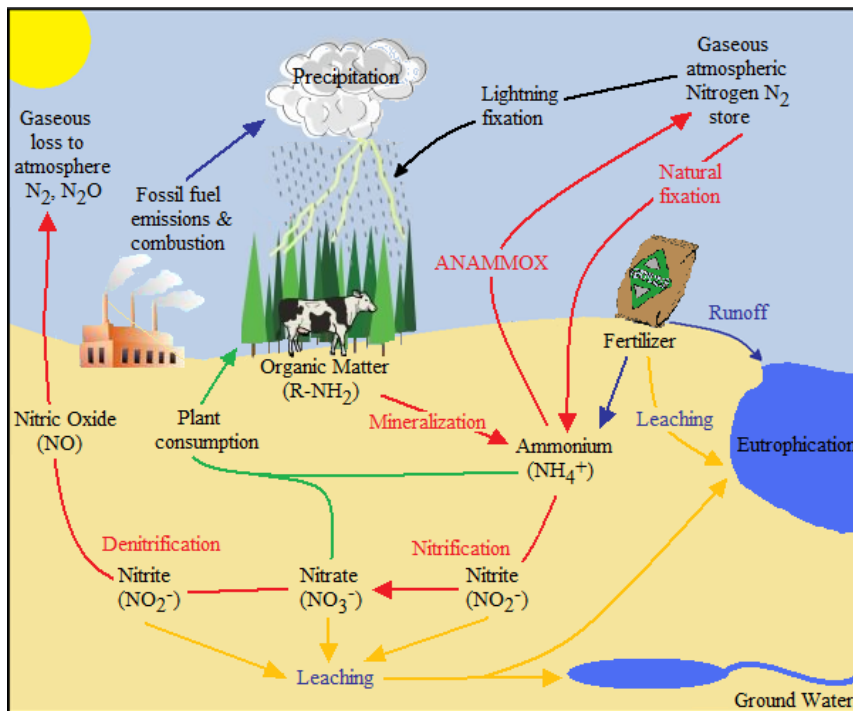
Figure 5: The nitrogen cycle consists of 6 main processes which include nitrogen fixation, nitrogen assimilation and mineralization, nitrification, ANNAMOX and denitrification, modified from (Pidwirny 2009)

Human activity has caused an increase in the nitrogen loadings to both terrestrial and aquatic ecosystems worldwide these past two centuries. Terrestrial and aquatic (ocean) nitrogen fixation stood each for respectively 110 and 140 Tg N per year, but either of them alone did not measure up to the nitrogen deposition to the environment from food production (fertilizers) and fossil fuel combustion in the mid 1990's, which were the two anthropogenic nitrogen sources that alone stood for more than 160 Tg N per year to the environment (Galloway et al. 2008; Gruber & Galloway 2008) Humans in general are responsible of adding as much fixed nitrogen as all the natural sources combined to the terrestrial ecosystems. This greatly influences and alters the nitrogen cycle with severe atmospheric consequences from increased greenhouse gas emission to acid rain and photochemical smog (Vitousek et al. 1997).

The amount of nitrogen input to the biosphere has increased dramatically. Some researchers estimate that it has more than doubled since the pre-industrial period due to human activities (Elser et al. 2009), while others claim that it has in reality tripled (Elser et al. 2009; Galloway et al. 2008). This increase in human made nitrogen loading to the environment is largely due

to an ever increasing human population and its activity (Figure 6), with huge increase in food production, made possible by the Haber-Bosch process, to sustain the population boom over the last century. The use of fertilizers, accounts today for roughly 40% of the food production, and within just a time period of 35 years (1960-1995) the use of fertilizers had increased with 700% (Conrad 1996; Galloway et al. 2008; Gruber & Galloway 2008; Philippot et al. 2007) To the contrary, the natural nitrogen loading would be about 0.5 kg N ha^{-1} yr^{-1} in absence of human influence (Galloway et al. 2008).

GLOBAL POPULATION & REACTIVE NITROGEN TRENDS

Natural N Fixation

Legend: Global population | Total reactive N | N fixation in cropland | Fossil fuel N | Industrial N fixation
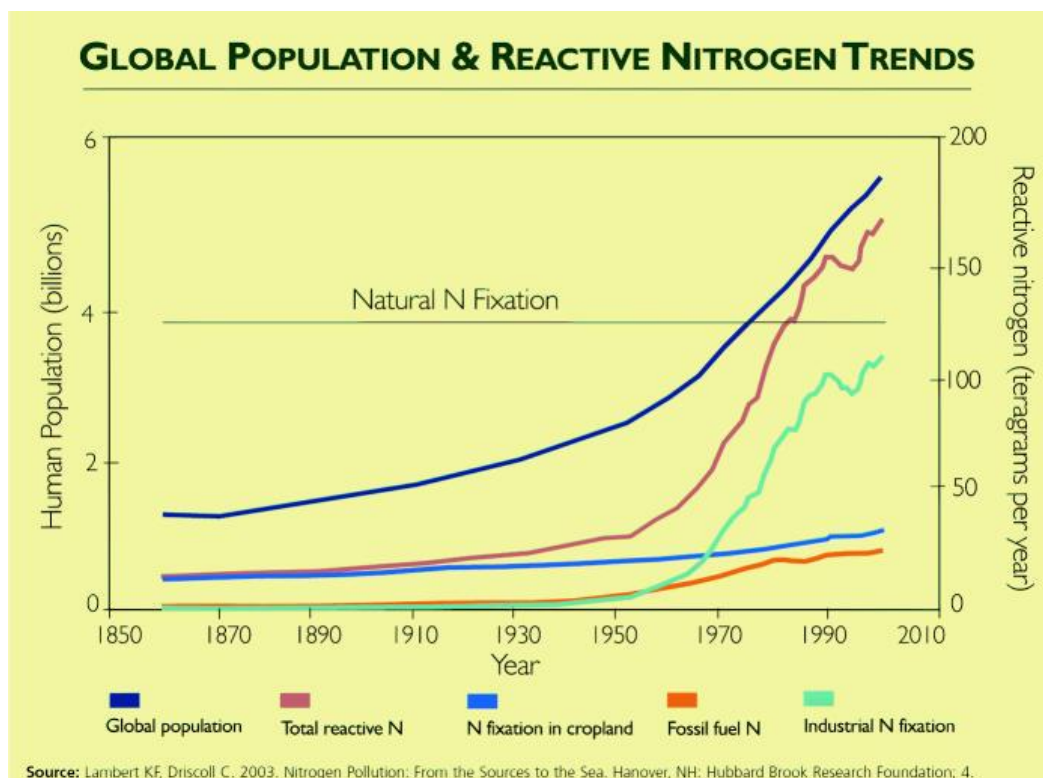
Figure 6: A overview of the reactive nitrogen trends in Tg pr year from 1850 to 2010 in relation to increasing human population (Fields 2004)

Human reactive nitrogen production has increased immensely since the 1860 (Galloway et al. 2008) with an annual increase of about 15 Tg per year (Fields 2004). This has in turn influenced all the major biochemical cycles in mainly all the ecosystems, but the consequences of this influence on the interaction between these cycles are poorly understood (Gruber & Galloway 2008). The environmental consequences, on the other hand, are well known and can easily sees first over the Arctic and Antarctic poles since these areas are more receptive to environmental changes compared to other parts of the world (Fields 2004; Vitousek et al. 1997).

The excess N in soil from e.g. overuse of fertilizers and other situations that causes a terrestrial ecosystem to become saturated with reactive nitrogen is carried away by surface runoffs to lakes, rivers to the costal oceans (Gruber & Galloway 2008; Peterson et al. 2001; Schlesinger 2006). This causes several environmental problems, ranging from Eutrophication of the aquatic ecosystems to acidification of both the terrestrial and aquatic systems (Fields 2004; Gruber & Galloway 2008). The excess anthropogenic nitrogen loading of reactive nitrogen, oxidized and reduced forms, in terrestrial and aquatic environment influences and affects not only the climate and the atmospheric chemistry but also human welfare and health as it may cause elevated levels of nitrate it the aquatic systems, which are toxic for humans in excess concentrations. The recommended and allowed concentrations in water by the health department range widely between the different countries from 10 mg in the USA (Knobeloch et al. 2000) to 50 mg per liter (Thorburn et al. 2003) in Australia (Galloway et al. 2008; Gruber & Galloway 2008).

1.6.1. Nitrogen Fixation

Nitrogen fixation ($N_2 \rightarrow NH_4^+$) is an energy consuming process that occurs in free-living organisms as well as in symbiosis with plants (legume root nodules) and to a lesser degree in lightning strikes (Fields 2004); it is a process that converts atmospheric N_2 to ammonium (NH_4^+), by an enzyme called nitrogenase. This biological process can take place in both oxic and anoxic surroundings, but since the enzyme is inactivated in the presence of oxygen, do create the need for protection. Bacteria and Archaea have therefore developed several different strategies to protect the enzyme from oxidizing conditions, e.g. in cyanobacteria the nitrogenase enzyme is protected from O_2 in specialized structures called heterocysts (Tortora et al. 2001; Willey et al. 2008).

1.6.2. Nitrogen Assimilation and Nitrogen Mineralization

Nitrogen assimilation ($NH_4^+ \rightarrow$ Organic N) is a non-energy yielding biological process used by heterotrophic organisms that are incapable of independent nitrogen fixation to obtain the nitrogen needed for the synthesis of nitrogen rich molecules, which are important for survival and growth (Willey et al. 2008).

The opposite biological process is nitrogen mineralization (Organic N → NH_4^+) also called ammonification, performed by bacteria and in some cases also fungi. In this process organic nitrogen from dead decomposing organisms or animal waste, are converted back to ammonium and release into the environment for reuse by other organisms (Tortora et al. 2001).

1.6.3. Anaerobic ammonium oxidation (ANNAMOX)

Microorganisms that can perform the anammox process are a quite newly discovered group of bacteria that anaerobically converts ammonium to nitrogen gas, all belonging to the phylum planctomycetes. Anammox along with denitrification are the two major sources of atmospheric N_2 from anoxic water column, sediments and soils respectively (Bothe et al. 2007; Gruber & Galloway 2008).

1.6.4. Nitrification

Nitrification ($NH_4^+ \rightarrow NO_2^- \rightarrow NO_3^-$) is an energy yielding biological process, divided into two steps which can only proceed under oxic conditions. The first step is in general the limiting step in nitrification which involves the oxidation of ammonium to nitrite performed by bacteria belonging to the genera Nitrosomonas and Nitrosococcus. The second step is the further oxidization of nitrite to nitrate done by bacterial species such as Nitrobacter, which is of great importance because accumulation of nitrite in excess levels is potentially toxic for most organisms, causing inhibition of growth (Bollag & Henninger 1978). The end product of nitrification is nitrate, very important in agriculture, used by both organisms and plants. It is a highly motile molecule that is quickly lost to either leaching or denitrification or to both (Bothe et al. 2007; Philippot et al. 2007; Tortora et al. 2001; Willey et al. 2008).

In human perspective high concentrations of nitrate in the groundwater is of considerable concern due to the health problems it can cause, especially to infants under the age of 6 months. To these infants even low concentrations of nitrate in the drinking water or food can cause a potentially fatal condition called blue baby syndrome, where the hemoglobin is

oxidized to methemoglobin, which is then unable to transport oxygen. The oxidation of hemoglobin happens because infants have a low concentration of the enzyme methemoglobin reductase (Fields 2004). But also long term consumption of low nitrate contaminated drinking water is believed and associated in several countries to cause gastric cancer and reproductive risks (Galloway et al. 2008; Knobeloch et al. 2000).

1.8. Denitrification

Denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) is a taxonomically widespread process used by more than 60 different genera of bacteria including archaea and some fungi. It is a multistep biological process where nitrate is reduced to dinitrogen gas (N_2) by four enzymes, listed in the next chapter. This process, along with the anammox are the two major players in the removal of nitrogen compounds from different soils and habitats, and returning it to the atmosphere as N_2, thus causing a net loss of ammonium, nitrate and nitrite which in turn have a negative effect on soil fertility (Conrad 1996; Zumft 1997). Houlton and Bai estimated that a substantial amount of nitrogen, roughly 28 Tg, in terrestrial ecosystems are converted to N_2 by denitrifying prokaryotes on a yearly basis (Houlton & Bai 2009).

Microorganisms that are able to perform both aerobic and anaerobic respiration will preferably use oxygen, if present, as the terminal electron acceptor mainly because the energy yield is greater and secondly because the denitrification process is inhibited (the synthesis of nitrate reductase) by the presence of oxygen. It may take up to 20 hours before full denitrification capacity is established after the introduction of anoxic condition (Kuypers et al. 2005). Even though anaerobic respiration doesn't yield nearly as much energy as when microorganisms respire under oxic condition, it is surely better than the alternative which is metabolic arrest due to the lack of ATP production (Willey et al. 2008).

Soil, sediments and water consists of communities with a large diversity of different bacteria species, all competing for the limited amount of nutrients available in these environments and around 5 to (Philippot et al. 2007) 15 percent (Bergaust 2009) of these bacteria species are denitrifiers. The end product of denitrification dependents on the different bacteria species ability to synthesize the required enzymes involved in the denitrification process, some

bacteria drive this process all the way to dinitrogen gas because they retain the genes necessary for the synthesis of all the four enzymes involved, while some only to N_2O and others emit a mixture of both N_2O and N_2 to the atmosphere (Houlton & Bai 2009). Terrestrial ecosystems, soil and freshwater system, are the main sites that account for 50 (Gruber & Galloway 2008) - 80 % (Galloway et al. 2008) of the global denitrification. When terrestrial nitrogen loadings overexcited the local denitrification population's capacity to process, it is carried off by e.g. runoffs to freshwater system as groundwater, rivers, lakes and reservoirs. And in the freshwater systems, excess nitrogen will eventually be denitrified and converted to N_2, but it would be carried further in the aquatic system then normally which would lead to Eutrophication of the system (Peterson et al. 2001).

Several natural events and human activities can cause an increase in emission of denitrification gasses from prokaryotes. Among the natural events of interest are the freeze-thaw and dry-wet cycles, which under certain conditions can be a major source of N_2O. When it comes to human activities has the excess use of fertilizers, especially mineral fertilizers in combination with intensive cultivation of agricultural soil the tendency to promote higher denitrification rate and emission (Philippot et al. 2007).

1.8.1. The different reductase in denitrification

Complete denitrification is a four step process consists of 4 enzymes responsible for the reduction of nitrate to dinitrogen gas, shown in Figure 7.

$$
\overset{1)}{NO_3^-} \;\rightarrow\; \overset{2)}{NO_2^-} \;\rightarrow\; \overset{3)}{NO} \;\rightarrow\; \overset{4)}{N_2O} \;\rightarrow\; N_2
$$

Figure 7: An overview of the denitrification process, with the different reductase involved and the intermediates produced. 1) Nitrate reductase (NAR & NAP), 2) Nitrite reductase (NIR), 3) Nitric oxide reductase (NOR) and 4) Nitrous oxide reductase (N$_2$OR).

The first step in the denitrification process is performed by NAR and/ or NAP, responsible for the reduction of nitrate to nitrite, located in the membrane and the periplasm respectively (Philippot et al. 2007; Zumft 1997).

The second reductase catalyzes the reduction of NO_2^- into NO and is the first reaction in the denitrification process that produces a gaseous intermediate. The enzymes, NirK which is a copper containing reductase and NirS (I and II) which are a cytochrome cd_1 containing reductase, are structural different in addition of having different prosthetic metal groups can be said to be evolutionary unrelated even though they catalyze the same reaction in the denitrification process (Conrad 1996; Zumft 1997). There are no known bacteria that contain both of the genes NirS and NirK and the Thauera strains are no exception, all the known strains consists of only the NirS nitrite reductase gene (Etchebehere & Tiedje 2005).

The third reaction in this denitrification cascade is the reduction of the gaseous intermediate NO further to N_2O by the enzyme NOR which contains of the three subclasses cNOR, qNOR and qCuNOR. This is also a very important step in the denitrification pathway due to the fact that NO is a potent cytotoxin, so in order for prokaryotes to survive and live, a rapid continued reduction of NO to N_2O is therefore vital (Bergaust et al. 2008; Conrad 1996; Philippot et al. 2007; Richardson et al. 2009).

The last and final step in the denitrification cascade is the reduction of N_2O to N_2 which effect the composition of our atmosphere profoundly. This reaction is catalyzed by the enzyme NosZ which is a multicopper homodimeric reductase (Philippot et al. 2007).

1.8.2. Intermediates of denitrification

The full denitrification process is the reduction of nitrate (NO_3^-) to dinitrogen gas (N_2), with nitrite, nitric oxide and nitrous oxide as intermediates.

Denitrification intermediates of human and environmental concern are nitrite, N_2O and nitrate. Although nitrate is not an intermediate in the denitrification process but works as a terminal electron acceptor, it is still important (Conrad 1996; Philippot et al. 2007). Accumulation of nitrite in the environment is not preferable since it leads to the formation of carcinogenic nitrosamines, and nitrate concentration higher than 10-50 mg/L (Knobeloch et al. 2000) in e.g. the groundwater due to leaching is of great concern to human health (Philippot et al. 2007; Willey et al. 2008).

The last two intermediates nitric oxide and nitrous oxide are gases, more precisely a signal molecule and a greenhouse gas, respectively. Nitrous oxide has long been used as an anesthetic under different types of surgeries, commonly called laughing gas. Not all bacteria can reduce nitrous oxide to dinitrogen gas which makes this process important for the turnover of N_2O in soil (Conrad 1996; Tortora et al. 2001). Another important gas in soil is the denitrification intermediate NO, which is also a powerful signal molecule in the human body, engaged from blood pressure regulation, immunity to memory (Cooper 2000; Tortora et al. 2001).

Release of the intermediates NO and N_2O are frequently observed for both the nitrification and the denitrification process when excess reactive nitrogen manages to cause an overload of the enzymatic system in these two processes, a phenomena known as "hole in the pipe", Figure 8 (Madsen 2008).
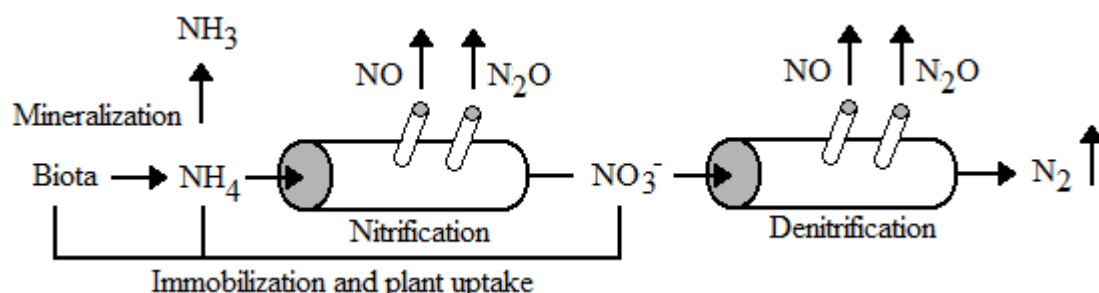
Figure 8: The "hole in the pipe" is a phenomena where the biochemical pathways, nitrification and denitrification, leaks N_2O and NO gases to the atmosphere, due to an overload of their systems enzymatic capacity (Madsen 2008).

1.8.3. Biochemistry and biological regulation of denitrification

The denitrification process is a complex interlacing system consisting of four different enzymes (nitrate-, nitrite-, nitric oxide-, and nitrous oxide reductase) which regulates and coordinates the reduction of ionic N-oxides to dinitrogen gas, with moderate or high accumulation of intermediates depending on how strict the regulation is in the different

bacterial species. Accumulation of some of the intermediates in the ecosystem is quite harmful to the microbial populations in general and to the stratospheric ozone layer. Therefore are moderate or tight control of NO and N_2O production vital for microbial survival and to the climate respectively (Seitzinger et al. 2006). Not only internal factors influence, regulate and control the denitrification process but also several external environmental factors are of great importance to the denitrification process as oxygen, nitrate and nitric oxide (Conrad 1996). Regular environmental events like the seasonal cycle and also water status and pH in soil influence the denitrification process indicated by the highest measured rates of trace gases in the spring and fall (Philippot et al. 2007).

There are three general requirements for the denitrification process to occur in soil and other habitats, which are anoxic condition or limited oxygen supply, the presence of suitable electron donors and acceptors and of course microbial populations in the ecosystem that have the metabolic ability to perform denitrification (Philippot et al. 2007).

The intermediates in the denitrification process need to be tightly controlled and regulated since both nitrite and NO in excess levels are toxic (Rodionov et al. 2005), which would have an undesirable effect on the prokaryotic population itself (Zumft 1997). However, a moderate accumulation of the intermediates may have an antibacterial effect on the other microbial species in the same ecosystem, not involved in the denitrification process, which would give the denitrifying population a tactical advantage by impeding or inhibiting the competition (Choi et al. 2006). Human activities are also an influencing factor on the denitrification process, as the use of fertilizers to air, water and soil pollutions. Some types of pollution have the tendency to inhibit denitrification as some heavy metals tend to do (SAKADEVAN et al. 1999).

2. Experimental procedures

The five bacteria strains used in this study belong to the genus *Thauera,* acquired from different countries and origin, Table 1. All of them were isolated from different types of sludge. *T. aminoaromatica* and *T. linaloolentis* were purchased from Deutsche Samlung für Mikroorganismen (DSMZ), while the other strains were kindly provided from Prof. J. Tiedje, USA and Shinoda, Japan.

Table 1: A short description of the different *Thauera* strains used, acquired and modified from Mao's manuscript in prep.

Strain	Place/ Country	Origin	Acquired from
Thauera aminoaromatica	Konstanz, Germany	Anoxic ditch sludge	DSMZ
Thauera linaloolentis	Germany	Activated sludge	DSMZ
Thauera sp. 27	Michigan, USA	Anaerobic sludge	(Etchebehere & Tiedje 2005)
Thauera sp. 28	Michigan, USA	Anaerobic sludge	(Etchebehere & Tiedje 2005)
Thauera sp. 63	Michigan, USA	Anaerobic sludge	(Etchebehere & Tiedje 2005)
Thauera sp. DNT-1	Japan	Anaerobic sludge	(Shinoda et al. 2004)

2.1. Minimal medium supplemented with 10% complex medium

We knew from an earlier study (Mao et al., manuscript in prep.), that all *Thauera* strains used in the present investigation can be cultured in a minimal medium supplemented with 10% complex medium. Now we were interested in finding a well defined medium where the amount of carbon and nitrogen were known and could be controlled, before comparative phenotypic analysis of *Thauera* were performed. The other mediums tested were Sistrom's and medium 586 recommended by DSMZ.

Minimal medium supplemented with 10% complex medium contained (g l$^-$): NH$_4$Cl 0.900g, MgCl$_2$*6H$_2$O 0.200g, K$_2$SO$_4$ 0.100g, CaCl$_2$*2H$_2$O 0.050g, NaHCO$_3$ 0.080g, NaAC 0.400g,

Peptone 1.00g, Meat extract 0.600g. 0.1M phosphate buffer ($Na_2HPO_4 + NAH_2PO_4$) was added and the pH adjusted to 7.5.

Stock cultures of all the strains were made and frozen in -80°C, except *Thauera sp. DNT-1*. The strains were cultivated in 120 ml serum bottles prepared with 50 ml minimal medium supplemented with 10% complex medium and a magnetic stirrer (950 rpm) to ensure aerobic conditions, at a temperature of 28 °C. The optical density (600 nm) of these cultures was measured after a couple of days due to rapid growth.

2.1.1. Growth rates

All the *Thauera* strains (shown in Table 1) except for *Thauera sp. DNT-1*, were raised from frozen stocks in 120 ml serum bottles each containing 50 ml of 10% complex medium with a pH of 7.5. The incubations took place under oxic conditions at 28 °C with vigorous stirring (950 rpm) using a magnetic stirring (Cowie 25 x 8 mm) to ensure adequate gas exchange between the liquid and the gas phase, and also to prevent aggregation. When these first pure cultures reached late exponential phase they were inoculated into new bottles with fresh 50 ml of minimal medium supplemented with 10% complex medium and incubated under the same conditions. These second batch cultures were then used in the measurements of optical density (600 nm) to calculate growth rate. *Thauera linaloolentis* was the only strain also used to estimate growth rate by gas measurements, based on electron transport rates.

2.1.2. ERIC (Enterobacterial Repetitive Intergeneric Consensus) PCR

ERIC-PCR is a method to obtain a genomic fingerprint by amplifying the DNA sequence between the small repeated sequences found in the genome of most gram negative- and also in some gram positive bacteria, generating strain specific fragments. It is a sensitive method for distinguishing between different strains of the same bacterial species. In this context the method was used as a verification of the cultures purity (Debruijn 1992; Vinuesa et al. 1998; Wang et al. 2009).

The solutions used for ERIC-PCR, and the PCR program used, are listed below. After amplification, the ERIC-PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. 10 µl PCR products from each strain was mixed with 2 µl loading buffer and added to each well in duplicates. A 100 bp ladder (Biolabs) was loaded in one well as a molecular marker (Debruijn 1992).

Genomic fingerprinting (ERIC)

Solutions:

- 5x PCR buffer   5.0 µl
- 25mM Mg^{2+} 2.0 µl
- 2mM dNTP   2.5 µl
- 50µM Eric R1 (primer P1) 0.5 µl
- 50µM Eric R2 (primer P2) 0.5 µl
- 5 U/ul Taq polymerase   0.35 µl
  (Acquired from Promega)
- ddH$_2$O   X µl
- DNA   40 ng (2µl)

  Total   25 µl

PCR program (takes about 6 hours):

95 ℃ 7 min

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94 ℃ 1 min   These 3 steps
52 ℃ 1 min   are repeated
65 ℃ 8 min   35 times

----

65 ℃ 8 min

4 ℃   ∞

2.2. Testing of different growth media

Before a comparative phenotypic analysis of the different *Thauera* strains were performed, it was necessary and important to ensure that all the strains were able to grow on the same medium and, if possible, with only one carbon source. A number of different growth media were therefore tested to assess if there were another medium the *Thauera* strains would thrive and grow in other than the minimal medium supplemented with 10% complex medium. This type of medium consists of more than one source of carbon and is therefore referred to as a complex medium which contains either or a combination of these elements like yeast extract, meat extract and peptone (Willey et al. 2008). The two other media tested were Sistrom's

medium (Lueking et al. 1978; SISTROM 1962) and medium 586, the later being a mineral medium recommended by the DSMZ Company for the growth of *Thauera aminoaromatica*.

2.2.1. Sistrom's medium

Two strains were tested for growth on Sistrom's medium (Lueking et al. 1978; SISTROM 1962); *Thauera aminoaromatica* and *Thauera sp. DNT-1*. The latter strain was only used when testing growth of different *Thauera* species in Sistrom's medium, and was not used in other experiments. Sistrom's medium, contained the following (g l^{-1}): K_2HPO_4 3.48 g, NH_4Cl 0.195 g, succinic acid 4.00 g, L-glutamic acid 0.100 g, L-aspartic acid 0.040 g, NaCl 0.500 g, nitrolotriacetic acid 0.200 g, $MgSO_4*7H_2O$ 0.300 g, $CaCl_2*7H_2O$ 0.015 g, $FeSO_2*7H_2O$ 0.007 g. In addition, trace elements and vitamins were added (g l^{-1}): EDTA (triplex 3) 1.765 mg, $ZnSO_4*7H_2O$ 10.95 mg, $FeSO_4*7H_2O$ 0.005 g, $MnSO_4*H_2O$ 1.54 mg, $CuSO_4*5H_2O$ 0.392 mg, $Co(NO3)_2*6H_2O$ 0.248 mg, H_3BO_3 0.114 mg, nicotinic acid 0.0010 g, thiamine HCl 0.0005 g and biotin 0.010 mg. The medium was autoclaved after adjusting the pH to 7.0 using KOH.

To test these two strains ability to grow on Sistrom's medium, four 120 ml serum bottles were each prepared with 50 ml medium and a magnetic stirrer (950 rpm) to ensure aerobic conditions. In the first part of this experiment, frozen stocks of both *T. aminoaromatica* and *Thauera sp. DNT-1* were inoculated directly into separate bottles containing 50 ml Sistrom's medium and cultivated at 28 °C. The optical density (600 nm) of these two cultures was measured after one week. In the second part of the experiment frozen stock of both strains were first cultivated overnight in minimal medium supplemented with 10% complex medium under vigorous stirring (950 rpm) at a temperature of 28 °C. They were then inoculated into separate bottle containing 50 ml Sistrom's medium. The optical density (600 nm) of these two cultures was measured after one week.

2.2.2. Medium 586 (*Thauera aminoaromatica*)

Two experiments were performed to assess if the five *Thauera* species would grow on medium 586 (DSMZ), which consists of two main solutions A and B, with additional trace element (SL-10) and vitamin solution.

The main solution A consists of KH_2PO_4 0.816 g, K_2HPO_4 5.920 g, 500 ml distilled milliQ water, and solution B consists of NH_4Cl 0.530 g, $MgSO_4*7H_2O$ 0.200 g, KNO_3 2.00 g, $CaCl_2*2H_2O$ 0.025 g, Na-benzoate 0.720 g and 500 ml distilled milliQ water. These two solutions are made prepared and autoclaved separately with a pH of 7.5, and combined after cooling. This medium also contains 10 ml of sterile filtrated trace elements (SL-10) and 5 ml of sterile filtrated vitamin solution. The trace element solution SL-10 consists of HCl (25 %; 7,7 M) 10 ml, $FeCl_2*4H_2O$ 1.50 g, $ZnCl_2$ 0.070 g, $MnCl_2*4H_2O$ 0.100 g, H_3BO_3 0.006 g, $CoCl_2*6H_2O$ 0.190 g, $CuCl_2*2H_2O$ 0.002 g, $NiCl_2*6H_2O$ 0.024 g, $Na_2MoO_4*H_2O$ 0.036 g, distilled water 990 ml, and the vitamin solution consists of vitamin B_{12} 0.050 g, pantothenic acid 0.050 g, riboflavin 0.050 g, pyridoxamine – HCl 0.010 g, biotin 0.020 g, folic acid 0.020 g, nicotinic acid 0.025 g, nicotine acid 0.025 g, A-lipoic acid 0.050 g, P-aminobenzoic acid 0.050 g, thiamine – $HCl*2H_2O$ 0.050 g and 1000 ml distilled water.

The media were prepared according to the protocol, except for a few changes: Hoagland's A-Z solution (Hoagland's A-Z trace element solution 2009) was used instead of the trace element solution in the protocol. The vitamin solution followed the manual except for the three vitamins, pyridoxamine-HCL, a-lipoic acid and p-aminobenzoic acid, which were excluded after consultation with Åsa Frostegård. The Hoagland's A-Z-solution consist of LiCl 0.050 g, $CuSO_4*5H_2O$ 0.100 g, $ZnSO_4*7H_2O$ 0.100 g, H_3BO_3 1.10 g, $Al_2(SO_4)_3$ 0.100 g, $SnCl_2*2H_2O$ 0.050 g, $MnCl_2*4H_2O$ 0.700 g, $NiSO_4*6H_2O$ 0.100 g, $Co(NO_3)_2*6H_2O$ 0.100 g, TiO_2 0.100 g, KI 0.050 g, KBR 0.050 g, and 1.8 L distilled milliQ water.

In the first part of the experiment stock cultures (-80 ℃ freezer) of *Thauera aminoaromatica*, *Thauera linaloolentis*, *Thauera sp. 27*, *Thauera sp. 28* and *Thauera sp. 63* were each transferred to a separate 120 ml serum bottle containing 50 ml of medium 586, with the same incubation conditions as listed above (vigorous stirring, 950 rpm, at 28 °C). The optical density (600 nm) of these cultures was measured after one week and then again after the second week of cultivation.

In the second part of the experiment stock cultures of only *Thauera aminoaromatica*, *Thauera linaloolentis* and *Thauera sp. 63* were first cultivated overnight in minimal medium supplemented with 10 % complex medium at 28 °C and under vigorous stirring (950 rpm) to ensure aerobic conditions and also to minimize aggregation, before being transferred to five separate bottles each containing both 50 ml of medium 586 and a yeast concentration ranging from 12.4 mg^{-1} to 349.9 mg^{-1} with the same incubation conditions (vigorous stirring, 950 rpm, at 28 °C). The optical density (600 nm) of these cultures was measured after one week of cultivation. After one week the cultures containing medium 586 and yeast were then re-inoculated into flasks with 50 ml of fresh medium 586. Only 100μl were transferred from each culture to ensure that the yeast extract from the previous medium was heavily diluted so that it would not support any significant growth. The cultures were incubated for another week before optical density was measured again.

2.3. Gas kinetic experiments; an introduction to the robotized incubation system

In this thesis the instrument of importance is a robotized incubation system in which phenotypic characteristics of bacteria, especially denitrifying bacteria, is studied by measuring the amounts of gaseous metabolites and end products at the transition from oxic to anoxic conditions.

The incubation system (Molstad et al. 2007) can accommodate fifteen 120 ml crimp sealed serum bottles with magnetic stirring (850 rpm) and also three positions without magnetic stirring for different standard or control bottles. The bottles are incubated in a temperature regulated water bath 0-40°C. The headspace of the bottles is sampled regularly by a Gilson Model 222 autosampler coupled to a peristaltic pump (Gilson Minipuls 3) for measurement of the gases produced by the bacteria. To minimize the risk for mechanical leakage the needle never pierces the septa of any given serum bottle at the same place twice when sampling. This is controlled by a computerized program called Python which also controls other aspects of this robotized incubation system as the GC, integrating the NO peaks and also organizes the data.

The different gas concentrations of N_2O, N_2, CO_2 and O_2 were sampled from the headspace of the serum bottles, but before being analyzed by the Varian CP4900 microGC it had to go through a filter placed between the peristaltic pump and the injector of the GC to ensure that no particles entered the GC. The microGC itself consists of two columns, a 10 m poraPLOT and a 20 m 5 Å Molsieve with each a separate injector and thermal conductivity detector (TCD). After the gas is brought through the outlet loop of the GC it arrives to a T piece and from here the gas samples are divided and directed through two different loops by a helium flow of 15 ml min^{-1} to the NO analyzer, which is a Chemoluminescence NOx analyzer Model 200A (Fig. 9). The main loop carries 96% of the gas sample directly to the NO analyzer, while 4% goes through a split loop (SL), it takes a detour and arrives to the NO analyzer 60 seconds later than the main loop, creating a second NO reading. The linear range of NO detection is increased by a factor of 100 with this second NO peak because the second peak is broader than the first.

The headspace of every serum bottle is subject to a constant dilution after each sampling and every sampling involves the outtake of 4.2 ml headspace gas. But not the whole gas volume sampled is used in the analyzing process and that fraction is returned back to the same bottle after GC injection by down pumping. The actual volume taken out of the headspace of each bottle is replaced with helium gas to maintain a stable pressure of about one atmosphere, which will with every sampling cause a dilution of 1.6%, equivalent to 1.9 ml with helium. A further in-depth detailed description of this instrument can be found in the journal by Molstad et al., 2007 (Molstad et al. 2007).
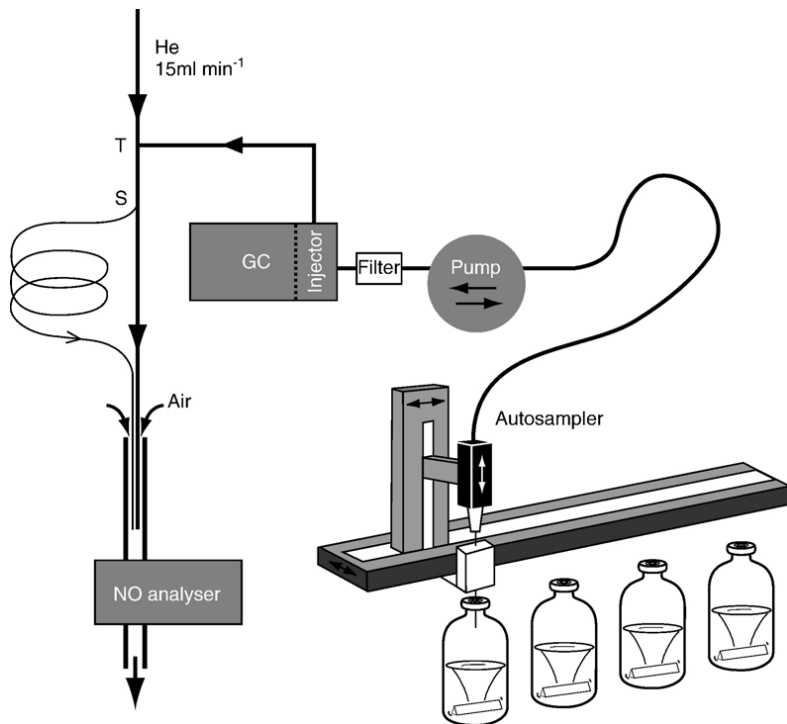
Figure 9: Incubation system for gas measurements of pure cultures, to characterize phenotypic traits (Molstad et al. 2007)

2.3.1. Bacterial strain, medium and incubation conditions

The *Thauera* strain used in these three experiments (two gas kinetic- and nitrite measurements on the robotized incubation system and the estimation of F_{den} using nalidixic acid) was *Thauera linaloolentis* grown on a minimal medium supplemented with 10% complex medium.

The medium and KNO_3^- solution were sterile filtrated, and 50 ml medium supplemented with 2 mM KNO_3^- were added to each autoclaved 120 ml serum bottle already containing a triangular magnetic stirrer (Cowie 25 x 8 mm).The bottles were then sealed tightly with rubber septa and aluminum caps. The air retained in the headspace and medium of each bottle was removed and replaced with helium during six repeated cycles of evacuation and He-filling, by a semi-automated system. During this process the medium was stirred at maximum rotation to ensure adequate gas exchange between the liquid phase and the headspace. The overpressure in each bottle after the He-wash was relieved by using a piston free syringe filled with alcohol, to ensure that no contamination would occur. Before inoculation a volume of

either 0, 1 or 7% of helium was removed from each bottle with a gas syringe and replaced with pure oxygen. The bottles were then put in the robotized incubation system designed to measure gas kinetics in pure batch cultures at a constant temperature of 28°C and stirring (850 rpm).

2.3.2. Preparation of inoculum

The cultures to be used as inocula in these three experiments were raised from frozen stocks and inoculated into minimal medium supplemented with 2.5% complex medium. These cultures were grown overnight with vigorous stirring (950 rpm) to ensure aerobic conditions and also to minimize the chance of aggregation formation, which would cause micro anoxic zones. The overnight inocula were incubated in a water bath at 28°C.

In the second experiment (Gas kinetic- and nitrite measurements on the robotized incubation system) I wanted to ensure that the bacterial cultures to be used as inocula did not carry any remnants of a denitrification proteome. The cell numbers in these cultures were kept low by growing them with only ¼ of the carbon content used in the other experiments. Moreover, the growth of these pre-cultures was followed closely, and as soon as the OD_{600} approached 0.1, a portion of the culture was transferred to fresh medium. This cycle was repeated eight times under aerobic conditions (with vigorous stirring) giving a total of about 40 - 50 generations.

2.3.3. Gas kinetic- and nitrite measurements on the robotized incubation system

Two gas kinetic experiments were performed where different gasses and nitrite were measured before and during denitrification. These two experiments were set up with three different oxygen concentrations (0, 1 and 7%) with two parallels for gas measurements and three for nitrite measurements in each oxygen level in addition to the three standards.

The concentrations of the different gases (NO, N_2O, CO_2, O_2 and N_2) in the headspace of each bottle were normally measured every 2 -3 hours, but in these two experiments it were set in a continuous loop. Samples (1 ml) for nitrite measurement where taken from three of the

replicate flasks at each oxygen level at regular intervals of 30 to 120 minutes, and stored in a -20°C freezer without additives.

2.4. Estimation of F_{den} using Nalidixic acid

2.4.1. Condition of the storage experiment, the use of glutardialdehyde

During early rudimentary gas measurements with nalidixic acid, samples were taken from different bottles for cell counts and for determination of the fraction of cells that switched to denitrification at oxygen depletion (F_{den}). These samples were mixed with glutardialdehyde (1.25%) and stored for a couple of days before microscopy. It was then I encountered the problem, after a couple of days some kind of reaction had happened causing either precipitation or a gel based structure to form, in the samples with added glutardialdehyde. It was therefore decided to do a couple of experiments to see if the problem could be resolved or at least find the reason why this type of reaction happened.

All the bacteria strains of *Thauera* were utilized in different combination in these three experiments except of one bacteria strain, *Thauera sp. 28* which was not used. The bacteria were raised from frozen stocks in 120 ml serum bottles each containing 50 ml of 10% complex medium with a pH of 7,5. They were incubated at 28 °C under oxic conditions and the medium was under constant magnetic stirring (950 rpm) to ensure adequate gas exchange between the liquid and the gas phase, and to prevent aggregation. When the cultures reached late exponential phase a portion (0.3 ml) was withdrawn from each bottles and inoculated to new bottles with fresh 50 ml of minimal medium supplemented with 10% complex medium. These new cultures were incubated under the same conditions. This second set of batch cultures was used in the experiment described below, which was divided into three sub-experiments.

Table 2: Overview of the different experiments (1-3) and treatments, consisting of glutardialdehyde concentrations ranging from (0.32 to 1.25%) and the final medium concentrations from (23.75 to 95.00%)

Bottle nr.	Glutardialdehyde concentration in the medium in %	Preliminary experiment Part 1	Experiment, Part 2		Experiment, Part 3	
		Medium concentration with added glutardialdehyde in %	Medium concentration with added glutardialdehyde in %	Medium concentration with added Nalidixic acid and glutardialdehyde in %	Medium concentration with added glutardialdehyde in %	Medium concentration with added Nalidixic acid and glutardialdehyde in %
1	1,25	95.00	95.00	95.00	-	-
2	1,25	71.25	71.25	71.25	-	-
3	1,25	47.50	47.50	47.50	-	-
4	1,25	-	23.75	23.75	23.75	23.75
5	0,95	-	96.20	96.20	-	-
6	0,95	-	72.15	72.15	-	-
7	0,95	-	48.10	48.10	-	-
8	0,95	-	24.05	24.05	24.05	24.05
9	0,64	97.44	97.44	97.43	-	-
10	0,64	73.08	73.08	73.07	-	-
11	0,64	48.72	48.72	48.72	-	-
12	0,64	-	24.36	24.36	24.36	24.36
13	0,32	-	98.70	98.7	-	-
14	0,32	-	74.03	74.02	-	-
15	0,32	-	49.35	49.35	-	-
16	0,32	-	24.68	24.67	24.68	24.67

To begin with a rudimentary experiment was set up with three series consisting of I) only medium (minimal medium supplemented with 10% complex medium), II) *Thauera sp. 27* and III) *Thauera sp. 63* each contained six bottles were either or both the medium and the conservation additive glutardialdehyde were diluted to assess the magnitude of the problem, shown in the table above. Nalidixic acid was not utilized in this part of the experiment.

Due to inconclusive results in the rudimentary experiment, it was necessary to perform a more extensive experiment. This time seven series consisting of A) Only medium (minimal medium supplemented with 10% complex medium), B) *Thauera sp. 63*, C) *Thauera linaloolentis*, D) *Thauera aminoaromatica*, E) *Thauera sp. 63* with nalidixic acid, F) *Thauera linaloolentis* with nalidixic acid, G) *Thauera aminoaromatica* with nalidixic acid and each series contained sixteen bottles were either or both the medium and glutardialdehyde were diluted, this time with even a higher dilution factor, also with and without the use of nalidixic acid (20 mg^{-1}). OD was measured right before nalidixic acid was added to the three bacteria cultures, and the OD for *Thauera linaloolentis* was measured to 0,049, *Thauera aminoaromatica* to 0,070 and *Thauera sp. 63* to 0,169.

The last part of a series of experiments on the conservation additive glutardialdehyde was done to determine if there were any difference between using sterile filtered milliQ water (V) or 0.1M phosphate buffer (B) to dilute the bacteria cultures, with and without added nalidixic acid (20 mg^{-1}). This last experiment therefore consisted of only four series, a) *Thauera linaloolentis*, b) *Thauera aminoaromatica*, c) *Thauera linaloolentis* with Nalidixic acid, d) *Thauera aminoaromatica* with nalidixic acid and each series contained four bottles were either or both the medium and glutardialdehyde were diluted, Table 2.

2.4.2. Nalidixic acid experiments

Different types of antibiotics are often used to investigate and dissect the metabolic pathways of microorganisms. The antibiotics act either by blocking or inhibiting a specific step, or several steps, in the metabolic pathway, depending on what kind of antibacterial drug is used, and the consequences can then be observed.

In this case I chose nalidixic acid, which inhibits the DNA gyrase. Nalidixic acid at a bactericidal concentration will cause a inhibition of the DNA synthesis but not RNA or protein synthesis, while at even higher concentrations the nalidixic acid becomes bacteriostatic rather than bactericidal which will not only cause inhibition of the DNA synthesis but also the RNA- and protein synthesis (Crumplin & Smith 1975). It is therefore important that this kind of antibiotic is used at the right concentration to get the desired effect, 20mg^{-1} (Kogure et al. 1984) does not appear to affect the cells metabolism other then

preventing the cell from dividing, which means that the cells will become elongated when metabolizing nutrients (Joux & Lebaron 1997). The effect nalidixic acid has on the cells can be view in a microscope after staining the cells with e.g. SYBR Green or acridine orange.

To view the effect nalidixic acid has and also be able to count the number of cells before a possible lysis, they need to be inactivated. The inactivation of cells in a suspension is often done by using either the chemical glutardialdehyde or formaldehyde, which consists of very reactive molecules that inactivate the nucleic acids and proteins in the cells. In these experiments glutardialdehyde was used for this purpose (Willey et al. 2008).

The robotized incubation system was used to determine the moment at which the oxygen depletion curve was at its lowest with the onset of NO accumulation. This time point, which was regarded as onset of anaerobic respiration, was selected to add the nalidixic acid. This experiment was set up with three different oxygen concentrations (0, 1 and 7%) with four parallels in each (A - D) and with three standards for calibration and to monitor gas leakages. The concentrations of the different gases (NO, N_2O, CO_2, O_2 and N_2) in the headspace of each bottle were normally measured every 2-3 hours, but in this experiment it was measured more frequently, due to rapid growth and reduction of the gaseous intermediates, to ensure that the peaks of the NO and N_2O curves were captured.

A sample for cellular counts was taken from every bottle right before 20 mg^{-1} nalidixic acid was added to the cultures. Additional samples were taken out after 3 and 8 hours of incubation with nalidixic acid. The samples for cellular counts were stored on 1.25% glutardialdehyde, either at room temperature or at 4°C.

3. Results

The main purpose of this thesis was to determine how big a fraction of a population of the denitrifying bacterium, *Thauera linaloolentis* that managed the transition from oxic to anoxic respiration, when incubated under defined conditions where an initial amount of oxygen was depleted. This was achieved through two different methodological approaches; estimation of electron transport rates based on gas kinetic measurements on a robotized incubation system, and the use of nalidixic acid for quantification of growing cells by microscopic counts.

Initial, preparatory work included the choice of bacterial strain and appropriate growth medium, as well as determination of oxic and anoxic growth rates for different *Thauera* strains. Problems associated with the use of glutardialdehyde as storage preservative were also addressed. The results from these initial studies are presented in the first sub-chapters below.

3.1. Minimal medium supplemented with 10% complex medium

We knew from an earlier study (Mao et al., manuscript in prep.), that all *Thauera* strains used in the present investigation can be cultured in a minimal medium supplemented with 2.5 or 10% complex medium. Based on this, I chose minimal medium supplemented with 10% complex medium for the following experiments and stock cultures, if nothing else is specified.

3.1.1. Growth rates

The oxic growth rate of the 5 different *Thauera* strains varies between 0.166-0.345, where *Thauera aminoaromatica* had the lowest while *Thauera strain 63* had the highest growth rate closely followed by *Thauera strain 28*. *Thauera linaloolentis* were grown on either or both minimal medium supplemented with 2.5 and 10% complex medium, which had no significant effect on the growth rate, Table 3.

Table 3: An overview of the oxic growth rates estimated from optical density measurements at the time of linear increase of the different *Thauera* strains grown in minimal medium supplemented with either 2.5 or 10% complex medium.

Growth rates	Minimal medium supplemented with X% complex medium	Optical density ($_{600nm}$) measurements
Thauera linaloolentis		0,232
Thauera aminoaromatica		0,166
Thauera strain 27	2.5 %	0,241
Thauera strain 28		0,326
Thauera strain 63		0,345
Thauera linaloolentis	10 %	0,229

3.1.2. ERIC-PCR

ERIC-PCR is a quick, cheap and easy method to assess if a culture is pure or contaminated, but this is not an absolute or exact method. There will always be some day-to-day variation between different PCR runs, where some bands will appear weaker-, or stronger, or completely disappear. Picture A and B show the PCR products of the different *Thauera* strains after preparation of stock cultures, while picture C shows the PCR product of *Thauera linaloolentis* after an experiment, all the bands are located at the same place with minute variation in intensity of the bands, indicating pure cultures.
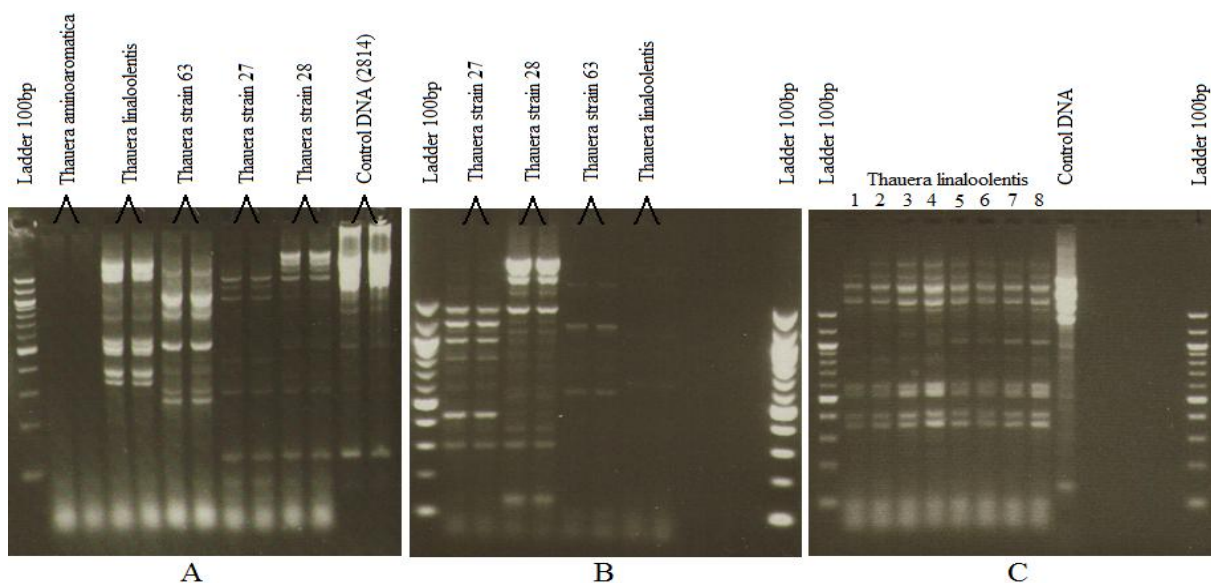

Figure 10: ERIC-PCR was performed after each time a new batch of stock cultures were made and after certain experiments to control that the pure cultures in fact was pure and not contaminated, with a 100 bp ladder for comparison.

3.2. Testing of different media

Now we were interested in finding a well defined medium where the amount of carbon and nitrogen were known and could be controlled, before a comparative phenotypic analysis of *Thauera* were performed. The other mediums tested were Sistrom's and medium 586 recommended by DSMZ.

3.2.1. Sistrom's medium

Aerobic growth on Sistrom's medium, which is a defined medium with only one carbon source (succinate), was tested for two of the strains, *Thauera aminoaromatica* and *Thauera sp. DNT-1*. The bacteria were inoculated from frozen stock directly into separate bottles containing 50 ml Sistrom's medium. The optical density (600 nm) of these two cultures was measured after one week of incubation, but no significant increase in the optical density could be observed in either of the cultures.

It may be because the strains had difficulties in adjusting after being frozen -80 °C or are in fact unable to utilize succinate as carbon source, which is more defined than the minimal medium supplemented with 10 % complex medium, consisting of meat extract (0.600g) and peptone (1.0g), the later being the most likely explanation.

A second experiment was performed where frozen stocks of both strains were first cultivated overnight in minimal medium supplemented with 10% complex medium. Growth in this pre-culture reached late exponential growth before being re-inoculated into separate bottle containing 50 ml Sistrom's medium. The optical density (600 nm) of these two cultures were again measured after one week, but still no significant increase in optical density could be observed, which would indicate that these *Thauera* strains are unable to utilize succinate, which is the sole carbon source in Sistrom's medium

3.2.2. Medium 586 (*Thauera aminoaromatica*)

The second medium tested was recommended by DSMZ (Deutsche Samlung für Mikrroorganismen).

Table 4: List of *Thauera* strain tested on medium 586 and optical density (600nm) measured after seven and fourteen days of incubation.

Optical density measurements 600nm		
Growth days	7 days	14 days
Thauera aminoaromatica (TAA)	0.112	0.213
Thauera strain 63 (T63)	0.004	0.007
Thauera strain 28 (T28)	0.007	0.009
Thauera strain 27 (T27)	0.003	0.008
Thauera linaloolentis (T. lina)	0.011	0.017

All the strains listed in Table 4 were tested on medium 586, which is also defined medium with only one carbon source (Na-Benzoate). Cells were inoculated from frozen stock directly into bottles containing 50 ml of medium 586, and the optical density (600 nm) was measured after one week and two weeks of incubation. No significant increase in optical density in any of the cultures was detected, except for *Thauera aminoaromatica*. Even though *T. aminoaromatica* was able to grow on this medium, it had a low growth rate, as indicated by the optical density measurement of only 0.213 after two weeks of incubation.

Therefore in the second experiment stock cultures of *Thauera aminoaromatica*, *Thauera linaloolentis* and *Thauera sp. 63* were transferred to five separate bottles each containing both 50 ml of medium 586 and of 5 different yeast concentrations ranging from 12.4 mg^{-1} to 349.9 mg^{-1} to see if this would boost growth. The optical density (600 nm) of these cultures was measured after one week of cultivation, but only a minute increase in the OD values was observed for the two strains *Thauera strain 63* and *Thauera linaloolentis*. The only exception was one bottle containing *Thauera linaloolentis* with 12.4 mg^{-1} of yeast which had increased significantly in optical density, Table 5. This was not due to growth of *Thauera linaloolentis,* but rather a contamination of the culture since none of the other cultures containing higher concentration of yeast had grown this significantly and the color of this culture was golden yellow and not a milky white, the latter being typical for the *Thauera* strains.

After one week 100 μl of the cultures containing medium 586 with yeast were transferred to fresh medium (50 ml in medical flasks) without yeast. This ensured that the yeast from the previous medium was strongly diluted so it would not boost growth in these new bottles. The optical density was measured after one week. No growth could be observed in either of the cultures containing *Thauera strain 63* or *Thauera linaloolentis*, only *Thauera aminoaromatica* appeared to have the metabolic capacity to utilize this single carbon source and grow, although slowly, Table 5.

Table 5: Overview of two experiments, where oxic growth of three of the *Thauera* strains was tested in medium 586, with and without yeast. The *Thauera* strains were first grown in medium 586 with different yeast concentrations to boost growth after being taken from the -80°C freezer and incubated for one week, the optical density (600nm) was then measured, and a portion of the culture was re-inoculated into fresh medium 586 without yeast. The optical density was measured again after another week of incubation.

Yeast concentration in medium	With yeast			Without yeast		
	T63	*T. lina*	*TAA*	*T63*	*T. lina*	*TAA*
12.4 mg⁻l	-0.013	0.276	0.101	-	0.028	0.069
49.2 mg⁻l	0.003	0.005	0.181	-	-	0.181
121.3 mg⁻l	0.021	0.021	0.276	-	-	-
236.8 mg⁻l	0.035	0.039	0.318	0.000	-	-
349.9 mg⁻l	0.046	0.055	0.539	0.000	0.002	-

(-) Optical density measurements not performed.

3.3. Gas kinetic experiments

Two separate gas kinetic experiments were performed on the robotized incubation system; both the gas kinetic measurements and e-flow estimations showed a unified picture of *Thauera linaloolentis* ability to perform a balanced transition from oxic to anoxic respiration in each of the initial oxygen concentrations, with good reproducibility between parallels and experiments. Figure 11 and 13 (close-ups are exhibited in the appendix) shows the results of the two gas kinetic experiments, with close-ups of the time frame during oxygen depletion for 1 and 7% initial oxygen, Figure 12 and 14. The gas kinetics is displayed on the left side, while the e-flow estimations are displayed on the right side in both of Figures 11 and 13.
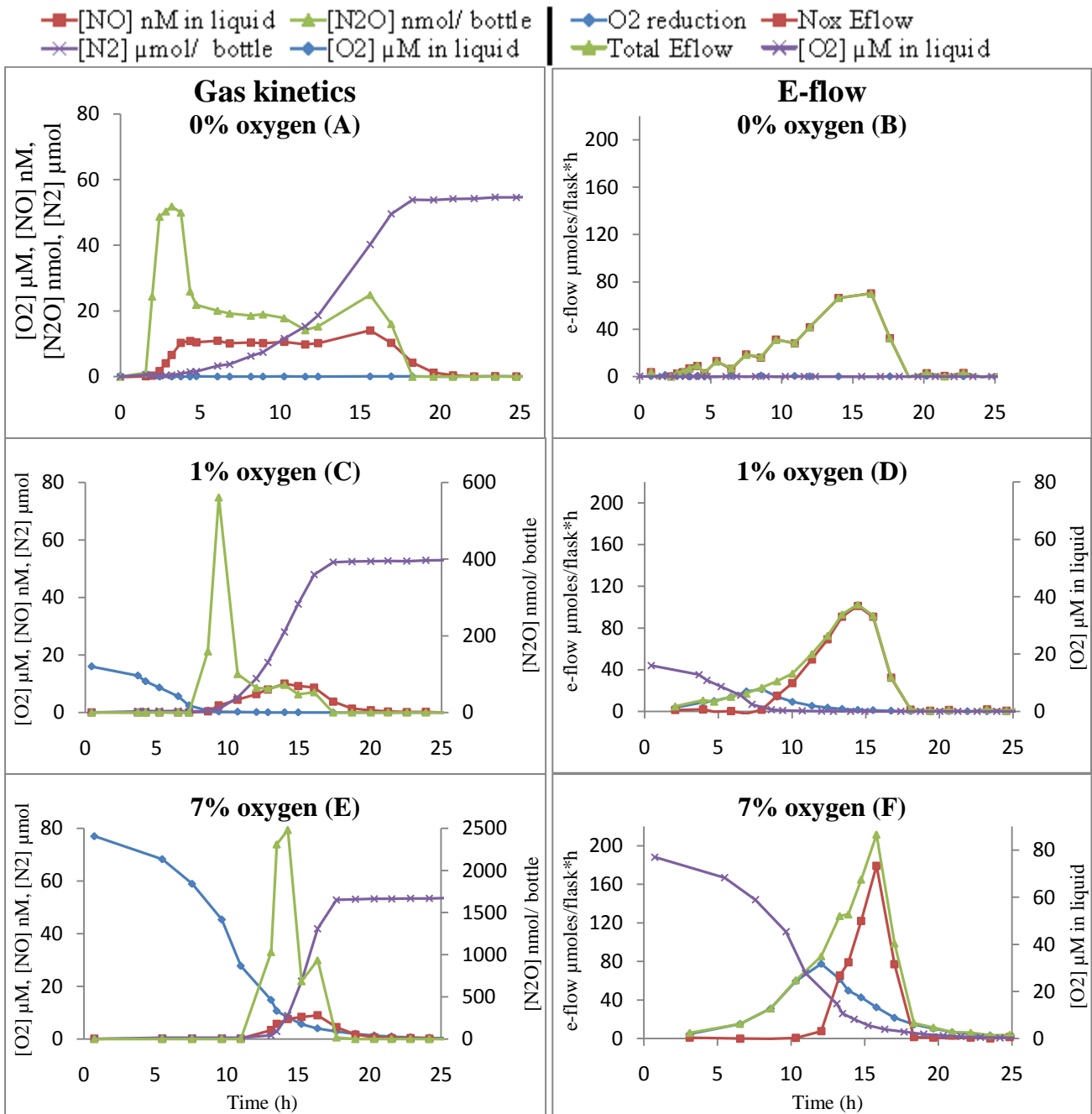
Figure 11: Overview of the gas levels and kinetics during oxygen depletion, denitrification (pictures A, C and E) of *Thauera linaloolentis* and also of the electron transport rates under the initial oxygen concentrations 0, 1 and 7% (B, D and F). Each initial oxygen concentrations consisted of the measurements of four gases; [NO] nM in liquid, [N2O] nmol/ bottle, [N2] μmol/ bottle and [O2] μM in liquid. The e-flow was estimated by monitoring all the available electron acceptors before and during the denitrification process.

The *Thauera linaloolentis* strain was cultivated in minimal medium supplemented with 10% complex medium and 2mM nitrate (KNO_3^-) with the initial oxygen concentrations of 0, 1 and 7%. Normally is NO expected to increase before N_2O but in 0% initial oxygen it appeared to

increase almost at the same time. A closer look of the NO data revealed that NO increased in the pM concentration a while before N_2O started to increase. NO accumulated to about 10 nM and stayed at this concentration for about 13 hours. N_2O increased rapidly in a short time span, with a peak at 50nmol pr bottle before stabilizing on a concentration of about 20 nmol for 10hours, with a secondary peak at 15 hours into the experiment. Molecular nitrogen increased gradually, after initial N_2O was detection, through the whole denitrification process and ended at a concentration of about 50 µmol after 16-18 hours, when all nitrate was converted.

The oxygen was depleted in 1% initial oxygen after 8-9 hours of incubation, while NO started to increase after 7 hours of incubation in the pM concentration, this time in the presence of 0.61-2.3µM oxygen. NO accumulated to about 10 nM and stayed at this concentration for about 12 hours. N_2O increased rapidly in a short time span, with a peak at 560 nmol pr bottle before stabilizing at a concentration of about 50-60 nmol for 4 hours. Molecular nitrogen increased rapidly, after initial N_2O detection, through the whole denitrification process and ended at a concentration of about 50 µmol after 17-18 hours.

The oxygen was depleted in 7% initial oxygen after 22-23 hours of incubation. NO increased after10-11 hours of incubation in the pM concentration and this time in the presence of even higher oxygen concentration (30 µM). NO accumulates to about 10nM and stayed at this concentration for about 11 hours. N_2O increases rapidly in a very short time span, with a peak at 2310 nmol pr bottle. N_2O concentration was measured for duration of 7 hours. Molecular nitrogen started to increases at an oxygen concentration of about 20µM and increased even more rapidly after initial N2O was detected through the whole denitrification process and ended at a concentration of about 50 µmol after 17-18 hours.

The denitrification process ended when all added nitrate (2mM) was reduced to N_2, which happens within 17, 10 and 6.5 hours for 0, 1 and 7% initial oxygen respectively. The end concentration of N_2 was almost identical in all the treatments for both experiments (about 50µmol) which indicate that the amount of nitrate added was the same in all the treatments.

The e-flow results from both the experiments showed the same patterns in all three initial oxygen concentrations. The e-flow estimations from Figure 11 and 13 displays only a small reduction of F_{den} with increasing initial oxygen concentration in the electron transport during

the transition to anoxic respiration, indicating that a large fraction of population were able to produce a denitrification proteome and initiate denitrification. This was confirmed by the results obtained from the nalidixic acid experiment, Table 8, which also showed that F_{den} decreased with increasing initial oxygen concentration.

A phenomenon seen in both experiment, but more clearly in the second experiment, Figure 13, is that the total electron transport rate at the transition to anoxic respiration of initial 7% oxygen stalls a moment before resuming. Higher cell density would cause a more rapid and sudden depletion of oxygen, which might be the reason for this phenomena.
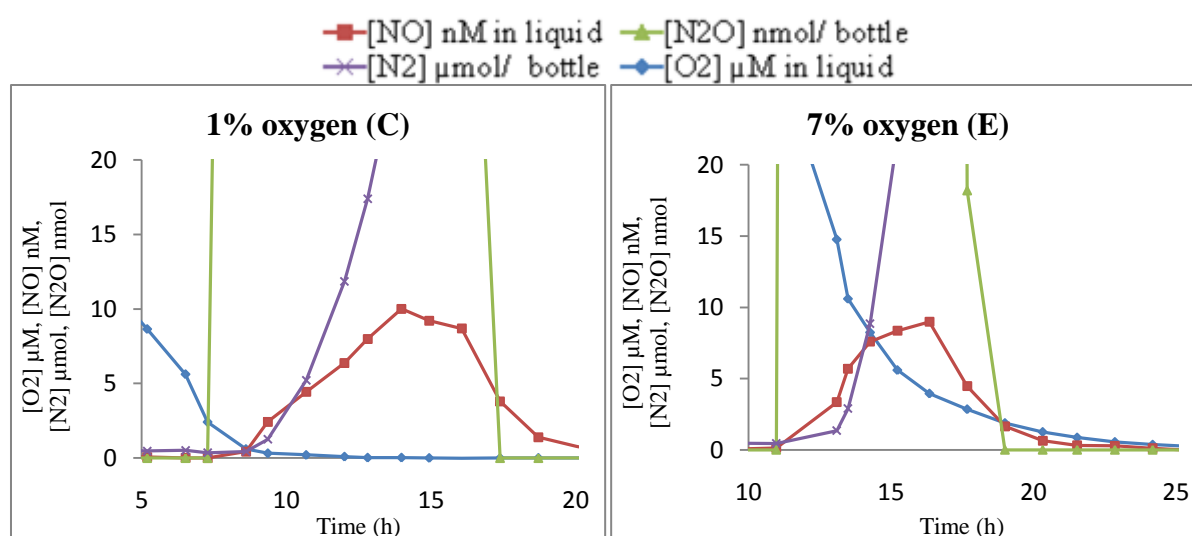
Figure 12: Close-up of pictures C and E from figure 11, to get a better and clearer view of the transition from oxic to anoxic respiration during oxygen depletion and onset of denitrification by the increase of NO in the pM range.

We started to speculate that the bacteria might harbor some old denitrification proteome which could explain why the bacteria managed the transition so well under rapid oxygen depletion in the first gas kinetic experiment. That is the reason why a secondary gas kinetic experiment was performed with a small alteration compared to the first experiment which was to let the inoculum grow over several generations (40-50) in minimal medium supplemented with just 2.5% complex medium and never letting the inoculum reach an optical density of more than 0.1.
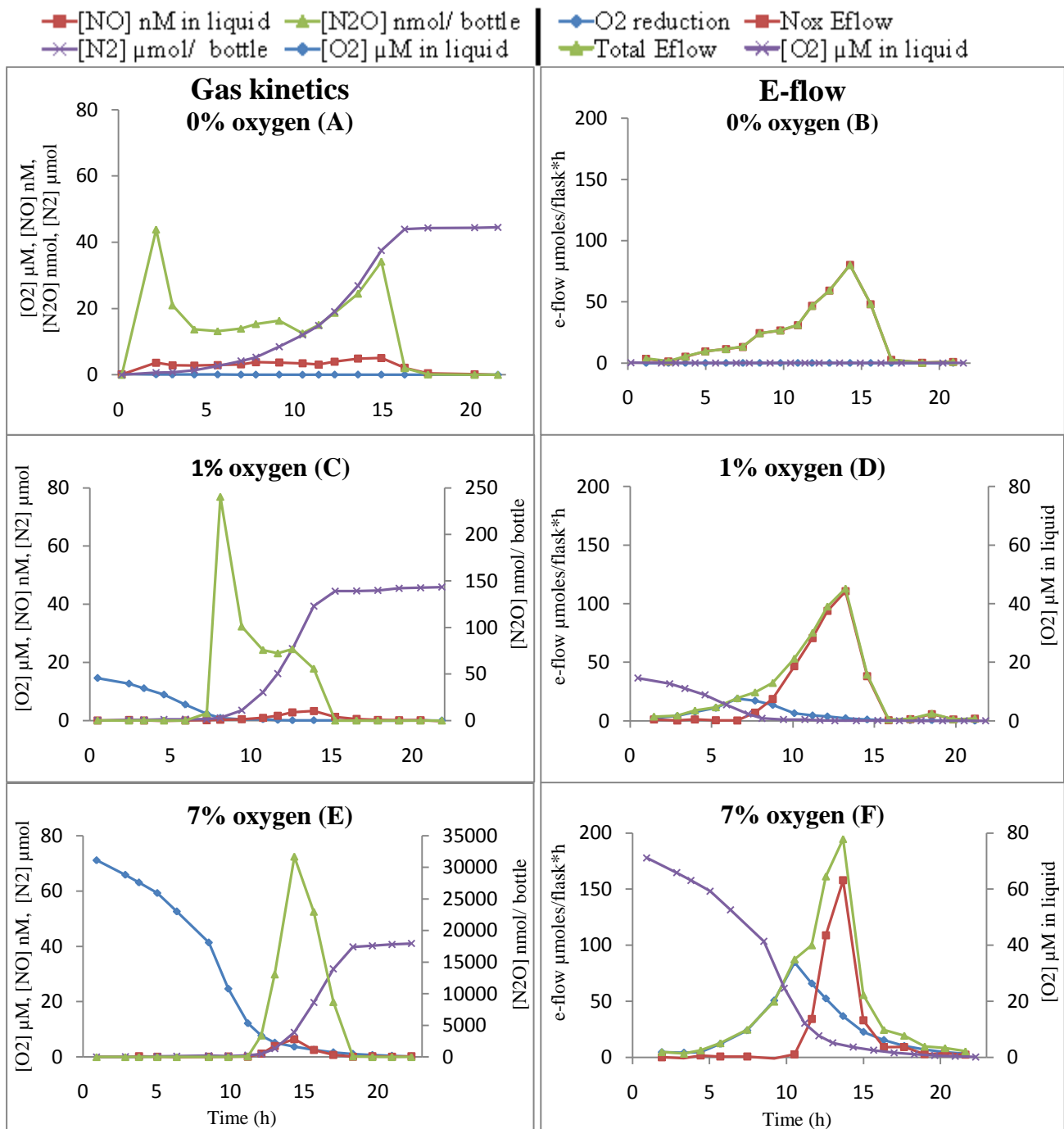
Figure 13: The second gas kinetic experiment, a duplicate of the experiment shown in Figure 11, except that the inoculum for this experiment were grown over several generations (40 – 50 generations) with the optical density (600 nm) of the cultures never going over 0.100. This was done to ensure that the cultures were well aerated and that if a proteome existed it would be heavily diluted.

The results of this second attempt were more or less identical to the results achieved in the first experiment with variations in time and concentration levels of the gases detected in the batch cultures. An interesting phenomenon was detected in both of the gas kinetic

experiments for initial oxygen concentrations 1 and 7%. It seems that the denitrification process was initiated before all the oxygen was completely depleted, more visible in 7% than 1%, Figure 12 and 14. The biggest difference between the two experiments was that the initiation of denitrification in 1 and 7% initial oxygen happened at lower oxygen concentrations in the second experiment and it was also in this second experiment the lowest concentration of NO was measured. N_2O varied considerable more than NO both between the parallels for the different treatments and also between the two experiments, summarized in Table 6.
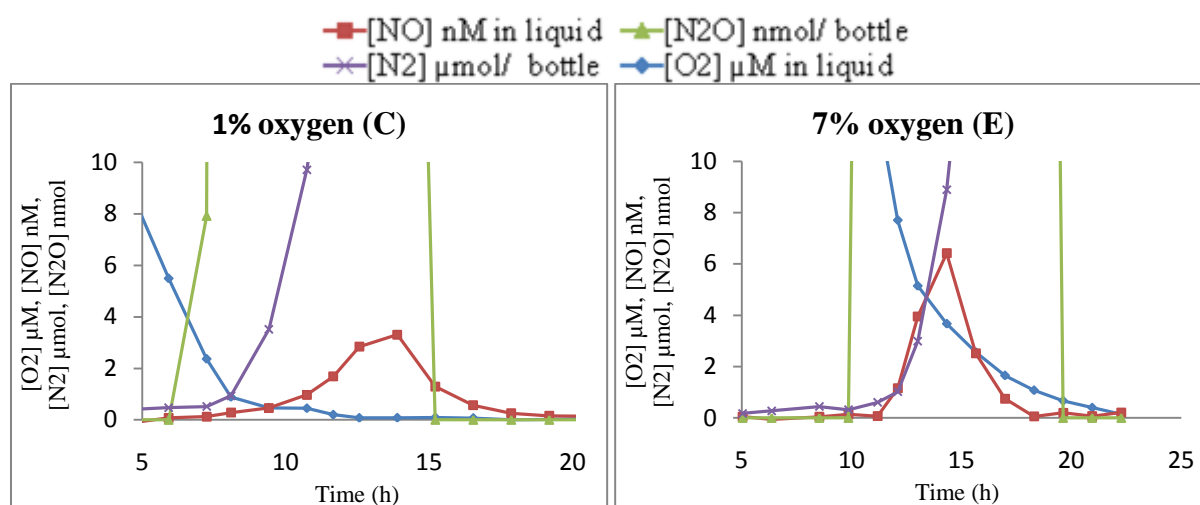
Figure 14: Close-up of pictures C and E from Figure 13, to get a better and clearer view of the transition oxic to anoxic respiration during oxygen depletion to onset of denitrification, defined by the start of NO accumulation.

The highlights in Table 6 shows that the oxygen concentration increases at first detection of NO and at first detection N_2O of the initial oxygen concentration 0 to 7%, from 0.03 to 27.2 µM and 0.03 to 27.7 µM respectively, while the duration of NO in hours decreases with increased initial oxygen concentration.

Table 6: Summarizes the two gas kinetic experiments performed on *Thauera linaloolentis* which are displayed in Figures 11-14. The medium which *Thauera linaloolentis* was grown in for these experiments was a minimal medium supplemented with 10% complex medium with a pH of 7.50 and a KNO_3^- concentration of 2mM, with different initial oxygen concentrations in headspace (0, 1 and 7%). The variables displayed for both experiments are the oxygen concentration at first detection of NO and N_2O, and also maximum concentrations of NO, N_2O and NO_2^-. The duration of detectable NO concentrations in hours were also estimated.

	Experiment 4						Experiment 5					
Initial O2 (vol. %)	0		1		7		0		1		7	
Parallel	1	2	1	2	1	2	1	2	1	2	1	2
μM O_2 at first detection of NO	0.03	0.06	1.4	0.61	27.2	26.5	0.22	0.24	0.89	1.43	4.67	12.2
$[NO]_{max}$ nM in liquid	14.1	14.8	8.8	10.0	9.0	9.5	5.5	5.1	3.3	1.4	2.4	6.4
Duration of [NO] nM in hours	18.8	19.2	12.7	12.6	11.9	9.4	18.7	20.0	9.8	9.7	7.1	7.1
μM O_2 at first detection of N2O	0.03	0.06	2.3	2.4	27.7	26.5	0.22	0.24	5.49	7.74	22.0	22.0
$[N_2O]_{max}$ μmol/ bottle	0.05	0.05	0.37	0.56	2.5	9.8	0.04	0.04	0.24	0.05	0.10	31.7
$[NO_2^-]_{max}$ μM/ bottle	0.06	0.06	0.02	0.02	0.55	0.54	0.04	0.04	0.06	0.05	0.05	0.92

The production and pseudo-fermentation of PHB could explain why *Thauera linaloolentis* had the ability to manage a balanced transition from oxic to anoxic respiration under oxygen depletion, displayed in the e-flow graphs in Figure 11 and 13. A PHB test was therefore performed to assess if *Thauera linaloolentis* cells produced PHB by staining the cells with Nile red and controlled in a fluorescence microscope for PHB granules. The test revealed that *Thauera linaloolentis* did not produce any PHB.

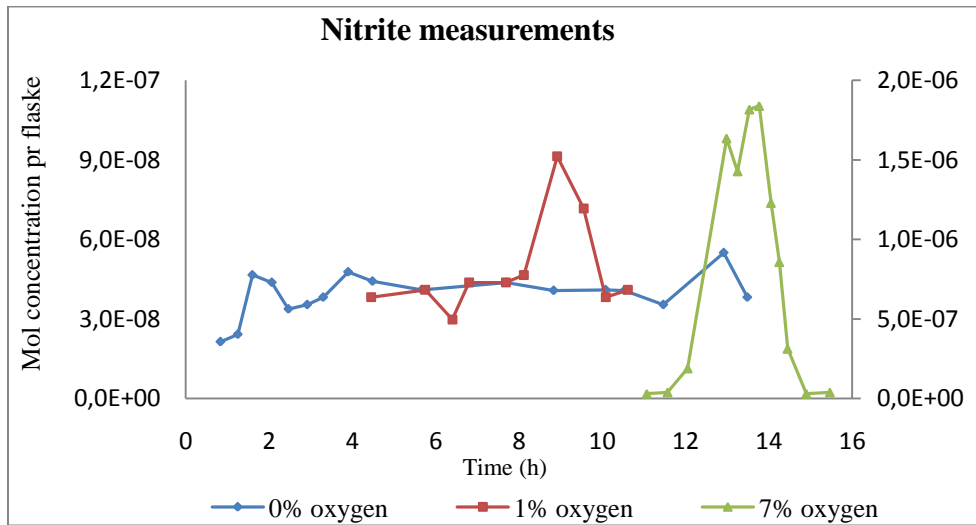### 3.3.1. Nitrite from wet chemistry measurements

Figure 15: A overview of nitrite levels for the 3 initial oxygen concentrations 0, 1 and 7% during denitrification by *Thauera linaloolentis* in molar concentration pr flask.

The observed nitrite values in all the treatments included in the experiment are very low, however we see an increasing amount of nitrite being produced the higher the initial oxygen concentration. In 0% oxygen concentration the first levels of nitrite can sees within 1 hour of experimental start, and lasts for the duration of 12.5 hours. In samples with 1and 7 % oxygen concentration the first levels of nitrite can sees after 4.5 and 11 hours of experimental start, and lasts for the duration of 6 and 4.5 hours, with a concentration peak of 91.3 and 1840 nM pr flask after 8.9 and 13.8 hours, respectively. *Thauera linaloolentis* grown in 7% oxygen concentration produces about 20 times more nitrite compared to 1% oxygen.

3.3.2. Growth rates

Growth rates are normally a bit higher for aerobe then anaerobe respiration, because the energy yield is lower in anaerobic metabolism. Growth rates calculated from e-flow of O_2 and NOx, gave slightly higher values than when growth rates were calculated from total e-flow. Suggesting that aerobe growth rates are slightly underestimated while anaerobe growth rates are overestimated not so much for 0% oxygen but clearly for 1 and 7% oxygen, Figure 16.

Figure 16: Overview of oxic and anoxic growth rates for *Thauera linaloolentis* when incubated at three initial oxygen levels; 0, 1 and 7%. Growth rates during oxic and anoxic phase are estimated from regression analyses from O_2 and NOx, respectively, and also of total e-flow at the time of linear increase in the electron transport rates. A more detailed list of growth rates for both the gas kinetic experiments is presented in Table 7.

Cultures of Thauera linaloolentis grown under strictly oxic condition had a growth rate that ranged between 0.229-0.232, Table 3, while cultures grown under initial oxygen concentrations of 1 and 7% showed elevated growth rate, Table 7.
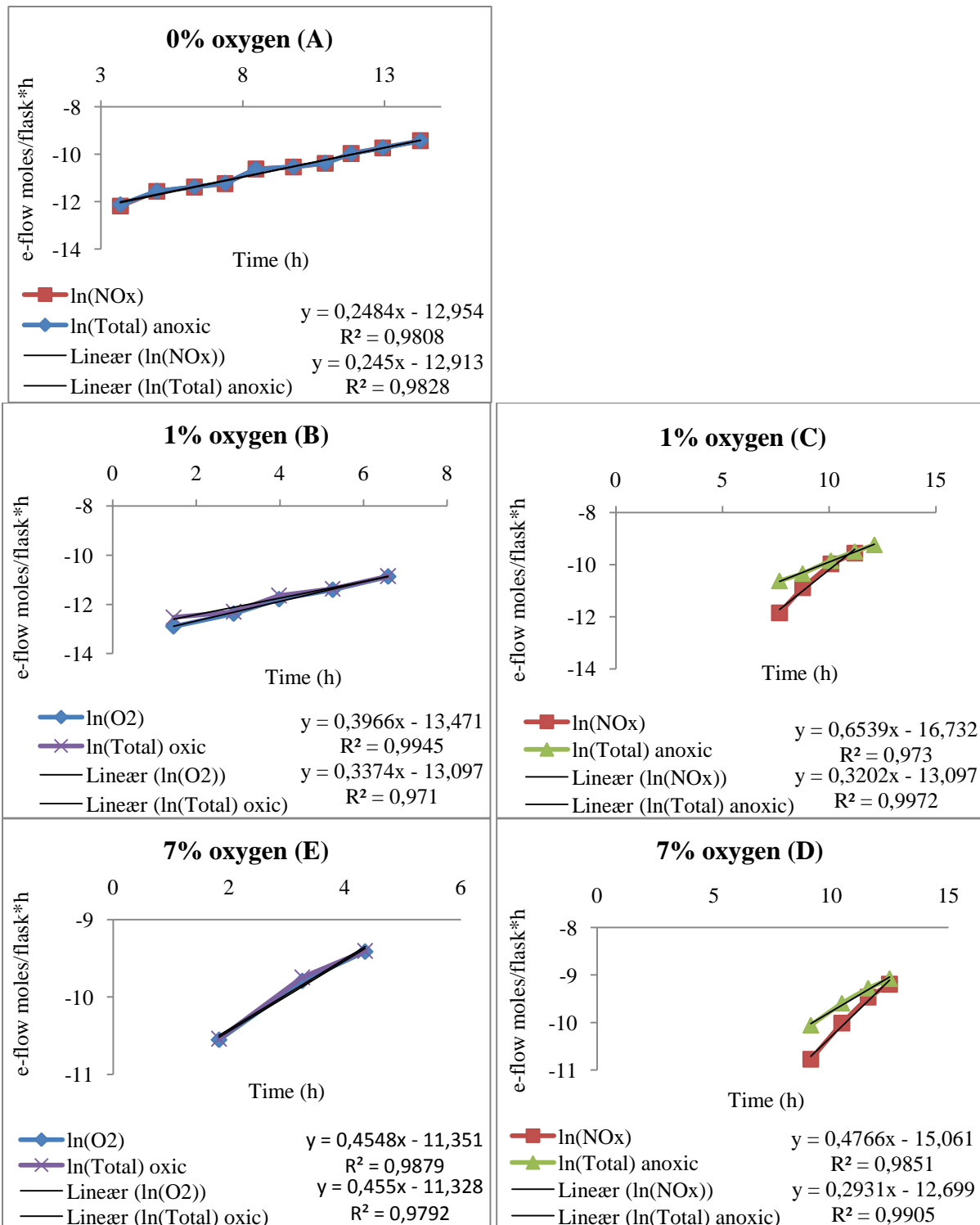
Table 7: Oxic and anoxic growth rates, -for *Thauera linaloolentis* when incubated at three initial oxygen levels; 0, 1 and 7%. The values presented were determined based on the gas emission values from two separate experiments; Exp. 4 and Exp. 5. Growth rates during oxic and anoxic phase were estimated from regression analyses from O_2 and NOx, respectively, and also of total e-flow at the time of linear increase in the electron transport rates.

	Initial O_2 vol. %	NO_3^- mM	Ln (O_2)		Ln (Total E-flow)		Ln (NO_x)		Ln (Total E-flow)	
			Oxic				Anoxic			
Parallel			1	2	1	2	1	2	1	2
Exp. 4	0		-	-	-	-	**0.207**	0.209	**0.206**	0.210
Exp. 4	1		**0.295**	0.272	**0.301**	0.264	**0.378**	0.292	**0.276**	0.270
Exp. 4	7	2	**0.320**	0.534	**0.310**	0.544	**0.415**	0.527	**0.260**	0.275
Exp. 5	0		**-**	-	**-**	-	**0.212**	0.248	**0.207**	0.245
Exp. 5	1		**0.397**	0.433	**0.337**	0.395	**0.654**	0.337	**0.320**	0.249
Exp. 5	7		**0.455**	0.408	**0.455**	0.449	**0.477**	1.829	**0.293**	0.275

The calculation of oxic growth rate by the use of ln(O_2) have a tendency to be a fraction higher then when using ln(total e-flow), but the difference is quite small. The difference becomes more apparent and a lot bigger, during the calculation of anoxic growth rate, Table 7.

3.4. Estimation of F_{den} using Nalidixic acid

A preliminary experiment was first performed where growth of *T. linaloolentis* under aerobic conditions was measured after addition of three different concentrations (10, 20 and 30 mg [-1]) of nalidixic acid. The result showed no clear differences in cellular counts of the three different treatments, and therefore it was decided to use the concentration 20 mg [-1] recommended by Joux and Lebaron (Joux & Lebaron 1997).
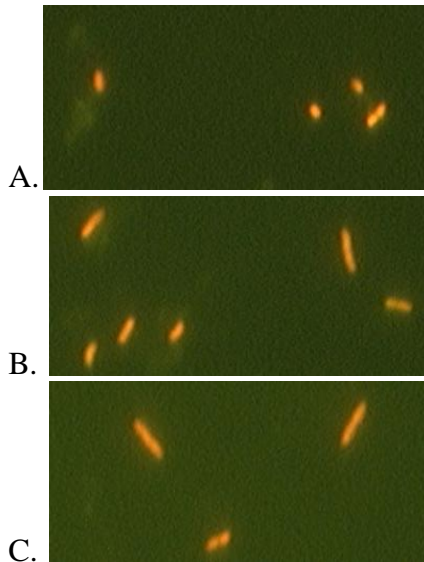
Figure 17: Pictures of the *Thauera linaloolentis* stained with acridine orange, either treated with or without nalidixic acid. Picture A) shows *Thauera linaloolentis* grown under oxic conditions without being treated with nalidixic acid. Pictures B) and C) on the other hand shows *Thauera linaloolentis* grown under oxic and anoxic conditions respectively, treated with nalidixic acid and cultivated for 8 hours before being stained. (All three pictures are of same magnification and size)

The *Thauera linaloolentis* cells that have the ability to produce the denitrification proteome under anoxic condition, after the initial oxygen concentration of 0, 1 and 7% in headspace are depleted will become elongated after 8 hours of incubation with nalidixic acid. Since the cells were in different stages of the transcription phase when nalidixic acid was added to the pure culture, the cells will become elongated with different lengths. The cells became visible elongated for the most parts compared to cells that were not treated with nalidixic acid, clearly shown in pictures A - C, in Figure 20.

To verify the results of the e-flow estimations obtained from the two gas kinetics experiments (exp. 4 and 5) on the robotized incubation system another experiment was performed where nalidixic acid was utilized for detection and quantification of growing cells. The two gas kinetic experiments performed with initial oxygen concentration of 0, 1 and 7% and the single nalidixic acid experiment gave some interesting and matching results in regard to the cell F_{den} fraction. It showed that almost all the cells managed a balanced transition from oxic to anoxic respiration.

The Nalidixic acid experiment was performed on the robotized incubation system to pin-point the moment anaerobic denitrification started, when oxygen neared depletion simultaneously with the onset of NO, Figure 21.
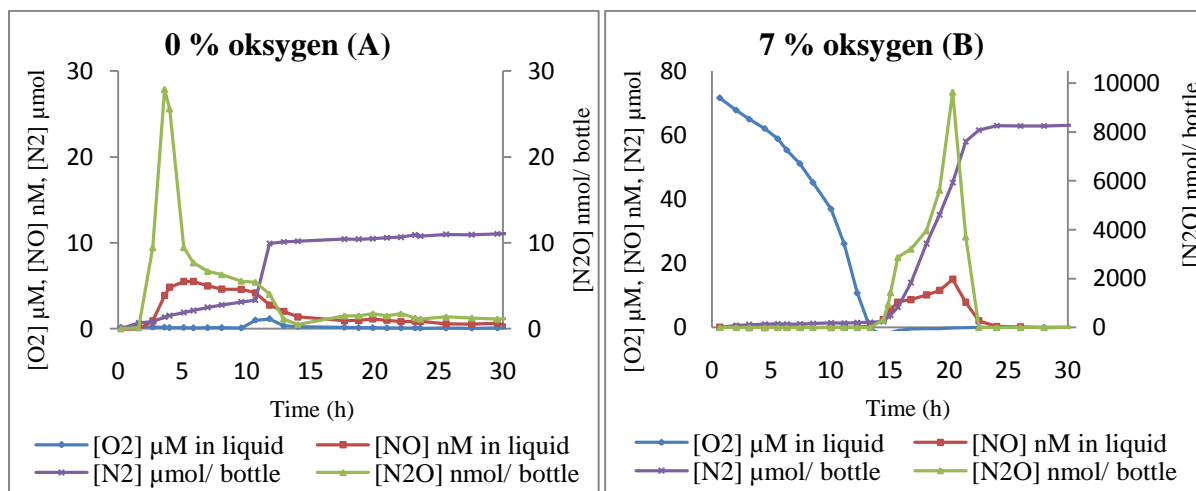

Figure 18: Gas kinetics of *Thauera linaloolentis* during denitrification. Only the oxygen levels 0 and 7% are shown. Nalidixic acid (20 mg $^{-l}$) was added at different time points for each initial oxygen concentration, when the oxygen concentrations approached depletion and at the first sign of NO accumulation. Nalidixic acid was in this experiment added after 2.5, 9.1 and 14.8 hours after inoculation, depending on initial oxygen concentrations, in the bottles.

Samples were taken before nalidixic acid was added and after 8 hours of incubation for the initial oxygen concentration of 0, 1 and 7%. The cells were stored on glutardialdehyde before being stained with acridine orange and counted in a fluorescence microscope. The results are summarized in Table 8.

Table 8: Shows the fraction (F_{den}) of the *Thauera linaloolentis* population that managed to produce the denitrification proteome under anoxic conditions after the initial oxygen concentration of 0, 1 and 7% in the headspace are depleted with 2 mM NO_3^- added to the medium.

2 mM NO_3^- added	Initial O$_2$ vol. %			
	Preliminary test			
	0	**0**	**1**	**7**
Cells that managed the switch	90,8	84,2	77,5	73,7
Cells that did not managed the switch	9,2	15,8	22,5	26,3

A comparison of the three experiments with and without the use of nalidixic acid gave concurring results. The e-flow graphs in figures 11 and 13 shows that both experiments have a relatively high F_{den} which can also sees in Nalidixic acid experiment shown in table 8.

3.1.1. Optimization of storage conditions; the use of glutardialdehyde

A rudimentary experiment was set up, due to some unexpected problems in earlier gas kinetic experiments on the robotized incubation system with the use of Nalidixic acid. The first experiment consisted of the following 3 series; I) just medium, II) *Thauera sp. 27* and III) *Thauera sp. 63* each containing 6 bottles were either or both the medium and the conservation additive glutardialdehyde are diluted to assess the magnitude of the problem, shown in Table 2. Nalidixic acid was not utilized in this part of the experiment. A visual assessment of the ependorf tubes were done, which showed that some kind of reaction had occurred, from light to heavy precipitation which can sees in all the tubes, Figure 17.
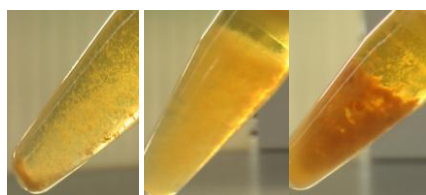

Figure 19: Medium (minimal medium supplemented with 10% complex medium) and pure cultures of *Thauera sp. 27* and *Thauera sp. 63* stored on 1.25% glutardialdehyde.

To assess the problem future a second experiment was decided to be necessary. This time seven series consisting of A) Only medium (minimal medium supplemented with 10% complex medium), B) *Thauera sp. 63*, C) *Thauera linaloolentis*, D) *Thauera aminoaromatica*, E) *Thauera sp. 63* with Nalidixic acid, F) *Thauera linaloolentis* with Nalidixic acid, G) *Thauera aminoaromatica* with Nalidixic acid each containing 16 bottles were either or both the medium and glutardialdehyde are diluted. This time with even higher dilution factors of both the medium and glutardialdehyde, shown in Table 2. Again a visual assessment of the ependorf tubes was done, which showed that some kind of reaction had occurred, from light to heavy precipitation which can sees in all the ependorf tubes except for the tubes 4, 8, 12 and 16 in all series (A-G) which had none to minimal precipitation, Figure 18. These tubes

contained 23.7 – 24.7% pure cultures with decreasing amount of glutardialdehyde from 1.25 to 0.32 %, shown in table 2.

Figure 20: The three first pictures shows pure cultures of *Thauera linaloolentis*, *Thauera sp. 63* and *Thauera aminoaromatica* stored on 1.25% glutardialdehyde. The four last pictures shows the cultures diluted down to 23.7-24.7% of original strength with varied glutardialdehyde concentrations (0.32 to 1.25%) added.

Prepared some filters for microscopic assessment after a couple of days of storage and once again after a week, could only see minimal interference from the precipitation and slime formation, especially when Nalidixic acid was added (data not shown). But on the other hand only few cells could be seen in the microscope due to the high dilution factor. The optical density for these cultures where between 0,049 and 0,169.

The last experiment on the conservation additive glutardialdehyde were to determine if there was any difference between using sterile filtrated milliQ water (V) or 0.1M phosphate buffer (B) to dilute the bacteria cultures, with and without added Nalidixic acid. This last experiment therefore consists of the following 4 series; a) *Thauera linaloolentis,* b) *Thauera aminoaromatica*, c) *Thauera linaloolentis* with Nalidixic acid, d) *Thauera aminoaromatica* with nalidixic acid. Proceeded only with the treatments that diluted the pure cultures the most (down to 23.7%), which consist of the following tubes 4, 8, 12 and 16, due to heavy precipitation in the three least diluted pure cultures, shown in Table 2. A visual inspection of the tubes that are diluted with 0.1 M phosphate buffer (B) shows increased precipitation compared with the samples diluted with sterile filtered milliQ water (V), Figure 19.

Figure 21: *Thauera linaloolentis* grown in minimal medium supplemented with 10% complex medium which are diluted with either water (V) or 0.1M phosphate buffer (B) down to 23.7% of original strength.

4. Discussion

Denitrification is a taxonomically widespread process used by more than 60 different genera of bacteria including archaea and some fungi. The survival and fitness of an organism located in an environment that experiences periodic anoxic flux is largely dependent on the organisms ability to regulate the transcription of the various genes involved in denitrification, which consists of a enzymatic network of four enzymes, nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase responsible for the reduction of nitrate to dinitrogen gas. There are no known bacteria that contain both of the genes NirS and NirK and the Thauera strains are no exception, all the known strains of Thauera consists of only the NirS nitrite reductase gene (Etchebehere & Tiedje 2005; Macy et al. 1993; Rodionov et al. 2005). Essentially, we have little understanding of how the regulation of denitrification occurs in *Thauera*, but NO is for most microorganisms a signal molecule and also a potent cytotoxin, so in order for prokaryotes to survive and live, a rapid continued reduction of NO to N_2O is therefore vital, this can also sees in *T. linaloolentis*, NO accumulated never to more than 15nM (Bergaust et al. 2008; Conrad 1996; Philippot et al. 2007; Richardson et al. 2009). Another survival strategy some prokaryotes have when the habitat they live in becomes inhospitable due to the lack of e.g. oxygen, nutrients or both, would be the ability to form endospores to survive (Veening et al. 2008)

4.1. Testing of different media

The work presented in this thesis is part of a larger study where denitrification regulatory patterns in different Thauera strains will be compared. We therefore needed to find a medium that supported growth of several Thauera strains. Ideally, this should be a medium where the amount of carbon and nitrogen are specified and controlled. A couple of different media were therefore tested for growth characteristics in relation to the Thauera strains, before a comparative phenotypic analysis were performed on the robotized incubation system under varying conditions.

Extensive testing was performed on Sistrom's medium (succinate) and medium 586 (Na-benzoate) to valuate if any of the Thauera strains would be able to utilize succinate or Na-benzoate. It revealed that both of these mediums were unsuitable since none of the Thauera

strains were able to grow in Sistrom's and only T. aminoaromatica was able to grow in medium 586. It was therefore decided to use a minimal medium supplemented with 10% complex medium chosen on the basis of previous work done in this lab by Mao (Mao *et al.*, manuscript in preparation). This is a medium in which all the Thauera strains would grow in, but extra precautions were taken to ensure no contaminations of the pure cultures occurred.

ERIC-PCR was utilized to control the produced stock cultures and also used in between larger experiments that lasted over a longer period of time, which increases the chance of contamination (Debruijn 1992)

4.2. Gas kinetics experiment

Denitrification has been studied in several different model organisms as *P. denitrificans*, while only a few studies of phenotypic analysis have been performed on *Thauera*, which is not a model organism. Therefore are little known and understood about how the regulation of denitrification occurs in *Thauera*. Earlier studies of *Thauera* performed by Mao (Mao *et al.*, manuscript in preparation) indicate that denitrification is initiated at low oxygen concentration. We have seen several times during denitrification that the concentration of NO for the different *Thauera* strains stays generally low and the experiments done in this study show the same for *T. linaloolentis*. NO never accumulated to more than 15 nM, indicating that NO is tightly regulated, this is also seen for *P. denitrificans*. N_2O on the other hand has a tendency to vary greatly not only between experiments but also between parallels, accumulating from 100 to 31700 nM in experiment 5, and 2500 to 9800 nM in experiment 4, Table 6. *P. denitrificans* seems to have a better control over the production and reduction of the N_2O intermediate then *T. linaloolentis,* Figure 22.

We could clearly see in experiment 4, Figure 11, that denitrification started before all the oxygen was depleted, in 1 and 7% initial oxygen. The reasons for this could be many; old proteome, cell aggregation, production of PHB as well as PHB fermentation during low oxygen concentration to ease the cells transition to anoxic conditions or that *Thauera* does a partial aerobe denitrification before a complete anaerobic environment is established.

To investigate if the cells harbored an old proteome, a second experiment was performed where the inocula was strictly controlled, both the amount of carbon, cell density and number of cell generation. The culture was also tested for PHB (poly-β-hydroxybutyrate) which is a polymer created and stored in some organisms, when excess substrate is present. Pseudo-fermentation of PHB might ease an organism's transition from aerobic to anaerobic respiration (Ciggin et al. 2009) or sustain the organism under an anoxic spell, which would otherwise be left without energy at O_2 depletion, and thus be unable to produce a denitrification proteome. This is what we speculate is the case for *Paracoccus*, but it does not appear that Thauera produce PHB due to the negative results obtained with the Nile-red test. The gas kinetics and e-flow patterns for the second experiment were almost identical to experiment 4, which also rules out the theory about *Thauera* harbored on an old denitrification proteome (proteins expressed from denitrification genes).

 Small amount of aggregation can never be ruled out but highly unlikely even thought the inocula in the last experiment performed was strictly regulated and controlled. The last theory would be that *T. linaloolentis* do a partial aerobe denitrification which is also highly unlikely due to the fact that nitrate uptake is inhibited by O_2. Figure 15 shows that nitrate is reduced to nitrite for 7% initial oxygen in the period where oxygen is still present. This phenomena has more likely to do with increased cell density in the culture which causes a more rapid take down and abrupt depletion of oxygen.
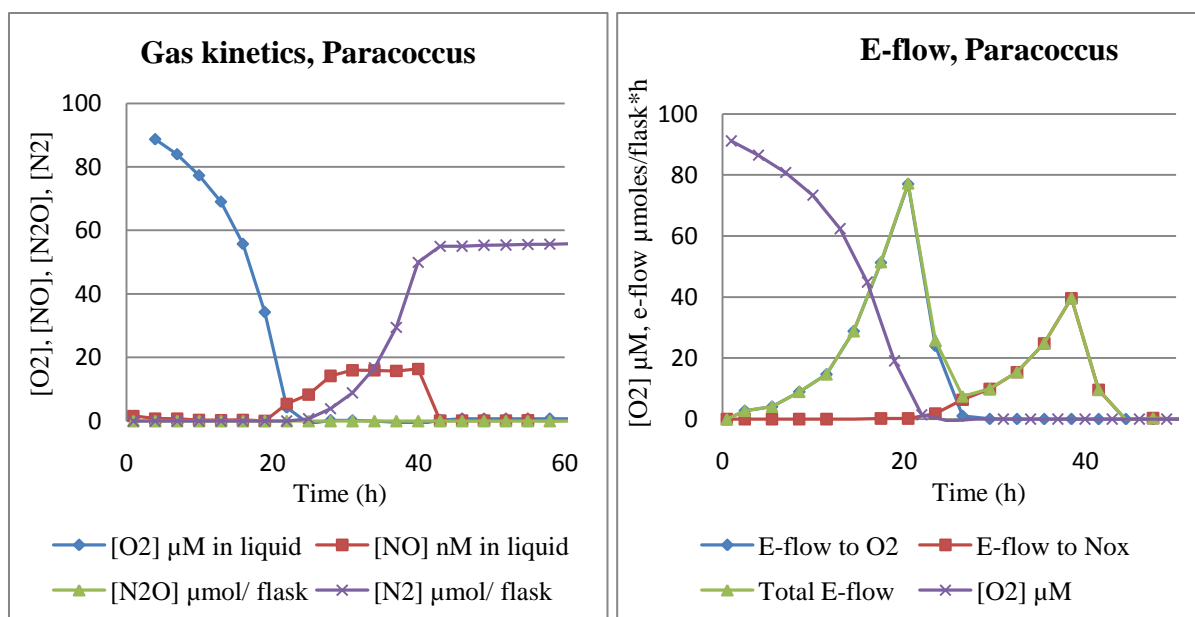
Figure 22: *Paracoccus denitrificans* grown in 50 ml Sistrom's medium with 2mM nitrate and 7% initial oxygen. Gas kinetics during oxygen depletion, denitrification of *Paracoccus denitrificans* and the electron transport rates (e-flow), consisted of the measurements of the four gases; [NO] nM in liquid, [N2O] nmol/ bottle, [N2] μmol/ bottle and [O2] μM in liquid, and the monitoring of all the available electron acceptors before and during the denitrification process. (The results for Paracoccus denitrificans are from the article Transcription and expression of NOx reductases in Paracoccus denitrificans driven by oxygen depletion; inhibitory effects of suboptimal pH, by Linda Bergaust et al.)

The onset of nitrite accumulation preceded that of NOx gas production in Paracoccus denitrificans, while the opposite pattern was observed in T. linaloolentis (data not shown). *T. linaloolentis* did not reduce all the nitrate to nitrite before continuing to the first committed step in the denitrification process as *Paracoccus denitrificans* did, but instead continuously reduces nitrate to N$_2$. An increasing amount of nitrite was produced at higher initial oxygen levels, but still this was three orders of magnitude lower than the values obtained in *P. denitrificans*. This indicates that *T. linaloolentis* handles denitrification quite differently than *P. denitrificans*.

4.3. Estimation of F_{den} using Nalidixic acid

A nalidixic acid experiment was performed to confirm the F_{den} results obtained by gas kinetics on the robotized incubation system. The result obtained from the nalidixic acid experiment confirms and reinforces the result obtained from the gas kinetic experiment. It showed that a large fraction of about 84 to 74 % managed the transition from oxic to anoxic respiration of respectively 0 and 7% initial oxygen. This indicates as mention in Linda Bergaust paper (*Paracoccus species*, manuscript in preparation) that F_{den} may be dependent on the speed of oxygen depletion. F_{den} is high where oxygen is depleted slowly as in 0%, while it is lower in 7% oxygen where the oxygen depletion rate is much higher due to higher cell density in the cultures. A very low fraction of *Paracoccus denitrificans* population managed the transition (19 to 30%) compared to *T. linaloolentis.* It also appears that Thauera actively channels electrons from oxygen to NOx, at oxygen depletion. Total electron flow shows possible a small stagnation in connection with the transition from oxic to anoxic respiration, most evident in sample with high initial oxygen levels figure 13(F). A bit before eminent oxygen depletion the Thauera chooses to direct the electron flow towards denitrification (partially aerobe at 7%), which appears to be more energetically favorable at the time.

During early rudimentary gas measurements with Nalidixic acid, samples were taken from different bottles for determination of the fraction of cells that switched to denitrification at oxygen depletion (F_{den}) by cell count. It was then I encountered a problem, when cell samples from the nalidixic experiment were mixed with glutardialdehyde (1.25%) and stored for a couple of days, some kind of reaction had happened causing either precipitation or a gel based structure to form, in the samples with added glutardialdehyde. This made it impossible to do a cellular count in the microscope for determination of the fraction of cells that switched to denitrification at oxygen depletion (F_{den}). The reason for this is still unknown, but after some testing I found a solution to the problem, and that was to dilute the samples to 25% before adding 1.25% glutardialdehyde. Even thought this solves the problem, it is not a perfect solution, and it is therefore recommended that another preservative is used.

5. Conclusions and future perspectives

The main purpose of this study was to determine how big a fraction of the *Thauera linaloolentis* population that manage the transition from oxic to anoxic respiration, at 0,1 and 7% initial oxygen concentration during oxygen depletion. The results were achieved through the two different methodological approaches which displayed a unified picture of *Thauera linaloolentis* ability to manage a balanced transition from oxic to anoxic respiration, while *P. denitrificans* were unable to do so. Although similar results were obtained in both the methods, a clear difference exists between the two methods in terms of cost and time efficiency in achieving the same results. The first method was the gas kinetic measurements performed on a robotized incubation system and the second method was the use of nalidixic acid for quantification of growing cells by microscopic count. The first method is both cost effective and less time is spent to achieve the result, while the second method are both expensive and time consuming.

Little is known about the regulatory network and the factors that initiate the transcription of the different reductase in the denitrification process of the different *Thauera* strains. Therefore is more research on the genotypic and phenotypic regulation of denitrification needed to get a better understanding of how *Thauera* manages a balanced transition from oxic to anoxic respiration?

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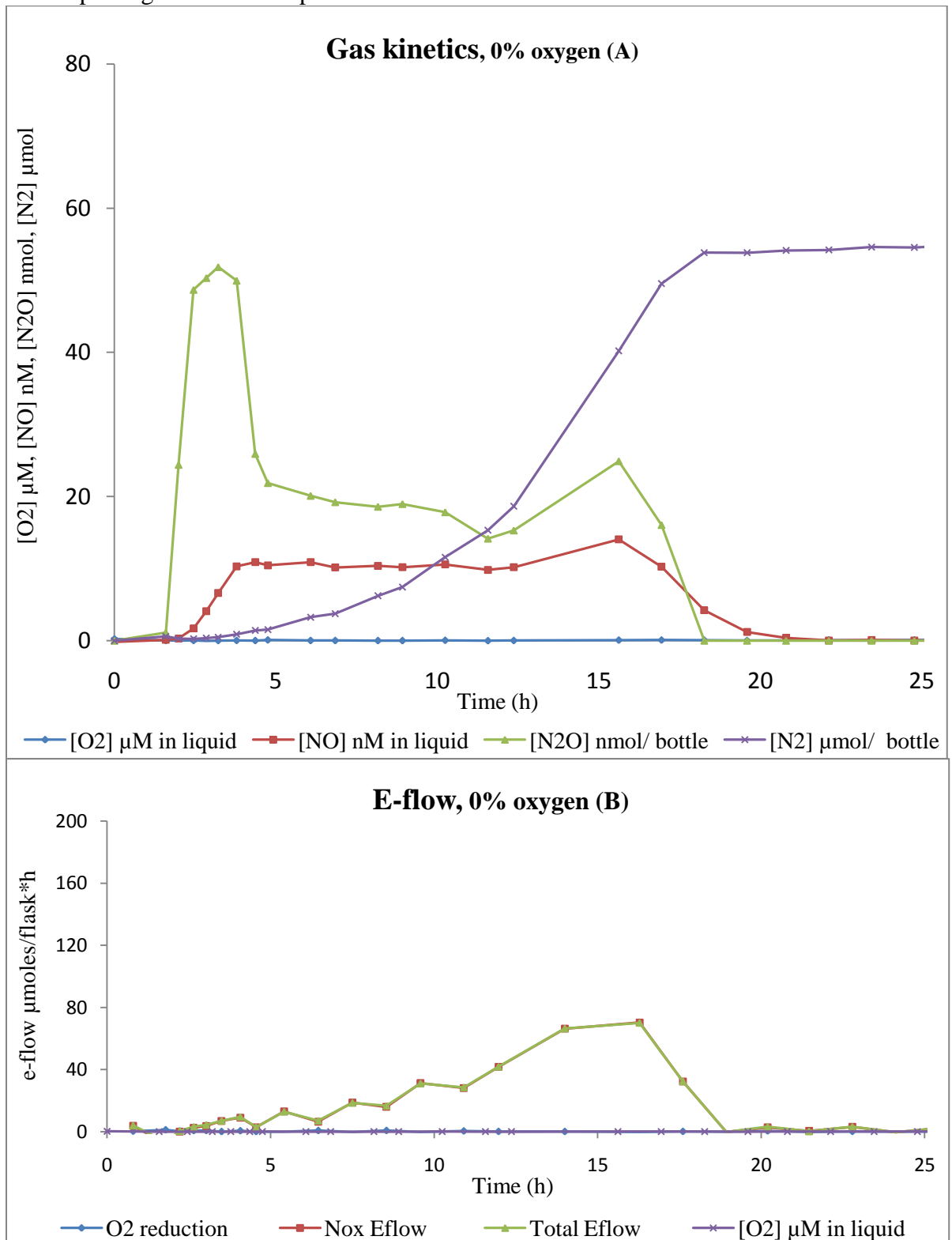
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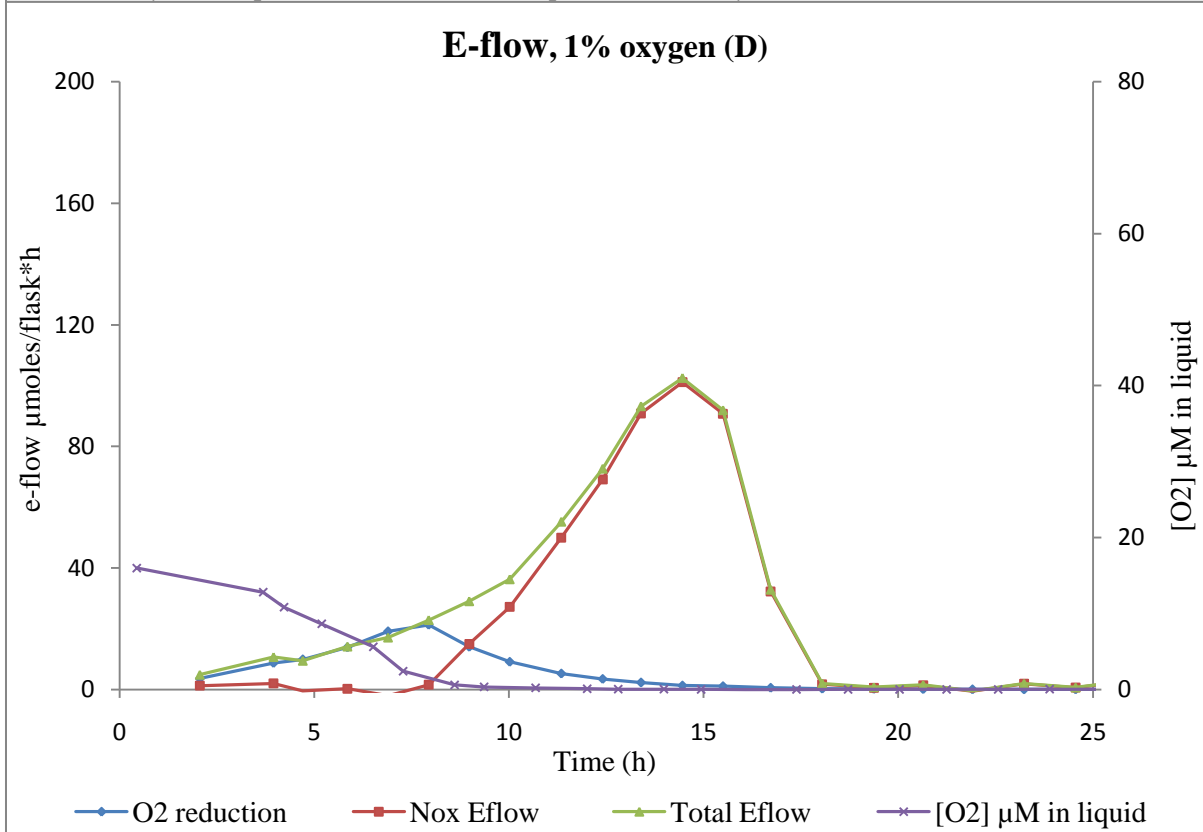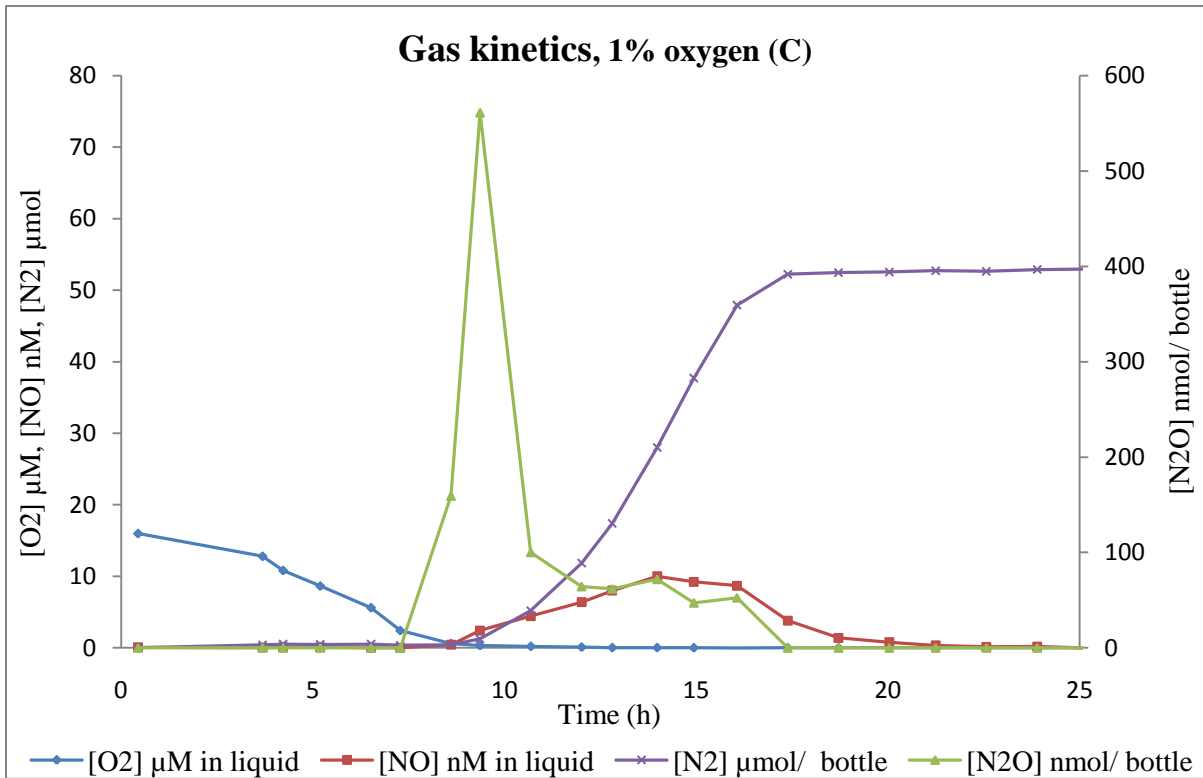
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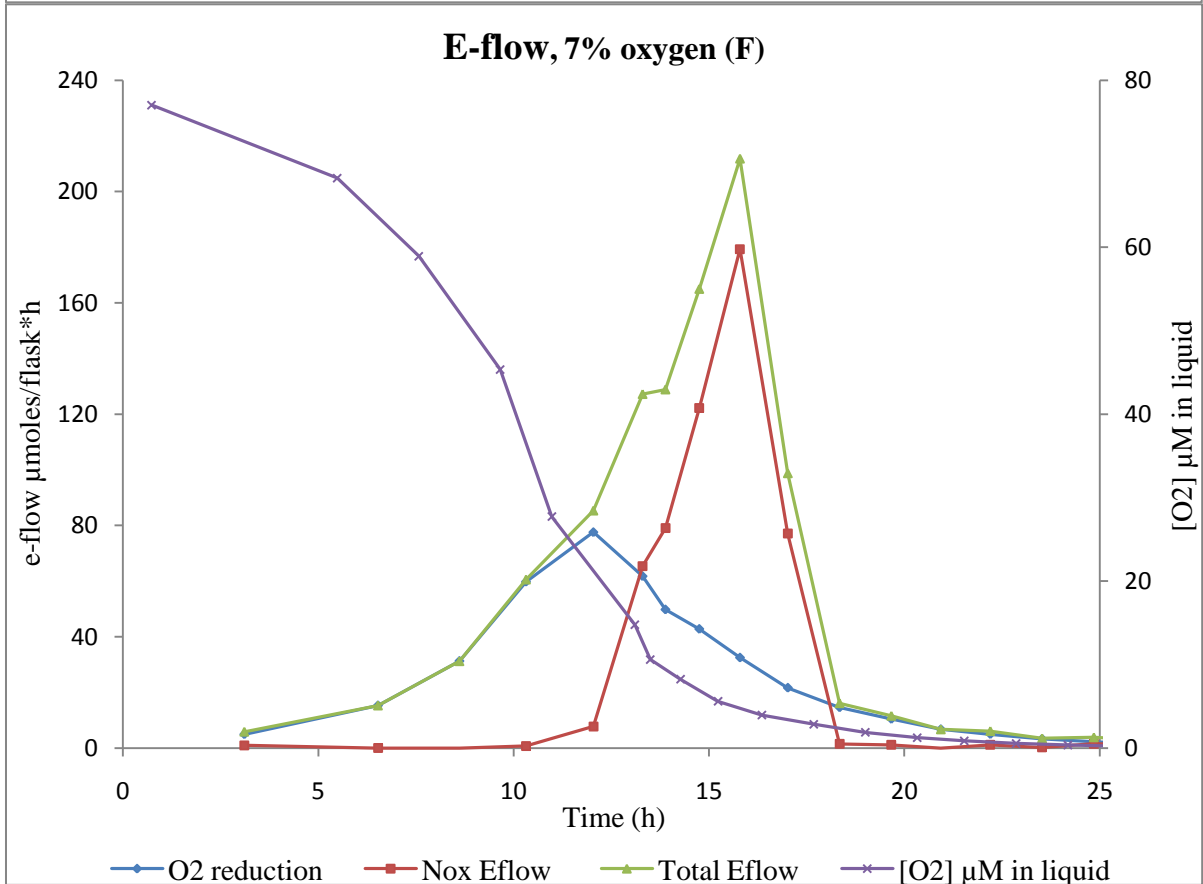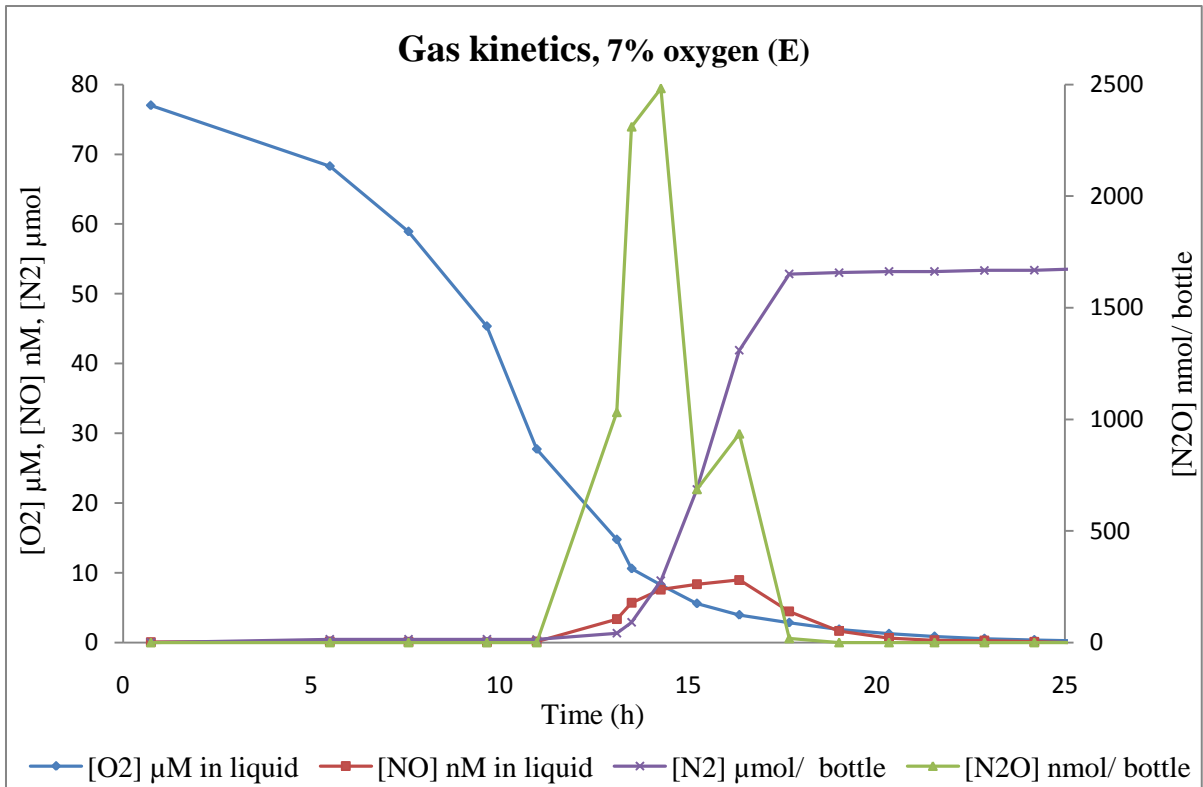
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Appendix

Close up of figures A-F in experiment 4.

Gas kinetics, 1% oxygen (C)

Legend: [O2] µM in liquid — [NO] nM in liquid — [N2] µmol/ bottle — [N2O] nmol/ bottle

E-flow, 1% oxygen (D)

Legend: O2 reduction — Nox Eflow — Total Eflow — [O2] µM in liquid

Gas kinetics, 7% oxygen (E)

Legend: [O2] µM in liquid, [NO] nM in liquid, [N2] µmol/ bottle, [N2O] nmol/ bottle

E-flow, 7% oxygen (F)

Legend: O2 reduction, Nox Eflow, Total Eflow, [O2] µM in liquid

Close up of figures A-F in experiment 5.

Gas kinetics, 0% oxygen (A)

Legend: [O2] µM in liquid — [NO] nM in liquid — [N2O] nmol/ bottle — [N2] µmol/ bottle

E-flow, 0% oxygen (B)

Legend: O2 reduction — Nox Eflow — Total Eflow — [O2] µM in liquid

Gas kinetics, 1% oxygen (C)

Legend: [O2] µM in liquid — [NO] nM in liquid — [N2] µmol/ bottle — [N2O] nmol/ bottle

E-flow, 1% oxygen (D)

Legend: O2 reduction — Nox Eflow — Total Eflow — [O2] µM in liquid

Gas kinetics, 7% oxygen (E)

Legend: [O2] μM in liquid — [NO] nM in liquid — [N2] μmol/ bottle — [N2O] nmol/ bottle

E-flow, 7% oxygen (F)

Legend: O2 reduction — Nox Eflow — Total Eflow — [O2] μM in liquid