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Development of an enrichment protocol for isolation of N_2O reducing bacteria from highly weathered, acidic African soils

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Abstract

N_2O is known to be one of the most potent greenhouse gases due to its long residence time in the atmosphere and with a global warming potential almost 300 times larger than CO_2. Emissions of N_2O are one of the biggest contributors to global warming and so developing methods to mitigate N_2O emissions is therefore needed.

Denitrification in agricultural soils is one of the major sources of anthropogenically produced N_2O. Denitrification is a respiratory process of many organisms where NO_3^- is reduced to N_2 through several redox reactions and intermediates. The process is performed by a wide range of organisms carrying a full-fledged or truncated set of denitrification enzymes. Soil pH is one of the major controllers of the denitrification process, and particularly the N_2O reduction step. At soil pH < 6.1 the synthesis of functional N_2O reductase is significantly hampered thus contributing to increased N_2O emissions.

Acidic soils are found in over 30 % of the world's ice-free land and are formed through the influence of climate and geology, but soil acidification can also be strongly enhanced by human activities. Africa holds large regions of old, highly weathered acidic soil with low nutrient contents. Increasing the crop yields is necessary here to feed a growing population, and although fertilizers are generally unaffordable to small-scale farmers, subsidizing programs have increased fertilizer use, mostly of ammonium-based fertilizers. This has led to enhanced soil acidification and increased N_2O emissions in many areas.

In this study, I developed a protocol to enrich and isolate N_2O reducing bacteria from acidic soils. Until now, only bacteria in the genus *Rhodanobacter* found in acidic European soils have shown the ability reduce N_2O at low pH. One main goal of this work was to find out if this capacity is found also among other bacterial groups, and to obtain isolates that could be used for further studies of pH effects on N_2O reduction. Such bacteria could potentially be used for the development of novel methods for N_2O mitigation in acidic environments.

In the current work, two acidic African soils from North Ghana (Bush Fallow, BF and Kpaliga 1, K1), were sampled and used for experimental work. To increase the chance of isolating the desired organisms, enrichments provided with N_2O as the sole electron acceptor were incubated anoxically. In a first experiment, glutamate was provided as the carbon source, but consumption of the glutamate caused pH to rise above 7. Several options were tested to maintain pH below

the threshold of 6.1 through the entire incubation period. These included the use of buffering (phosphate buffer, PB) and the use of different carbon sources as electron donors. A mix of hexoses and 150 mM PB turned out to be the best solution and was used in the following enrichments.

Unexpectedly high amounts of CO_2 were produced compared to the N_2 produced from N_2O reduction in enrichments of both soils. Since these soils were rich in iron, it was hypothesized that the high CO_2 production could be due to activity of Fe(III) reducing bacteria. Supporting this, it was found that the concentration of Fe(II) increased in anaerobically incubated slurries. However, incubations of bacterial cells extracted from the soils using a Nycodenz gradient, which would remove the Fe, showed that CO_2 production remained high while still no reduction of N_2O was observed. Based on this, and the identification of several dominant isolates (see below) it was concluded that the CO_2 production was predominantly caused by fermentative activity.

The enrichment with hexoses and 150 mM PB efficiently kept pH below 6.1 in soil K1 during the entire incubation and was used for further enrichments over successive cycles where portions of the enrichments were transferred to new medium. An increase in the N_2O reduction rates throughout the cycles confirmed that this strategy successfully increased the abundance of N_2O reducing organisms in the slurries. Attempts to isolate the N_2O reducing bacteria were performed using selective and differential agar media at low pH and by providing N_2O as the sole electron acceptor, but these were unsuccessful and no acid tolerant N_2O reducing organisms were isolated. Sanger sequencing of the 16S rRNA gene was performed for some of the isolates, but no known denitrifying bacteria were identified. 16S rRNA gene amplicon sequencing of the microbial community in the enrichment slurries and native soil was also performed and gave interesting and promising results. Species from the genera *Bacillus* and *Desulfitobacterium*, both which are known to include species that harbor the *nosZ* gene, dominated in the enriched soils. An interesting finding was that no *Rhodanobacter* species were enriched, currently the only known species capable of N_2O reduction at low pH. Thus, this study provides the first indication that other bacterial genera may contain a functional N_2OR enzyme at acidic pH, thereby suggesting that this quality is more widespread than previously thought. Increasing our understanding of such organisms is of great importance as acidic soils are a growing issue in the world and are further exacerbated with the expected increase of fertilizer. Low pH N_2O reducing organisms could be used in the development of novel methods to fight N_2O emissions from agriculture, for example by including them in mixtures of plant

growth promoting bacterial inoculants or enriched in digestates to be spread on farmland and could thus be an important contributor in the battle against global warming.

Sammendrag

En av de viktigste årsakene til global oppvarming i dagens samfunn er utslipp av N_2O. Gassen er regnet som en av de farligste klimagassene på grunn av sin lange levetid i atmosfæren og et globalt oppvarmingspotensial nesten 300 ganger større enn CO_2.

Denitrifikasjon i landbruksjord er en av de viktigste årsakene til N_2O utslipp. Dette er en respiratorisk prosess hvor NO_3^- blir redusert til N_2 gjennom flere redoksreaksjoner og mellomprodukter. Prosessen utføres av et bredt spekter av organismer som kan inneha et fullt eller trunkert sett med denitrifikasjonsenzymer. Jord pH er en faktor som påvirker denitrifikasjonsprosessen, og spesielt reduksjonen av N_2O. En jord pH lavere enn 6.1 fører til at syntesen av det funksjonelle N_2O reduktase enzymet blir hemmet og dermed økte utslipp av drivhusgassen N_2O.

Jord med lav pH er et problem i over 30 % av verdens is-frie land og er forårsaket av flere naturlige årsaker, blant annet av klimaet og geologi, men også menneskelige aktiviteter som overbruk av kunstgjødsel som bidrar til ytterligere forsuring. Flere regioner i Afrika har sur jord ettersom den afrikanske jorden er gammel og forvitret og består av lite næringsstoffer. Økte avlinger i kontinentet er nødvendig for å tilfredsstille et økende behov for matproduksjon til en voksende populasjon, og selv om gjødsel generelt er for dyrt for småbønder, bidrar substituerings programmer med å øke bruken av gjødsel, hovedsakelig bestående av ammonium-basert kunstgjødsel. Dette har ført til forsuring av jordene og dermed økt utslipp av N_2O i mange regioner.

I denne studien var målet å utvikle en protokoll for å anrike og isolere organismer som kan redusere N_2O ved lav pH fra sur jord. Foreløpig er det kun bakterier fra slekten *Rhodanobater* som er kjent for å utføre N_2O reduksjon ved lav pH. Et mål i denne studien var derfor å undersøke om flere bakterier kan utføre denne reduksjonen, og i så fall identifisere isolater som kan brukes til å utvikle nye metoder for å redusere N_2O utslippene.

Jord fra Nord-Ghana (Bush Fallow, BF, Kpaliga 1, K1) ble brukt i disse forsøkene. For å øke sjansen for å isolere de ønskede organismene, ble anrikningsforsøk utført ved å tilsette rikelige mengder karbon og med N_2O som den eneste elektronakseptoren til jordslurries (blanding av jord og vann) som ble inkubert anaerobisk. I det første anrikningsforsøket ble glutamat gitt som karbonkilde, men glutamat nedbrytningen førte til at pH økte til over 7. Anrikningsprotokollen

måtte derfor forbedres slik at pH forble under grensen på 6.1 gjennom hele inkubasjonsperioden. Disse forbedringene innebar å tilsette buffer (fosfatbuffer, PB) i tillegg til å bruke en annen karbonkilde som elektrondonor. En blanding av heksoser med 150 mM PB viste seg å være den beste løsningen og ble brukt i følgende anrikningene.

Overraskende store mengder CO_2 ble produsert sammenlignet med N_2 produksjonen fra N_2O reduksjon under begge anrikningsforsøkene. Ettersom jorden fra Afrika hadde et betydelig jerninnhold ble det antatt at den høye CO_2 produksjonen kunne stamme fra reduksjon av Fe(II). Videre testing viste at konsentrasjonen av Fe(II) hadde økt i jordslurries som var inkubert anaerobisk med heksoser som karbonkilde. Likevel viste anrikningen av bakterier ekstrahert med Nycodens, som skulle ha fjernet jern fra jorden, at CO_2 produksjonen forble høy selv om ingen reduksjon av N_2O ble observert. Basert på dette, og artene funnet gjennom isolering (se under), ble det konkludert med at CO_2 produksjonen stammet fra fermentering.

Anrikningen med heksoser og 150 mM PB beholdt pH under 6.1 i K1 jorden under hele inkuberingsperioden og ble derfor brukt for videre anrikningsforsøk over tre sykluser. En økning i N_2O reduksjonsraten gjennom syklusene bekreftet at denne strategien var suksessfull og førte til økte mengder av N_2O reduserende organismer i jordslurriesene. Det ble også gjort forsøk på å isolere bakteriene som utfører denne N_2O reduksjonen ved bruk av selektiv og differensial isolasjon ved lav pH og ved å gi N_2O som den eneste elektronakseptoren, men ingen N_2O reduserende organismer ved lav pH ble isolert i dette forsøket. Sanger sekvensering av 16S rRNA genet ble utført for noen av isolatene, men ingen kjente denitrifiserende bakterier ble funnet. 16S rRNA gen amplicon sekvensering av det mikrobielle samfunnet ble også utført og ga interessante og lovende resultater. Arter fra slektene *Bacillus* og *Desulfitobacterium* var dominerende i de anrikete jordene, hvor begge slektene består av arter som innehar *nosZ* genet. Et interessant funn var at ingen *Rhodanobacter* arter ble anriket, og denne studien gir derfor den første indikasjonen på at andre bakterieslekter har et funksjonelt N_2OR enzym ved lav pH, og antyder dermed at denne egenskapen kan være mer spredt enn hva som er kjent. Å tilegne mer kunnskap om slike organismer er nødvendig ettersom sur jord allerede er et stort problem i verden, og den økte bruken av gjødsel i jordbruket er forventet å forsterke dette problemet. Bakterier som kan redusere N_2O ved lav pH vil være nyttig i utviklingen av nye metoder for å bekjempe N_2O utslippene fra jordbruk. Slike metoder innebærer blant annet å inkludere de N_2O reduserende organismene i blandinger med plantevekstfremmende bakterier eller anriket i digestater som kan spres på jordbruksland og dermed være en viktig bidragsyter i kampen mot global oppvarming.

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Abbreviations

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A	Acidic
BF	Bush Fallow
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
GC	Gas chromatograph
H_2	Hydrogen gas
K1	Kpalgia 1
N	Neutral
N_2	Dinitrogen gas
N_2O	Nitrous oxide gas
N_2OR	Nitrous oxide reductase
NAP	Periplasmic nitrate reductase
NAR	Membrane-bound nitrate reductase
NH_3	Ammonia
NH_4^+	Ammonium
NIR	Nitrite reductase
NO	Nitric oxide gas
NO_2^-	Nitrite
NO_3^-	Nitrate
NOR	Nitric oxide reductase
O_2	Gaseous oxygen
OTU	Operational taxonomic unit
PB	Phosphate buffer
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
TSB	Tryptic soy broth

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1. Introduction

1.1 The nitrogen cycle

Nitrogen is one of the most abundant elements in our atmosphere and an important compound to all living organisms as it serves as a necessary building block in several biological macromolecules such as amino acids and nucleic acids, in addition to being important in several energy generating processes (Robertson & Groffman, 2007). Nitrogen exists primarily as atmospheric nitrogen gas (N_2), unavailable for most organisms, and becomes available through the cycling of the gas. The nitrogen cycle is a network of reactions that describes the biogeochemical cycling of N-compounds through several oxidation and reductions steps mediated by a variety of organisms (Jetten, 2008; Schlesinger, 2009) (Figure 1.1). These steps span oxidation levels from +V for nitrate (NO_3^-) to ammonia (NH_3) at -III (Schneider et al., 2014). The cycle consists of several aerobic and anaerobic processes including assimilatory nitrate reduction, nitrification, anaerobic ammonium oxidation (anammox), nitrogen fixation, nitrification, dissimilatory nitrate reduction to ammonium (DNRA), and denitrification. These processes are performed by a diversity of organisms, including bacteria, archaea, and fungi (Bothe et al., 2006).

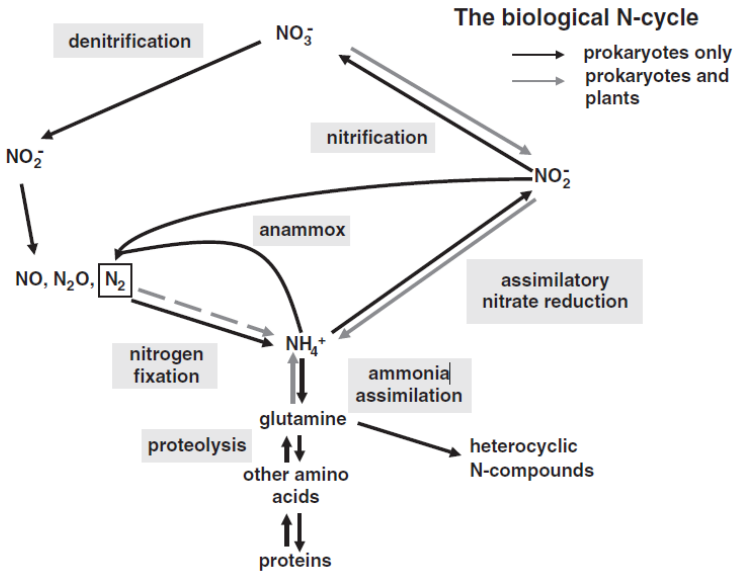

Figure 1.1 The biological nitrogen cycle. Processes in the biological nitrogen cycle where nitrogen undergoes several redox reactions from NO_3^- to NH_3. Figure from Bothe et al. (2006).

1.2 Denitrification

The denitrification step in the global nitrogen cycle is the respiratory process where NO_3^- through several intermediates is reduced to N_2. It is a key process in the cycle as denitrification is responsible for emitting nitrogen from the biosphere to the atmosphere (Zumft, 1997). This process is the most energetically favorable of all the anaerobic respiratory pathways for denitrifiers when oxygen is limited. When oxygen levels are low, denitrifiers can switch to nitrogen oxides as alternative terminal electron acceptors (Bothe et al., 2006). By performing this switch, microbes are able to continue respiration under anaerobic conditions, thereby allowing electron transport, ATP generation and so growth to continue (Kraft et al., 2011).

Denitrification is described as a modular pathway where different organisms possess different sets of denitrification enzymes (Graf et al., 2014). Complete denitrifiers are organisms capable of reduction of NO_3^- all the way to N_2 and these can function as sinks for N_2O emissions. In the natural environment, several organisms lack the genes for, or are due to other reasons, incapable of performing one or more denitrification steps (Lycus et al., 2017). Most common are denitrifiers incapable of performing the last step, reduction from N_2O to N_2 (Graf et al., 2014). Such truncated denitrifiers will emit N_2O into the atmosphere and thereby act as a source of N_2O emissions. This last denitrification step is therefore of great interest as it determines whether the process functions as a source or a sink for N_2O emissions.

Figure 1.2 Simplified overview of complete denitrification. Denitrification is the stepwise reduction of NO_3^- to N_2 catalyzed by specific denitrification reductase enzymes.

1.2.1 Global impact of denitrification

Emissions of nitrous oxide (N_2O) into the atmosphere are one of the major contributors to global warming today. N_2O is the third most abundant greenhouse gas and it is of particular concern as it has about 300 times the global warming potential of carbon dioxide (CO_2), in addition to its long global lifetime of 114 years in the atmosphere (IPCC, 2013; Ravishankara et al., 2009; United States Environmental Protection Agency, 2018). While there are many natural sources

of N_2O emissions, human activities such as intensive agriculture and in particular fertilization contribute significantly to the increased global N_2O emissions (Bakken & Frostegård, 2017). The United States Environmental Protection Agency (2018) predicts that N_2O emissions from agricultural soils will increase by 14 % from 2015 to 2030. This increase is driven by a need for increased food production to feed a growing human population, and consequently, of the use of more fertilizer and increased agricultural land use (Schlesinger, 2009; Tian et al., 2020).

Due to the expected rise of N_2O emissions, microbes able to perform the reduction of N_2O in soils are of great interest as they could work as potential sinks for N_2O emissions. 70 % of all N_2O emissions into the atmosphere are caused by soil processes, primarily incomplete denitrification (Mosier, 1998). Reduction of N_2O is carried out by only one known enzyme, Nitrous oxide reductase (N_2OR), and the reduction by microbes producing this enzyme is the only biological way N_2O can be broken down. Substantial research work into this phenomenon and the function of N_2OR has therefore been conducted in the last decades, as a better understanding of the denitrification process and the microbes performing it could potentially lead to N_2O emission mitigation options (Bakken & Frostegård, 2017; Bergaust et al., 2010; Liu et al., 2010).

1.2.2 Denitrifying microbes

The denitrifying microbial soil community is an important factor in determining the denitrification rates and thereby affecting emissions of N_2O. The denitrification process is carried out by a broad specter of soil bacteria, primarily heterotrophic but also chemo-and photolithotrophs (Robertson & Groffman, 2007; Zumft, 1997). All denitrifiers are facultative anaerobes who thrive under fluctuating oxygen levels, as the maturation and expression of the denitrification enzymes are triggered by low oxygen levels and available nitrogen oxides, which can be used as their alternative electron acceptors (Zumft, 1997).

The majority of studies on denitrifiers today are based on only a few model organisms. As denitrifiers include many taxonomically diverse groups of bacteria, getting knowledge about a wider range of organisms is needed to get a better understanding of how the denitrification process works (Lycus et al., 2017). The composition and variety of the denitrifying microbial community are of significance as it may regulate the denitrification process as well as the denitrification rates in response to environmental conditions (Bergaust et al., 2010; Van Den Heuvel et al., 2010). The denitrifying community is affected by several factors such as carbon

availability, O_2 concentrations, water content, availability of NO_3^-, and soil pH (Robertson & Groffman, 2007; Wallenstein et al., 2006). Carbon availability is important as heterotrophic denitrifiers require organic carbon as an electron donor, while oxygen concentrations are regulated by water and carbon content, in which carbon gets consumed rapidly thus, using up the available oxygen and resulting in anoxia in regions of the soil (Butterbach-Bahl et al., 2013; Robertson & Groffman, 2007). A major impactor of the denitrifying microbial soil community is soil pH, which affects both the composition, regulation, and activity of the community and its denitrification enzymes (Dörsch et al., 2012). pH exerts control over the last step in the denitrification process by affecting the function of the N_2OR and thereby affecting the $N_2O/(N_2O+N_2)$ product ratio in which decreasing pH leads to increasing N_2O emissions (Bakken et al., 2012). This demonstrates that the denitrification community is greatly affected by several environmental factors which in turn are affecting the denitrification process and the enzymes present and/or functional.

1.2.3 Denitrification enzymes

The denitrification enzymes catalyze each reaction in the denitrification process. They provide energy through the anaerobic respiration process and ensure that the amounts of intermediates stay below a toxic level for the cell (Zumft, 1997).

Denitrification and the maturation of the reductase enzymes are initiated once environmental signals such as concentrations of NO_3^-, NO_2^-, NO, O_2 and Cu are detected by members of the FNR/CRP superfamily of transcriptional regulators (Gaimster et al., 2018; Spiro, 2012). The first step of denitrification for Gram-negative bacteria is the reduction of NO_3^- mediated by nitrate reductase (NAR/NAP). NAR is a membrane-bound enzyme complex consisting of the three subunits NarG, NarH, and NarI. NarG contains the active site, NarI receives electrons and NarH connects the two other subunits. Many bacteria are not dependent on the presence of NarGHI as the nitrate reduction step may be performed by the alternative periplasmic nitrate reductase (NAP) instead. This complex consists of two subunits, NapA and NapB, where NapA contains the active binding site for NO_3^- (Kraft et al., 2011). The reduction from NO_2^- to NO is catalyzed by either NirK or NirS, both periplasmic enzymes, where only one of the enzymes is present in each organism. The two enzymes contain different metals in their active binding site where NirK is a copper-containing nitrite reductase and NirS is a *cytochrome cd1* containing nitrite reductase. The enzymes differ significantly in structure but are functionally similar where both are capable of reduction of NO_2^- to NO (Zumft, 1997). The further reduction of NO to

N_2O is performed by the membrane-bound periplasm facing nitric oxide reductase (NOR) consisting of subunits NorC and NorB. This is an important step as NO exists as a radical, and excess amounts are toxic. There are two known types of NOR, long-chain (lc)NOR and short-chain (sc)NOR. Short-chain NOR is a complex consisting of two subunits, NorC and NorB, where NorC is responsible for electron transfer to the catalytic NorB. Long-chain NOR consists of one unit that receives electrons from the quinone pool (Hendriks et al., 2000; Kraft et al., 2011). The final step in complete denitrification is the reduction of N_2O to N_2. This is catalyzed by the periplasmic enzyme N_2OR (also termed NOS) and ensures the last step of complete denitrification (Schneider et al., 2014). As mentioned in section 1.2, denitrification is a modular pathway, and consequently, not all denitrifiers include or have functional versions of all the denitrification enzymes (Lycus et al., 2017).

Figure 1.3 illustrates the complete denitrification pathway with all denitrification enzymes and their cellular localization in Gram-negative bacteria in addition to the electron transport pathway described briefly here. Electrons are transferred from NADH via NADH dehydrogenase to ubiquinone or ubiquinol, cytochrome *bc1*, or cytochrome *c* before being transferred to the denitrification reductases and used for the reduction of the nitrogen compounds (Chen & Strous, 2013).

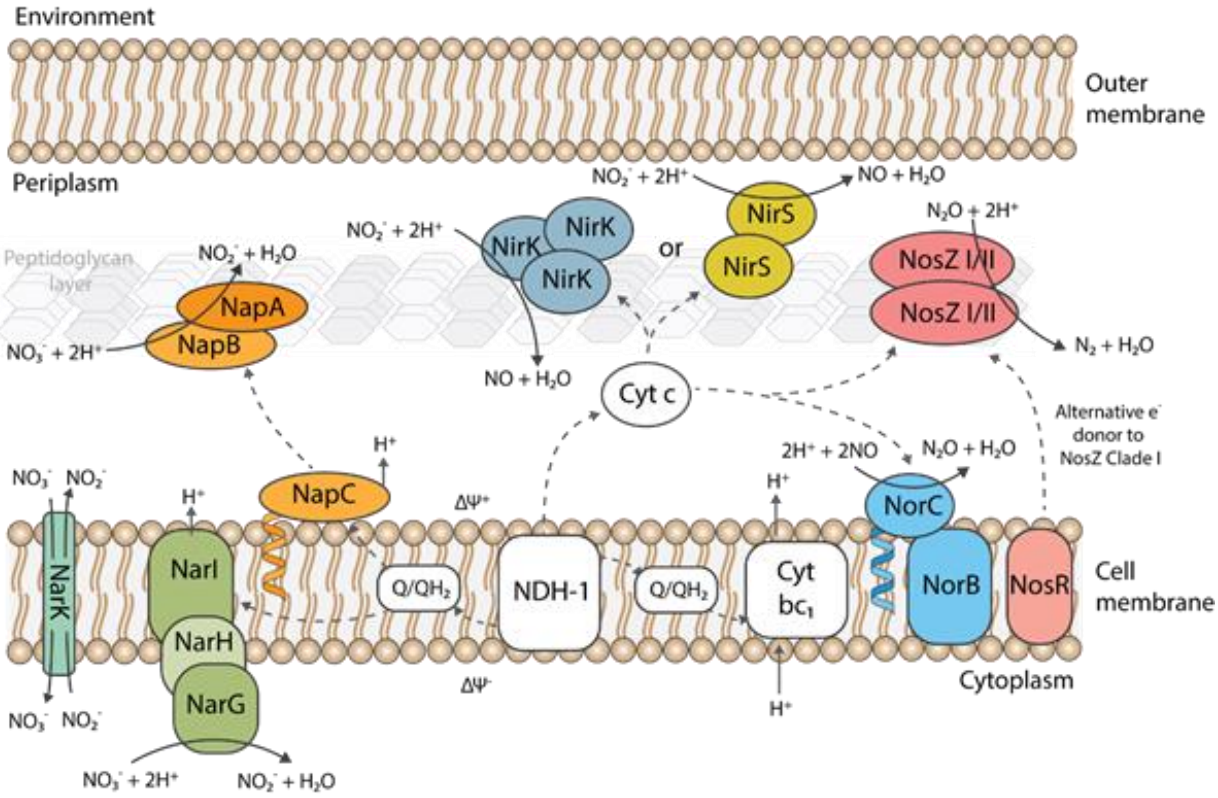

Figure 1.3 Detailed illustration of the enzymes and reactions in complete denitrification. The different denitrification enzymes and their subcellular localizations are shown and colored by the reaction catalyzed. The figure includes both nitrate reductases, NAR and NAP, nitrite reductases NirK and NirS, nitric oxide reductase NOR, nitrous oxide reductase NOS/N_2OR, and their accessory enzymes. The electron flow is illustrated in terms of H^+ ions (stoichiometrically unbalanced). Figure courtesy of Kjell Rune Jonassen (2021).

Not only Gram-negative bacteria are able to perform denitrification. Gram-positive bacteria are also capable of preforming denitrification and can carry all four denitrification enzymes, however, they differ in that all enzymes are membrane-bound as they lack a true periplasmic space. This is also the case for archaea where the whole denitrification process takes place on the outer side of the cytoplasmic membrane. Truncated denitrification has been observed in fungi, where the process is coupled to synthesis of ATP and located in the mitochondria, but no fungi are known to perform complete denitrification as none is known to carry N_2OR (Kraft et al., 2011).

1.3 The enzyme N_2O reductase

Many denitrifiers are incapable of performing the last step in the denitrification pathway as they lack N_2OR, the only enzyme capable of reducing N_2O to N_2. The presence and function of N_2OR is therefore crucial for determining whether denitrification will act as a source or a sink for N_2O (Suenaga et al., 2019).

The N_2OR enzyme is encoded by the *nosZ* gene and is located in the periplasm of Gram-negative bacteria. The periplasm is also where the maturation of the active copper sites of the enzyme takes place (Schneider et al., 2014). The reductase is a homodimer and consists of two copper-containing monomers, where each contains one catalytic CuA site and one catalytic CuZ site. CuA functions as the electron transfer site, while CuZ is where substrate reduction occurs. These sites react with each other and electron transfer from CuA leads to the reduction of N_2O to N_2 at the CuZ site (Kraft et al., 2011; Schneider et al., 2014).

The *nosZ* gene has been classified into two distinct groups termed clade I *nosZ* and clade II *nosZ* (Jones, C. M. et al., 2013). The main difference between the clades lies in their signal peptides, where clade I is encoded by a Tat peptide and clade II is encoded by a Sec peptide. Both groups are taxonomically diverse where clade I often exist in well-investigated genera such as *Bradyrhizobium*, *Pseudomonas*, and *Paracocus*, while clade II is more widely spread and present in over 14 bacterial phyla (Suenaga et al., 2019). Both clades are present in a range of different environments, but in similar abundance (Jones, C. M. et al., 2013). Suenaga et al. (2019) observed that clade II carrying bacteria had higher affinities for N_2O than clade I and was thus capable of utilizing it in lower concentrations than clade I. Additionally, clade II *nosZ* is more often seen with the absence of other denitrification enzymes e.g., in N_2OR only organisms, and could therefore serve as potential sinks for N_2O emissions (Suenaga et al., 2019).

1.3.1 pH control of nosZ

Truncated denitrification, caused by the loss of function of N_2OR, is a major source of N_2O emissions. pH is one of the most important controllers of the function of N_2OR, and several studies (Bakken et al., 2012; Bergaust et al., 2010; Šimek & Cooper, 2002) have demonstrated that the $N_2O/(N_2O+N_2)$ product ratio are affected by low pH leading to higher emissions of N_2O at low compared to neutral pH.

Šimek and Cooper (2002) concluded that both the rate of the denitrification process and the ratio of its gaseous products, including N_2O emissions, were highly dependent on pH. Investigations by Bergaust et al. (2010) demonstrated the pH control of N_2OR maturation using the model organism *Paracoccus denitrificans*. When grown under denitrifying conditions at pH 7, N_2O was reduced, and only small amounts of the gas were emitted. While when the organism developed the denitrification enzymes at pH 6, only N_2O was produced, and no reduction occurred. This was also observed in bacterial communities extracted from soil by Liu et al. (2014). Liu et al. (2014) also identified a threshold for the functional N_2OR at pH 6.1, as it was observed that the cells were not able to produce a functional N_2OR below that level. These results corroborate those seen in *Paracoccus denitrificans* by Bergaust et al. (2010) and demonstrate that the phenomenon is common to a wide range of diverse bacteria. Bergaust et al. (2010) also found that when cultures of *Paracoccus denitrificans* developed the N_2O reducing enzyme (N_2OR) at pH 7, the N_2OR was functional also at pH 6.1, but at lower rates and hypothesized that the loss of N_2OR activity was due to unsuccessful maturation of the protein due to low pH in the periplasm. This was further supported by Bakken et al. (2012) who hypothesized that the post-translational hindering was caused by interference with the assembly of the N_2OR enzyme in the periplasm, where the enzyme is more exposed to environmental effects than enzymes in the cytoplasm. Studies of the N_2OR enzyme from *Achromobacter cycloclastes* by Fujita and Dooley (2007) further corroborated this as they showed that H^+ ions interfered with the CuA center of the N_2OR enzyme.

1.3.2 N_2O reducing organisms at low pH

In addition to affecting the denitrification rate and the $N_2O/(N_2O + N_2)$ product ratio, as well as the function of N_2OR, pH also affects the composition of the denitrifying soil community (Wallenstein et al., 2006). It would therefore be interesting to explore the possibility of low pH N_2O reducers. A few studies have raised the interesting possibility that low pH tolerant N_2O reducing organisms exist (Hetz & Horn, 2021; Lim et al., 2018; Van Den Heuvel et al., 2010). Lim et al. (2018) showed some N_2O reduction in Norwegian soils under acidic, denitrifying conditions after an incubation of ~30 h. These observations could be the result of small populations growing up that produce functional N_2O reductase at low pH. Some studies corroborate this, e.g. Van Den Heuvel et al. (2010) performed experiments using soil slurries from a riparian buffer zone in the Netherlands where N_2O reduction at pH 4 was observed. They hypothesized that the observed reduction could be due to either adaptation to the low pH by the

microbial community present or enrichment of a low pH N_2O reducing community. The results confirmed the latter and showed that up to 70 % of the bacterial community consisted of *Rhodanobacter* species.

Lycus et al. (2017) worked on the isolation of denitrifiers from Norwegian peat soil and were able to identify one organism containing only N_2OR from low pH soil, as well as several organisms containing N_2OR in addition to other denitrification genes from the same soil. Lycus et al. (2017) also found that one organism was capable of performing N_2O reduction at low pH in pure culture. This organism was identified as a *Rhodanobacter* species which is so far the only organism which are implicated in low pH N_2O reduction.

These results indicate that low pH N_2O reducers exist and whilst observations have been made that some microbes can produce functional N_2OR at low pH, the mechanism of how they do this remains undetermined. Hypotheses for this phenomenon include the capability to assemble the enzyme after a longer period or making neutral/alkaline pH niches within the soil, in addition to microbes carrying a functional N_2O (Liu et al., 2014). The discovery and isolation of these organisms could provide more information about the way low pH inhibits N_2OR maturation, and what mechanisms some organisms use to be able to overcome it and thus get a greater understanding of this phenomenon from a scientific perspective. It is also interesting to aim at developing applied uses for these bacteria such as making fertilizers enriched in them to reduce N_2O emissions from acidic soils. Introducing such organisms to acidified agricultural soils or other similar environments would be of great interest as these microbes could serve as potential sinks for N_2O emissions under such environmental conditions.

1.4 Soil acidification

The low pH control over N_2OR is of special interest in acidic, agricultural soils. Acidic soils are a worldwide problem and occupy approximately 30 % of the world's ice-free land area (Figure 1.4). Acidic soils are found mainly within two global belts, the northern and the southern belt, where the northern belt is characterized by cold humid temperature, and the southern belt is characterized by tropical areas with high rainfall. (Von Uexküll & Mutert, 1995).

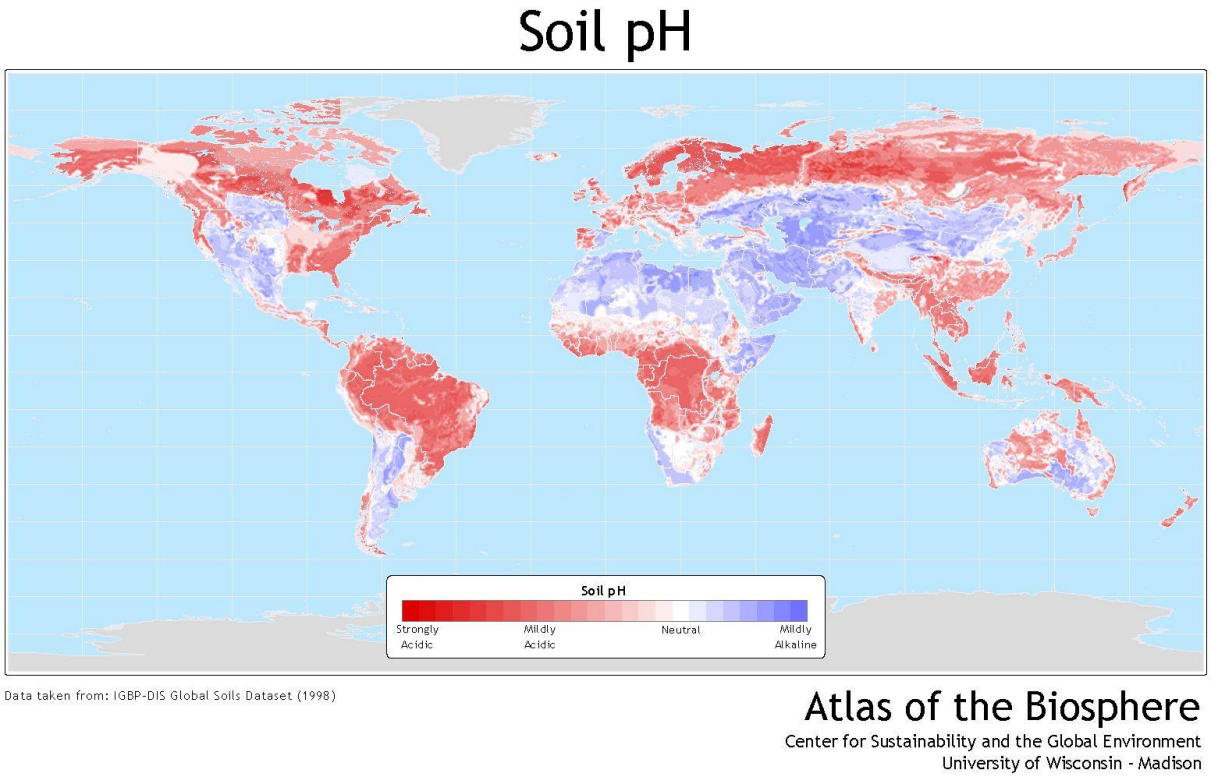

Figure 1.4. Global soil pH. Acidic pH is indicated by a strong red color, whereas alkaline regions are colored blue. There are two distinct areas with acidic pH, the northern belt and the southern belt, characterized by cold areas and tropical regions, respectively. Figure by IGBP-DIS (1998).

Soil acidification is a complex issue that as it is caused by several natural sources as well as being affected by human impact. Natural sources leading to soil acidification involve (but are not limited to) lack of essential nutrients and metals, carbon content, precipitation, and erosion and compaction (Bian et al., 2013; Von Uexküll & Mutert, 1995). In addition to acidity caused by natural sources, many industrial and agricultural process increases the acidity of soils. These processes include acidic rainfall, organic matter decay, application of both organic and synthetic fertilizer, as well as other cropping practices and burning of agricultural residues (Bian et al., 2013; Raut et al., 2012; United States Environmental Protection Agency, 2018). The use of nitrogen fertilizers has been shown to significantly increase the acidification of soils (Sumner & Noble, 2003; Tian & Niu, 2015; Vitousek et al., 1997) as seen in China where the overuse of fertilization caused the soil pH to decrease with almost one unit from the 1980s to the 2000s (Guo et al., 2010).

The increased agricultural management and fertilizer use are correlated with human population growth, especially in highly populated countries (Ravishankara et al., 2009). As the human population is growing the need for food grows correspondingly. This leads to the dilemma where fertilizer is needed as it provides nitrogen for plant uptake and growth and thus, contribute to increasing cropping yields (Adesemoye et al., 2010). While at the same time it causes soil acidification, and thus loss of soil nutrients, increasing atmospheric N_2O accumulation and thus, global warming (Vitousek et al., 1997). Developing nations especially suffer from this as many soils are naturally acidic and infertile which causes the yields to be low and therefore the need for fertilizer to be higher (Sumner & Noble, 2003).

1.4.1 Acidic African soils

Several regions of Africa suffer from low pH soils (Figure 1.5). Most African soils are old, highly weathered, and lack essential nutrients and organic matter, which contributes to low pH as well as poor fertility (Jones, A. et al., 2013). The tropical weathering with long rainy seasons also leads to increased acidification as rainfall causes leaching of base cations from the soil. The soils often contain high levels of iron and aluminium oxides further contributing to a poor soil state (Jones, A. et al., 2013).

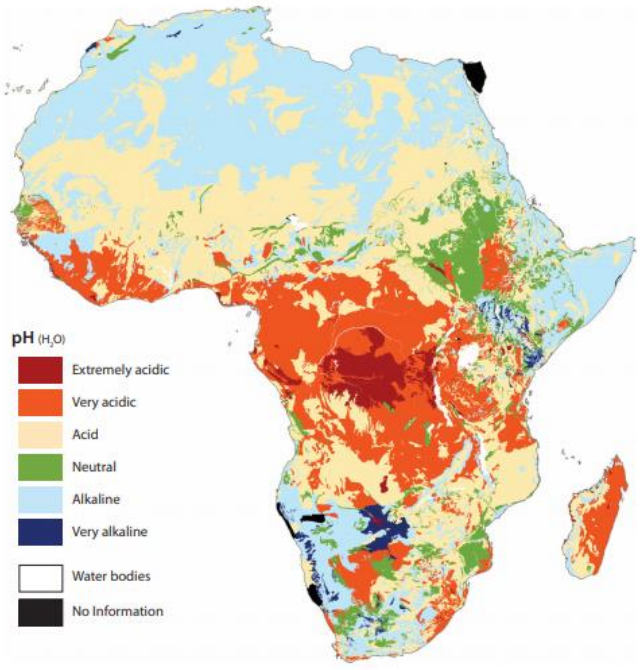

Figure 1.5. pH of soil in Africa. Acidic regions cover over half the continent's soils (burgundy, red and yellow regions). These acidic soil regions are caused by natural sources such as high weathering as well as poor agronomic practice and use of synthetic fertilizer. Figure by Jones, A. et al. (2013).

The poor soil state in the continent is aggravated by inappropriate land use and poor agriculture management. This leads to land degradation which is a serious issue in Africa as it affects more than half of all the African population who are directly dependent on locally grown crops (Bationo et al., 2006; Bationo et al., 2007). In developing continents such as Africa, limited use of fertilization is one of the main reasons for land degradation, in contrast to developed nations

where excess use of fertilizer and soil management has caused environmental damage. As the necessity for increased yields is rising due to a growing population, increased fertilization is needed to improve African agriculture, which is challenging as fertilization further increases soil acidification (Bationo et al., 2006; Hickman et al., 2011). This is problematic in regards of global warming as agricultural management involving fertilization causes N_2O emissions to rise and the emissions are further exacerbated by the low pH of the soils. It is therefore of pressing need to find a solution meeting both the demands for improved agricultural yields as well as limiting greenhouse gas emissions. Microbes capable of N_2O reduction in such low pH soils could be a potential solution that allows for both demands to be met and finding and isolating such organisms are therefore of great interest.

1.5 Aims

As N_2O emissions pose a large threat to global warming, it is of importance to understand the mechanisms behind this in order to address these emissions. In this study, the possibility of acidic tolerant N_2O reducers was investigated using two acidic African soils from north Ghana, Bush Fallow and Kpalgia 1. As previously mentioned, African soils are particularly suffering from low pH which is expected to be exacerbated by increasing use of fertilizers needed to overcome the low nutrients of the soils. Thus, methods to combat the low pH N_2O emissions in these soils are needed. There have been observations from low pH European soils of bacteria capable of low pH N_2O reduction, which leads to the question if it is possible to find such organisms adapted to growth in these unique African soils.

This study was inspired by observations by Lycus et al. (2017) (and other researchers) who have identified the existence of low pH N_2O reducers from several low pH European soils. The present project follows up on these observations, to see if low pH N_2O reducers can be identified and isolated from other types of soils, in this case, the physically and chemically distinct African soils of north Ghana. To increase the abundance of acid tolerant N_2O reducers up to a point where isolation was achievable an enrichment approach was used. Low pH African soils were given a surplus of electron donors which are likely to be available to a broad range of soil denitrifiers and N_2O was applied as the sole electron acceptor in order to perform a directed enrichment. Gas kinetics were monitored during enrichment before isolation was performed on a selective and differential growth medium and the isolates were identified through Sanger sequencing of the 16S rRNA gene. To get more knowledge about the microbial community

composition in the native and enriched soils, 16S rRNA amplicon sequencing was also performed.

When designing and developing an enrichment and isolation protocol there are several options to consider. These include deciding the soils to enrich from, which in this case were soil from North Ghana, as well as how to enrich the soil microbes. In the present study we chose to use soil slurries, but other options would be enriching intact soil or extracted bacterial cell cultures. Another decision was whether or not to buffer the soil slurries in order to keep pH stable, but at the same time avoid toxic impacts that could limit the microbes enriched. Choosing the electron donor and acceptor to provide was also necessary. The electron donor must be usable by most microbes to include a wide range of organisms, and the electron acceptor should select for the microbes we aim at enriching.

Low pH N_2O reducers native to African soils are of great interest as they are likely to make the best inoculants due to their adaptations to these soils. In addition, the low pH N_2O reducers identified in Europe are mainly from the genus *Rhodanobacter*, and by looking into these very different African soils, the aim is to find new taxa capable of performing N_2O reduction in acidic environments. This study aims to develop a better understanding of the mechanisms for N_2O reduction under acidic conditions in African soils through the enrichment and isolation of low pH N_2O reducers. Further, more long-term aims are to develop such bacteria as inoculants together with plant growth-promoting bacteria as a novel method for mitigation of N_2O.

2. Materials

2.1 Experimental solutions

1 M Na-glutamate stock

Components	Amount (g/L)
Na-glutamate ($C_5H_8NNaO_4*H_2O$)	187.2
Yeast extract	9.36

The volume was brought to 1 L using MilliQ water and the stock was filter sterilized (0.20 µm filter pore size) and stored at -20 ℃.

Phosphate buffering + glutamate stock to a concentration of ~ 30 mM

Components	Amount (g/L)					
	100 mM PB		150 mM PB		200 mM PB	
	pH 7.4	pH 5.8	pH 7.4	pH 5.8	pH 7.4	pH 5.8
$NaH_2PO_4*H_2O$	3.39	13.13	5.085	19.695	6.78	26.26
$Na_2HPO_4*2H_2O$	13.4	0.87	20.1	1.305	26.8	1.74
Glutamic acid	4.1	4.1	4.1	4.1	4.1	4.1
Yeast extract	0.2	0.2	0.2	0.2	0.2	0.2

The volume was brought to 1 L using MilliQ water and pH was adjusted to the specified pH using 5 or 1 mM NaOH or 4 mM HCl and autoclaved before use.

Modified Tryptic soy broth/agar (TSB/A)

Components	Amount (g/L)
Peptone from casein (pancreatically digested) (Merck Millipore, USA)	17
Peptone Hy-Soy ®T, Enzymatic hydrolysate (papain digest) (Sigma-Aldrich, USA)	3
NaCl	5
Glucose	2.5

The components were brought to a volume of 1 L with MilliQ water and the TSB without buffering was used for pH tests (3.5.2) while the TSA without buffering was used when agar plates were made for isolation (3.10). 0.015 g/L bromocresol purple and 15 g/L agar was also included in the solution when making agar plates. The solution was autoclaved and poured into petri dishes and left to set on the sterile bench.

Premade TSB (Sigma-Aldrich, USA) (including buffering) was used as the liquid medium during isolation (3.10).

1 M Hexose mixture

Components	Amount (g/L)
Glucose	90
Fructose	45
Xylose	45
Yeast extract	4.5
NH_4Cl	2.68

The components were brought to 1 L using MilliQ water before the solution was filter sterilized (0.20 µm filter pore size) and stored at -20 °C.

10X Sistrom's medium (without succinic acid)

Components	Amount (g/L)
K_2HPO_4 (or KH_2PO_4)	34.8 (27.2)
NH_4Cl (or $(NH_4)_2SO_4$)	1.95 (5.0)
L-Glutamic acid	1.0
L-Aspartic acid	0.4
NaCl	5.0
Nitrilotriacetic acid	2.0
$MgSO_4*7H_2O$	3.0
$CaCl_2*2H_2O$	0.15
$FeSO_4*7H_2O$	0.020
Trace elements solution*	1 mL
Vitamins solution**	1 mL
$(NH_4)_6MO_7O_{24}$ (1% solution)	0.2 mL

The components were brought to a total volume of 1 L with MilliQ water and stored at -20 ℃. When used it was thawed and diluted to 1X, pH was adjusted to 5.5 and the solution was autoclaved.

***Trace elements solution**

Components	Amount (g/L)
EDTA	17.65
$ZnSO_4*7H_2O$	109.5
$FeSO_4*7H_2O$	50
$MnSO_4*H_2O$	15.4
$CuSO_4*5H_2O$	3.92
$Co(NO_3)_2*6H_2O$	2.48
H_3BO_3	1.14

The volume was brought to 1 L with MilliQ water and stored at 4 ℃.

****Vitamins solutions**

Components	Amount (g/L)
Nicotinic Acid	10
Thiamine HCl	5
Biotin	0.10

The volume was brought to 1 L with MilliQ water, and the solution was stored at 4 ℃.

All pH measurements were performed using Delta 320 pH meter (Mettler Toledo AG, Switzerland).

3. Methods

3.1 The two African soils and their treatments

Two African soils, Bush Fallow (BF) and Kpaliga 1 (K1), were used in this experiment. They were both taken from cropping fields in north Ghana, located in the Guinea Savanna Zone, which is characterized by tropical savannah woodland and perennial grass species (Alhassan & Barnes, 1993). The soils have been exposed to highly variable rainfall and temperature, with both rainy and dry seasons from April to October and November until March, respectively. The annual mean temperature is ~28 ℃, however, this value ranges from 25 -36 ℃ between seasons. Humidity is seasonally variable with 65-85 % in the rainy season and down to 20 % during the dry season (SARI, 2014).

The Bush Fallow site has been fallowed with different perennial grasses for more than 10 years. From 2019, the soil was converted into a long-term legume crop and has been under cultivation since. The Kpaliga 1 site has been under cultivation for more than 10 years, where mineral fertilizers (NPK and urea) and urea have been applied every other year and maize and chili pepper have been cropped in rotation the last four years. Both soils are classified as Ferric Luvisols (FAO) and hold low amounts of carbon and nitrogen (see Table 3.1 for the soil characterization of the BF soil).

Table 3. 1. Soil characterization of soil from the Bush Fallow (BF) site.

Soil Property	**Value(s)**
SOC (g C kg^{-1})	3.81
Total N (g N kg-1)	0.604
NH_4^+- N (mg N kg^{-1})	5.32
NO_3-N (mg N kg^{-1})	0.36
Available N (NH_4+N+ NO_3-N) (mg kg^{-1})	5.68
Bray-1 P (mg kg^{-1})	10.04
Exchangeable Cation(s):	
K^+ (cmol $_{(+)}$ kg^{-1})	0.219
Ca^{2+} (cmol $_{(+)}$ kg^{-1})	1.388
Mg^{2+}(cmol $_{(+)}$ kg^{-1})	0.585
Na^+ (cmol $_{(+)}$ kg^{-1})	0.113
Total effective CEC (cmol $_{(+)}$ kg^{-1})	2.305
Soil pH (soil: H_2O; 1:5)	5.98
Textural Class	
Sand (%)	67.4
Silt (%)	27.2
Clay (%)	5.4
	Sandy loam

Samples from both soils were collected from 10-15 cm depth in November 2020 and transported to Norway. The soils were sieved through a 2 mm sieve and stored in the dark at 4 ℃.

3.1.1 Fumigation of the soils

To reduce the biomass of the soils, sections from both soils were fumigated using a chloroform fumigation protocol described in detail by Lim et al. (2016), with some modifications. A brief overview of the method is described here. The soils were transferred to aluminium containers, put into a glass vacuum desiccator, and treated with boiling chloroform vapor under vacuum for 1 minute. This was repeated three times with aeration to laboratory air in between. The desiccator was then left sealed with chloroform atmosphere for 24 h before it was opened, and chloroform was allowed to evaporate off. Variations from Lim et al. (2016) included that the chloroform was not water-washed and the chamber was not flushed 15 times before evaporation into air. The procedure was repeated three times before any residual chloroform was allowed to evaporate off and the soils transferred to 50 mL Falcon tubes and stored at 4 ℃ until use.

3.2 Enrichment protocol – general setup:

As the aim of this study was to enrich a low pH N_2O reducing bacterial population, a general enrichment protocol was set up. The enrichment was planned to go over 5 cycles, where each cycle would consist of 5 replicates from each soil at both neutral and acidic pH. As the experiment did not proceed as planned, the final enrichment went on for 3 cycles for acidic K1 soil slurries and for 1 cycle for acidic BF soil slurries. Neutral soil enrichment slurries were started for K1 soil, but not finished. No neutral slurries were started for BF soil.

Serum vials (120 mL) containing a stirring magnet were covered with aluminium and autoclaved for 20 min at 121 ℃ to ensure sterilization before use. Twenty-five mL liquid (specified in each section) was added to the vials together with 5 g fumigated soil, creating a soil slurry. This was done under sterile conditions by treating all equipment with 70 % ethanol and working in a sterile laminar flow hood. For the first cycle, 1 g native soil was used as an inoculum, while the next cycles were inoculated with 2.5 mL enrichment material from the previous cycle. A carbon source (specified in each section) was also added to the vials to provide a an electron donor. As the aim was to enrich a population of microbes a sufficient amount of a suitable carbon source was required. In these enrichments, 5x the amount of carbon needed to reduce 30 mL of N_2O was given to the slurries, in addition to 5 % yeast extract (based on the amount of carbon) to make sure that all vitamins needed were present. A brief overview of the general enrichment setup is shown in Figure 3.1. The vials were sealed with butyl rubber septa and aluminium crimps before the headspace was replaced with He through four cycles of

gas evacuation (180 s) and helium flushing (30 s) (Molstad et al., 2007). This treatment is referred to as He headspace throughout the thesis. After the headspace had been replaced with He, the vials were placed in a water bath at 23 °C with stirring at 500 rpm before overpressure was released. The vials were supplied with 0.7 mL O_2, to allow a smooth transition from oxic to anoxic metabolism, and an initial 5 mL N_2O before overpressure was released once more. A target total of 30 mL N_2O was to be reduced by each replica in each cycle, where the additional N_2O was to be given in doses of 5 or 10 mL. Measurements of gas kinetics were so performed (as described below in 3.3 and demonstrated in Figure 3.2).

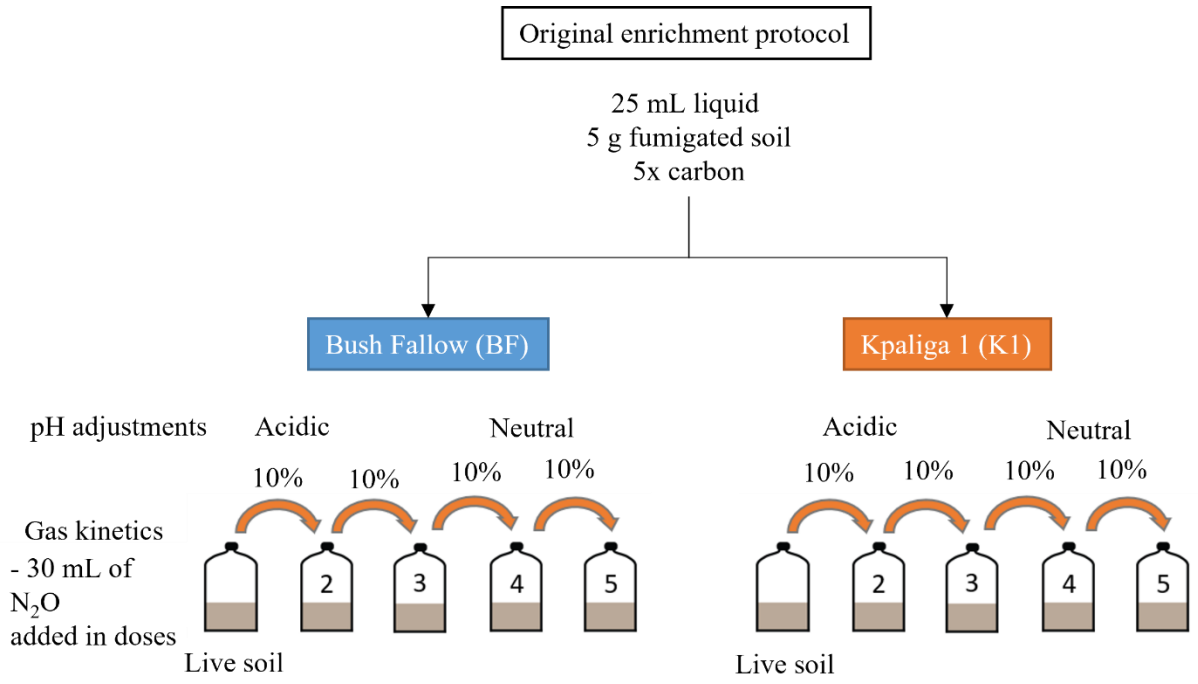

Figure 3. 1. Overview of the enrichment process. Originally, the soil slurries were planned to be enriched over 5 cycles, but this was reduced to 3 cycles. Each cycle consisted of 5 replicates. The first slurry was inoculated with native soil, while the next cycles were inoculated with 10 % of the enriched slurry from the previous cycle (2.5 mL). Each replicate was to be given a total of 30 mL of N_2O in doses and headspace gas kinetics were measured.

3.3 Measurements of headspace gas kinetics

Gas kinetics were measured using the robotized incubation system presented in Molstad et al. (2016) (Figure 3.2). This incubation system monitors, in real-time, concentrations of O_2, CO_2, H_2, NO, N_2O and N_2 in the headspace of the vials by frequent sampling. The incubation system consists of a water bath with magnetic stirring to ensure the desired temperature, avoid aggregates and provide continuous gas exchange between the slurries and headspace. A peristaltic pump was used to sample and transport gas from the vials to a Gas Chromatograph (GC) and a NOx analyzer. For each gas sample taken, He gas is returned to account for the

sample volume taken and avoid pressure change. Three gas standards were included, High, Low, and NO. The high gas standard contained 151 ppm of N_2O, 10 000 ppm CO_2 and 10 000 ppm CH_4, while the low standard contained 0.585 ppm of N_2O, 361 ppm CO_2, 1.89 ppm CH_4, 210 000ppm O_2 and 78 000 ppm N_2. The NO standard contained 25 ppm NO. These standard vials were used for calibration and to correct for leakage in and out during sampling.

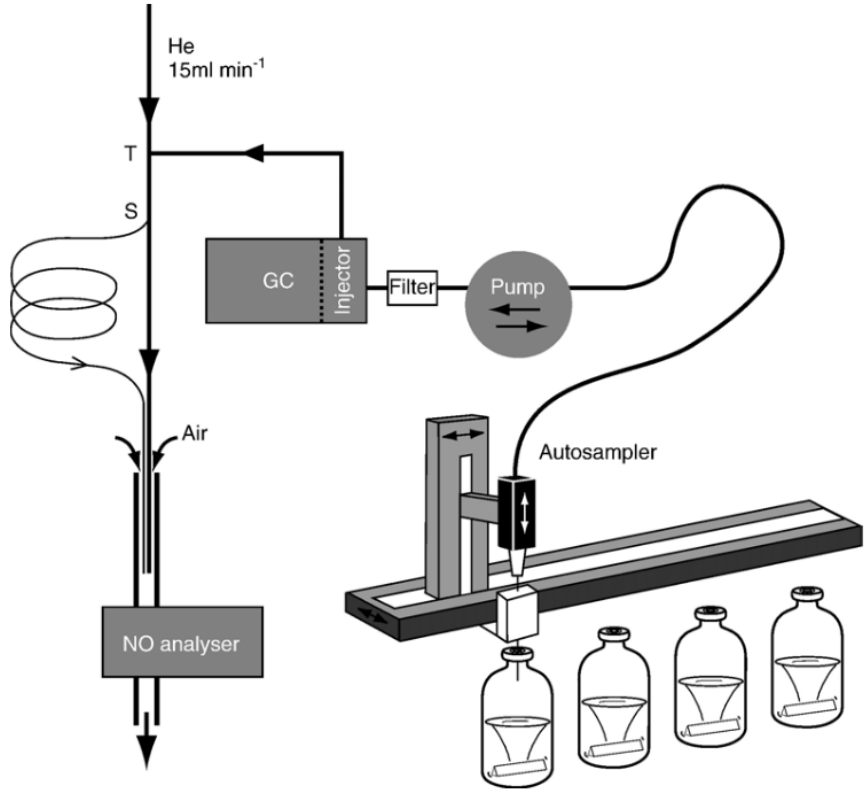

Figure 3. 2. The robotized incubation system designed by Molstad et al. (2007). The gases are sampled from vials using a peristaltic pump with He as the carrier gas and transported to a gas chromatograph (GC) and an NOx analyzer to analyze the headspace gas composition at different time points.

3.4 Enrichment 1: Enrichment experiment without buffering and with glutamate as the carbon source

In the first round of the enrichment experiment, a total of 20 soil slurries were prepared using the general enrichment protocol (see under 3.2) where unbuffered MilliQ water was the liquid used. These soil slurries included 10 vials from each soil where five were neutral and five were acidic. Due to the soil's natural pH, the acidic vials had a pH of 5.65 and only the neutral vials needed pH adjustment to pH 7.5. The soil slurries contained 25 mL MilliQ and 5 g fumigated soil and pH was adjusted by adding 100 µL 0.5 M NaOH to the neutral BF soil slurries and 50 µL 0.5 M NaOH to neutral K1 soil slurries. The carbon source used in this first experiment was

glutamate. Glutamate was chosen as an electron donor since it can be utilized by a broad range of bacteria. The amount of glutamate added was five times of that needed for the reduction of 30 mL of N_2O, to ensure that the carbon source was in excess and would not be depleted during the enrichment. For calculations see Appendix 6.2.1. In addition to glutamate, 5 % yeast extract (w/v) to provide nutrients was added.

The soil slurries were inoculated with 1 g of native soil before 700 µL of the 1 M Na-glutamate stock solution was added into each vial. The vials were made anoxic, placed in the incubation system with stirring and gas kinetics were measured. The stirring was at 650 rpm and the temperature at 22 ℃. After 73 h, the pH was measured using 1 mL of each slurry pulled out with a syringe, and it was observed that all the slurries had changed to a neutral pH. Their gas kinetics was measured until 260 h, where BF soil had received a total of 15 mL N_2O and K1 soil had received the full 30 mL. pH was measured at the completion of the enrichment and showed pH > 7.0 for all slurries. Based on the lack of pH control, these slurries were not used for further enrichment and the method had to be optimized.

3.5 Approaches to stabilize the pH of the slurries

To get a better understanding of what caused the pH to rise in the first enrichment experiment, several tests were conducted.

3.5.1 The effect of natural vs. fumigated soil on slurry pH

First, the effect of fumigated versus native soil on pH was tested. Soil slurries containing either 2 g of fumigated or native K1 soil mixed with 10 mL 0.01 M $CaCl_2$ solution were made using the same ratio as in the general enrichment setup. The slurries were incubated aerobically at room temperature with stirring at 600 rpm. The pH was measured with 10 minute intervals over the course of 1.5 h. Inoculating fumigated soil with native soil was also tested, where pH was measured before and after inoculating with 1 g native soil into the fumigated soil slurries.

3.5.2 The effect on pH of glutamate additions to soil slurries

To investigate if the addition of the 1 M Na-glutamate solution affected the pH, 280 µL was added into the vials containing 2 g fumigated K1 soil, 0.4 g native K1 soil and 10 mL 0.01 M $CaCl_2$ (3.5.1). pH measurements were done before addition, and immediately after. These

results showed an increase of over one pH unit just after addition and the pH continued to rise throughout the incubation of 48 h. Based on this observation it was necessary to change the enrichment protocol and find a way to keep the pH stable.

3.5.3 Phosphate buffering to keep pH stable

Buffering the slurries was tested as a solution to the pH problem. A buffered solution was made containing phosphate buffer, glutamic acid, and yeast extract. Phosphate buffering concentrations of 100 mM, 150 mM and 200 mM were made, with 30 mM glutamic acid and 0.2 g yeast extract added (2.1, Phosphate buffering + glutamate stock to a concentration of ~30 mM). The buffer solutions were autoclaved, and 10 mL was added to flasks containing 2 g native soil from either K1 or BF. Both neutral (pH 7.4) and acidic (pH 5.8) slurries were made. The flasks were incubated aerobically for 100 h, at room temperature, with vigorous stirring at 600 rpm and pH was measured at time intervals. None of the buffer concentrations kept pH stable during the aerobic incubation. The buffered solutions of 150 mM and 200 mM PB were also tested on acidic slurries (pH 5.8) during anaerobic incubation. After 100 h, pH was just below 6.0, which was borderline for the planned experiments since the pH was expected to rise more during the reduction of N_2O and growth on glutamate. Higher buffering concentrations were not tested as these might exclude phosphate sensitive organisms from being enriched.

3.5.4 The effect of carbon source on slurry pH

Since pH could not be kept stable with glutamate as the carbon source, two other carbon sources were tested. One was a Tryptic soy broth (TSB) solution (2.1, Modified Tryptic soy broth/agar (TSB/A)), and the other was a hexose mixture (2.1, 1M Hexose mixture). The hexose mix consisted of glucose, fructose, and xylose in a 2:1:1 ratio, 100 mM NH_4Cl, and 4.5 g yeast extract per L. Both carbon sources were made into a 1 M solution, filter-sterilized (0.20 µm), and stored at -20 ℃. pH of the solutions was also measured where TSB had a pH of 6.23 and the hexose mix had a pH of 6.47. Calculations for the amount hexose mixture needed were done by calculating the amount of a hexose (eg. Glucose) needed for the reduction of 30 mL of N_2O (these calculations are found in Appendix 6.2.2).

Soil slurries were made for both soils with 2 g native soil and 10 mL 150 mM PB pH adjusted to 5.8 and 7.4. Slurries of each soil and at each pH was given 225 µL of the 1 M TSB solution, and the same setup was used with the 1 M hexose mixture. Both carbon sources were added to

a final concentration of 30 mM. The flasks were incubated aerobically at room temperature (~22 ℃) with stirring (600 rpm). The pH of the slurries was measured at several time points over 40 h. To ensure that pH was not affected by the buffer itself, 150 mM PB in soil slurries without any carbon was also tested. Based on the tests described in 3.5 it was concluded that the 1 M hexose mixture was the best carbon source to ensure a stable pH for enrichment with acidic African soils.

3.6 Enrichment 2: Enrichment using 150 mM PB and hexose mixture

After hexoses were chosen as the carbon source for subsequent enrichments, vials were prepared using the general enrichment protocol for a second enrichment. 25 mL of 150 mM PB solution and 5 g fumigated soil was added to each vial. The vials were inoculated with 1 g of native soil and hexoses to a final concentration of 30 mM. The slurries made anaerobic and given a He headspace and was provided with 0.7 mL O_2 and 5 mL N_2O before being incubated at 22 ℃ with stirring (500 rpm). Shortly after the gas measurements were started, high production of CO_2 was observed, which did not correspond with equivalent N_2O reduction and was most likely caused by high fermentative activity. As an attempt to stimulate denitrification (after 96 h), and thus initiate N_2O reduction, 100 µL 1 M $NaNO_3$ and 200 µL of 1 M hexose mixture was added to BF and K1 replica 1-3. BF replica 4 and 5 and K1 replicate 9 got an extra addition of 200 µL of 1 M hexoses whereas K1 replica 5 did not receive anything. After 260 h the gas measurements were stopped, and the vials were left at 22 ℃ with stirring (500 rpm) for 48 h, and then stored without stirring and at 4 ℃ for 168 h.

3.7 Examination of the high CO_2 production

3.7.1 CO_2 production caused by iron reduction

The reduction of Fe(III) to Fe(II) by iron respiring bacteria could theoretically be a potential source of the high CO_2 production. To investigate if Fe(III) respiration took place in vials containing the hexose mixture, the amounts of Fe(II) produced in slurries with and without the hexose mixture was compared. The slurries without hexose mixture were freshly made and contained 5 g fumigated soil, 25 mL 150 mM PB, and 1 g native soil, while the slurries with hexose mixture was chosen from enrichment 2 (the enrichment with hexoses, see chapter 3.6, BFA-1-4 and K1A-1-4) and had been incubated anaerobically for 260 h. The pH was measured

to make sure they were similar before Fe(II) extraction was attempted. The slurries were transferred to 50 mL centrifugation tubes and centrifuged at 10000 g for 10 minutes at 20 ℃. The supernatant was transferred to new tubes and filter sterilized using a 0.20 µm filter. The Fe(II) concentration was measured using a spectrophotometer (Hach-Lange, USA) and a set of Lck 320 tubes that measures Fe(II) concentrations ranging from 0.2-6.0 mg/L. Portions of 2 mL of the samples were added to the tubes and mixed well. They were incubated on the bench for 5 minutes before being mixed again and Fe(II) was measured.

3.8 Enrichment 3: Enrichment of extracted bacterial cells using a Nycodenz density gradient

As it was confirmed that the soils were rich in iron and that reduction of Fe(III) to Fe(II) took place when carbon was provided, an attempt to avoid this issue was performed by extracting cells from soils instead of using of soil slurries in the enrichment. Portions of 20 g native soil from both BF and K1 was mixed with 200 mL MilliQ water using an Omni mixer (Omni international, USA) to dislodge cells from soil particles. This was done 5 times, resulting in a total of 1 L slurry from 100 g soil for each soil. The slurries were blended in the mixer for 3x1 minute at speed 6 m/s and kept on ice for 1 minute between rounds. The mixed soils were left on the bench for 30 minutes so that soil particles could settle.

The bacteria were separed from the soil using a Nycodenz density gradient (Bakken & Lindahl, 1995). A Nycodenz solution was made using 8 g Nycodenz in 10 mL MilliQ water, heated and stirred to dissolve, and then filter sterilized (0.45 µm). Centrifuge tubes (40 mL) were sprayed with ethanol to sterilize and allowed to evaporate off before 30 mL of the blended soil and 6 mL Nycodenz solution were added. The Nycodenz solution was added using a long syringe to carefully place the Nycodenz under the soil solution, making a Nycodenz cushion. The tubes were so centrifuged (Beckman Coulter, USA) with slow acceleration and no deceleration at 4 ℃ at 10000 g in a swingout rotor for 1 h. The supernatant was siphoned off using a vacuum pump (Figure 3.3.1) into a waste vial, before the ring of extracted bacterial cells floating on top of the Nycodenz layer, was siphoned off the same way into a fresh vial (Figure 3.3.2). The extracted bacterial cells were stored with 20 mL of 1 M Phosphate Buffered Saline at pH 7.4 and at 4 ℃ overnight, then transferred to 50 mL centrifuge tubes and centrifuged for 10 minutes at 10000 g and 4 ℃ with a fixed-angel rotor. The supernatant was poured off, and the pellet

was resuspended in autoclaved 5 mL Sistrom's medium (Sistrom, 1962) without succinic acid at pH 5.5 (2.1, 10X Sistrom's medium (without succinic acid).

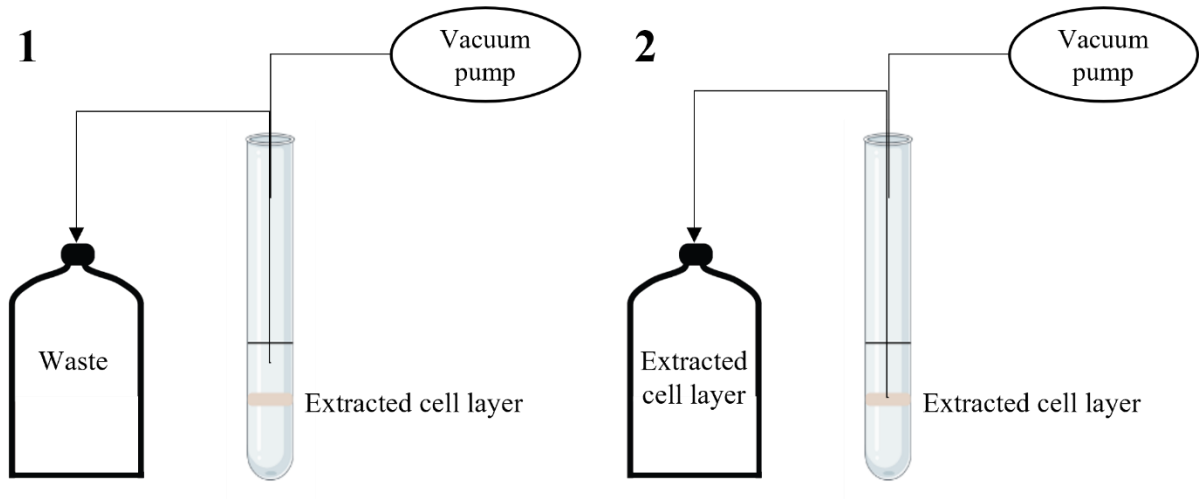

Figure 3. 3. Setup for siphoning off the extracted bacterial cell layer on top of the Nycodenz layer. 1) A vacuum pump was used to remove the supernatant above the extracted cell layer before **2)** the extracted cell layer was vacuumed off into a fresh vial.

Twelve vials (6 from each soil) containing 50 mL autoclaved Sistrom's medium without succinic acid and adjusted to pH 5.5 were made, and 500 µL of the extracted bacterial cell layer solution was added together with 560 µL of 1 M hexose mixture to a final concentration of 30 mM hexoses. The headspace was replaced with He and 0.7 mL O_2, and 5 mL N_2O were added. Measurements of gas kinetics were started at 23 °C with stirring at 500 rpm, and the vials were incubated for 1050 h.

3.9 Further enrichments using the hexose mixture as carbon source (Enrichment 2)

The enrichment vials containing soil slurries provided with a hexose mixture described in 3.6 were removed from the cold room and incubation was restarted. 1 mL of each soil slurry was pulled out using a syringe and pH was measured to ensure that the enrichment did not exceed the threshold of pH 6.1. The vials were incubated for 1260-1600 h and were given new doses of 5 mL N_2O once the previous dose had been reduced until a total of 30 mL N_2O had been reduced. Once the 30 mL N_2O had been reduced, pH was controlled, and a new cycle was started. BF soil slurries had a pH higher than 6.1 so a second cycle was not continued for this

soil. One K1 slurry had managed to reduce 30 mL N_2O after 1260 h and kept a low pH value. This slurry was therefore used as an inoculum for a second cycle.

A second cycle of the enrichment was performed using the general enrichment setup with 150 mM PB, 5 g fumigated K1 soil, and hexose mixture to a final concentration of 30 mM. The slurries were inoculated with 2.5 mL of the cycle 1 slurry material, before the headspace was replaced with He by sequential evacuations and He filling. Five replicas were made for cycle 2 using one of the slurries from cycle 1 as an inoculum. All the slurries received 5 mL of N_2O at the start of the incubation and gas kinetics were measured. The N_2O concentrations were monitored and new portions of N_2O were added in doses of 5 mL until 30 mL had been reduced by the slurries. pH was measured after the complete reduction and verified to be below 6.1. A third cycle was also performed. This was done using the same procedure as for cycle 2 but using a 2.5 mL inoculum from a cycle 2 slurry instead of from a cycle 1 slurry. This was the last cycle of the enrichments.

The final enrichment slurries from each cycle were stored at -20 ℃ in aliquots of 1 mL in 1.5 mL Eppendorf tubes. Moreover, 2-3 portions from each replica were stored as glycerol stocks where 500 µL slurry was mixed with 500 µL 50 % filter-sterilized (0.20 µm) glycerol for later isolation efforts.

3.10 Isolation of bacteria from the acidic African soils

Isolation was performed to acquire axenic cultures of low pH N_2O reducers from the African soils to study their physiology. The isolation process was adapted to select for low pH N_2O reducers and avoid fermenters.

The first round of isolation was performed using natural BF and K1 soil slurries prepared using an Omni mixer (see chapter 3.8). Dilution series were set up based on a conservative assumption that 1 g soil contained 10^8 cells, since it was expected that this was low-biomass soils. The blended soils were diluted in a 10-fold dilution series down to $1{:}10^7$ and 100 µL of the $1{:}10^5$, $1{:}10^6$, and $1{:}10^7$ dilutions were transferred to 1/10 dilution TSA plates without buffering (2.1, Modified Tryptic soy broth/agar (TSB/A) and spread with a sterile glass spatula. The TSA plates used had been pH adjusted to 5.8 and was made with a 1/10 dilution to allow for the isolation of slower-growing bacteria. The plates also included a pH indicator dye, bromocresol purple, to differentiate between fermenter and N_2O respiring colonies as fermenters would reduce the pH of the agar around the colony by producing acidic fermentation products. These

plates are further referred to as modified TSA or 1/10 TSA plates depending on their dilution. After inoculation of the plates with soil slurry dilutions, the plates were placed in an anaerobic chamber which was flushed with N_2 for a minimum of 15 minutes. The chamber was filled up with 18 mL of N_2O and the agar plates were incubated anaerobically with N_2O as the sole inorganic electron acceptor. An Oxoid™ AnaeroGen™ 3.5 L (ThermoFisher Scientific, USA) sachet was added to the chamber to remove any residual oxygen. For the first round of isolation, the chamber was incubated at room temperature for 13 days before opening. Colonies were selected based on their morphology, to achieve as high diversity as possible and were picked and streaked out on fresh modified TSA plates. These were then incubated aerobically at room temperature. Once colonies had grown, they were picked and re-streaked twice to avoid contaminants.

After the second round of plating, isolates were picked and transferred to sterile vials containing TSB at pH 7.0 and 5.8 (2.1 Premade TSB (Sigma-Aldrich, USA). The vials were incubated in a water bath at 23 ℃. Once turbidity was seen in the media, 100 µL of the culture was used to inoculate fresh TSB vials at pH 7.0 and 5.8. The vials were treated as described in 3.2 where they were made anaerobic by receiving He headspace, before 0.7 mL O_2 and 1 mL N_2O were given. The vials were incubated at 23 ℃ with stirring (500 rpm). The gas kinetics was monitored for 70 h after which the vials were incubated off-line for a total of one week. One round of headspace gas measurements was then performed, using the roboticized incubation system described in 3.3, to measure the concentrations of N_2O and N_2 in the headspace. This is referred to as endpoint analysis in the following sections.

The second isolation was performed using slurries from cycle 1 K1 and BF (K1A-1-5 and BFA-1-5). Portions of 1 mL slurry was taken from the vials after the first 5 mL N_2O was reduced in both. The following isolation and incubation of bacterial isolates was performed as previously described with the following changes. The second round of isolation was performed using dilutions of $1{:}10^3$, $1{:}10^{4,}$ and $1{:}10^5$ and the plates were incubated in the anaerobic chamber for 3 days. 14 K1 colonies were picked and inoculated into TSB vials and 14 BF colonies were inoculated into 1/10 TSB vials. All colonies were incubated at 23 ℃ with stirring (500 rpm) and after turbidity was visualized, 100 µL from each colony was used to inoculate fresh TSB or 1/10 TSB vials before they received a He headspace, 0.7 mL O_2 and 1 mL N_2O. K1 isolates were monitored for gas kinetics for 42 h, and then an endpoint measurement was done after a total incubation of 1 week. BF isolates were only measured at endpoint after being incubated for 1 week.

The third round of isolation was performed on one K1 slurry from cycle 2 (K1A-2-2). This was done the same way as for the BF soil in the second round of isolation, with the following modifications. This time, 100 µL of each dilution was transferred to both modified 1/10 TSA and full modified TSA plates. The plates were incubated in the anaerobic chamber until colonies was observed before being picked and isolated twice on 1/10 or full modified TSA plates. The colonies were then transferred to vials containing 1/10 TSB and incubated at 23 ℃ with stirring (500 rpm). Once growth was observed, 100 µL was used as inoculum into anoxic vials given a He headspace with 1 mL N_2O and 0.7 mL O_2 and endpoint measurements was done after 1 week incubation.

Glycerol stocks were prepared for all isolates, where 1 mL 50 % glycerol and 1 mL µL of bacterial culture were mixed well and stored at –80 ℃.

3.11 Sanger sequencing of the 16S rRNA gene in the African soil isolates

Sanger sequencing of the 16s rRNA genes was performed on the third round of isolates (3.10.3) (Sanger et al., 1977). The 16S rRNA genes from the isolates were amplified by PCR using the DreamTaq Green PCR Master Mix (2X) (ThermoFisher Scientific, USA) following the manufactures protocol using the components and thermo cycling conditions outlined in tables 3.2 and 3.3.

Table 3. 2. Components in PCR reactions.

Reagent	Volume
DreamTaq Green PCR Master Mix (2X)	25 µL
Forward primer (27F, 10mM)	5 µL
Reverse primer (1492R, 10mM)	5 µL
Template DNA	5 µL
Water, nuclease-free	To 50 µL
Total volume for each reaction	50 L

Table 3. 3. Thermal cycling conditions for PCR reactions.

Step	Temperature ℃	Time	Number of cycles
Initial denaturation	95	1-3 min	1
Denaturation	95	30s	30
Annealing	52	30s	
Extension	72	1 min	
Final extension	72	5-15min	1

The PCR products were verified on a 2 % (w/v) agarose gel with PeqGreen (VWR Peqlab, USA) (4 µL per 100 mL gel). 45 µL of each PCR product was loaded onto individual wells, in addition to a 100 bp DNA ladder (N32315 100bp DNA ladder) in a separate well. The PCR products were separated on the gel for 45 minutes at 80 V. The gel bands were visualized by a UV transilluminator (Gel Doc XR, Bio-Rad Laboratories, USA) to ensure that the reaction had amplified the correct region, before a scalpel was used to cut out the bands. The DNA was extracted from the gel using an E.Z.N.A.® Gel Extraction Kit (V-spin) (Omega Bio-tek, USA) following the manufacturers protocol.

The extracted DNA samples were prepared for Sanger sequencing of the 16S rRNA gene by preparing one tube with 5 µL sample and 5 µL 10 mM 27F primer (5'-AGA GTT TGA TCM TGG CTC AG-3') and one with 5 µL sample and another 5 µL 10 mM 1492R primer (5'-GGT TAC CTT GTT ACG ACT T-3') (Weisburg et al., 1991). The samples were then shipped to Eurofins Genomics for Sanger sequencing.

3.11.1 Sequencing data analyses

After receiving the results from the Sanger sequencing, some data processing needed to be done. A consensus sequence was made from the forward and reverse strand using Serial Cloner 2.6 (SerialBasics). Once all the isolates had a consensus sequence, they were run through nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/) to search for matching species against the reference RNA sequences database (Johnson et al., 2008). In addition to the isolate sequences, the two best hits for each isolate from BLAST and the 16S rRNA gene from *E. coli* as an outlier were used to make a phylogenetic tree. The software used to make the phylogenetic tree was Phylogeny.fr (http://www.phylogeny.fr/simple_phylogeny.cgi) (Dereeper et al., 2008) and the tree was subsequently visualized in EvolView (https://www.evolgenius.info/evolview/#login)

(Zhang et al., 2012). Phylogeny.fr was used to analyze molecular sequences and reconstruct the phylogenetic relationship between the sequences. This is done using MUSCL for multiple alignments, PhyML for building the tree, and TreeDyn for tree rendering and by using a confidence index (Dereeper et al., 2008). EvolView was used for tree visualization (Zhang et al., 2012). To classify the isolate sequences, the SILVA Alignment, Classification and Tree service (ACT) (https://www.arb-silva.de/aligner/) (Pruesse et al., 2012) was used. This software uses the least common ancestor (LCA) method to search and classify organisms.

3.12 DNA extraction and purification

The 1 mL portions of soil slurries that were stored frozen from the enrichment with hexoses (3.9) were used for DNA extraction, purification, and amplicon sequencing of the16S rRNA gene. These samples included frozen soil slurries from K1 cycle 1, 2 and 3, in addition to native K1 soil. The DNA extraction was performed using the kit DNeasy® Powersoil® Kit (Qiagen, Germany) by following the protocol provided with the following modifications. To get concentrated enough soils from the slurries, 2-3 of the 1 mL frozen slurries were thawed and mixed before being centrifuged at 10 000 g for 10 minutes at room temperature. The supernatant was poured off and the cell pellet was used instead of the soil sample. 1 mL soil slurry cell pellet was used for each sample. Instead of using a vortex adapter for homogenization and cell lysis, a FastPrep® 24 (M.P. Biomedicals, USA) was run for 2 x 40 sec at 6 m/s while resting on ice for 5 minutes in between runs.

To further improve the quality of the extracted DNA a purification kit was used (Genomic DNA Clean & Concentrator®-10 (Zymo Research, USA) according to the manufacturer's instructions. Through these steps, DNA from K1 native soil and K1 cycle 1, 2 and 3 soil slurries were extracted and purified.

3.12.1 Quantification of extracted DNA - NanoDrop and Qubit

A NanoDrop spectrometer (ThermoFisher Scientific, USA) was used to quantify the amount of DNA present in the extracted DNA samples based on UV-visible absorbance. NanoDrop measures a 260/280 ratio and a 260/230 ratio that both works as a measure of purification. DNA absorbs light at 260 nm, and pure samples will be indicated by a peak only at that wavelength. Proteins and other contaminants absorbs light at 280 nm and 230 nm and the ratio between

those wavelengths and 260 nm will therefore indicate the purity of the sample (Desjardins & Conklin, 2010).

To get more accurate measurements of the concentration of the DNA extracted, a Qubit™ fluorometer and Qubit™ dsDNA BR Assay Kit were used (ThermoFisher Scientific, USA). In this study, a broad range kit was used to include a range from 2-1000 ng DNA). Qubit measurements are based on fluorescent dye bound to the nucleic acids where the fluorescence is emitted only when bound to target molecules (here DNA). The fluorescence signal will then be directly proportional to the concentration of DNA in the solution. Qubit fluorescence is a more precise quantification method than NanoDrop, but both were performed to include the purity of the sample and the amount of DNA extracted (Simbolo et al., 2013).

3.13 16S rRNA gene amplicon sequencing

16S rRNA amplicon gene sequencing was performed by Novogene on the native soil and soil slurries from the DNA extraction and purification in chapter 3.12. This technique is used for insight into the microbial community composition and is based on the conserved 16S rRNA gene where the gene is used to classify and characterize reads using operational taxonomic units (OTUs) with a sequence similarity threshold of 97 % (Johnson et al., 2019).

To ensure that the extracted DNA samples met the quality demands from Novogene, the samples were concentrated using the Savant SpeedVac SVC 100H Centrifugal Evaporator. The centrifugation was done at 17 000 g and 36 ℃ with vacuum to evaporate water and leave a more concentrated DNA solution. Once the samples met the requirements, they were sent to Novogene for 16S rRNA gene amplicon sequencing where the V3-V4 region of the gene was amplified and sequenced using 250 bp paired-end Illumina sequencing.

The results from the 16S rRNA gene amplicon sequencing were processed through the in house GHAP pipeline built around tools from USEARCH and RDP (Cole et al., 2014; Edgar, 2013). GHAP works by clustering and classifying 16S rRNA gene sequences into OTUs at 97 % similarity and mapping their associated read counts across all samples. The pipeline classifies each OTU by finding their closest match in a set of reference 16S rRNA gene sequences from the databases RDP 16S Training Set and the RefSeq 16S reference sequence collection. The results are taxonomic classifications including confidence scores for the assignments.

4. Results

One of the main aims of this study was to develop a protocol for the enrichment of acid tolerant N_2O reducing bacteria from acidic African soils. Two soils were enriched in soil slurries and incubated anaerobically through several cycles by providing N_2O and a carbon source. Different carbon sources and buffer concentrations were tested to achieve a successful enrichment of acid tolerant N_2O reducing microbes from these soils. Bacteria were isolated from the enrichments of one of the soils (K1) and some isolates were identified by 16S rRNA gene sequence analysis. Moreover, a 16S rRNA gene amplicon sequencing was done of the total prokaryote soil community.

Throughout the thesis the slurries are named accordingly (Figure 4.1): 1) BF or K1 indicating which of the two soils that has been used (Bush Fallow, Kpaliga 1, respectively), 2) A or N indicating if they are acidic or neutral, 3) a number indicating the cycle of the enrichment and 4) the replicate number.

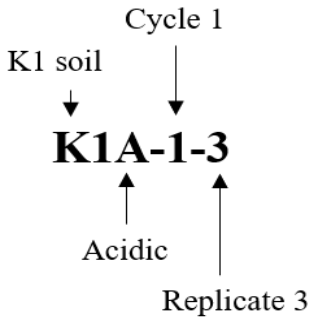

Figure 4. 1. Illustration of the naming of the soil slurries. K1 or BF indicating the soil, A or N indicating pH, the cycle of enrichment and the replicate

4.1 Initial enrichment experiment without buffering and with glutamate as the carbon source

The first attempt at enriching microbes from the two acidic African soils (Bush Fallow, BF, Kpaliga 1, K1) was done using soil slurries provided with glutamate as the carbon source. All slurries were given a He headspace, before receiving Na-glutamate to a final concentration of 30 mM, 0.7 mL O_2 and 5 mL N_2O. Both neutral (N) and acidic (A) soil slurries were prepared for both soils having an initial pH of 7.5 and 5.6, respectively. After 73 h of incubation at 22 °C with stirring (600 rpm), the pH was measured in 1 mL pulled out using a syringe from one neutral and one acidic BF replicate slurry and showed that the pH had risen in both vials to a neutral range (N: pH 7.62, A: pH 7.52). The incubation was ended after 160 h, and new pH measurements were performed in one vial from each treatment, where pH in the vials ranged between 7.2 and 7.5 (BF-N-3: 7.39, BF-A-1: 7.2, K1-N-4: 7.49 and K1-A-5: 7.46). The

measurements were done after the vials had been standing without stirring for a day, which may have affected the results. Gas kinetics and N_2O-N reduction rates and N_2-N and CO_2 production rates for this experiment are shown for K1 soil in Figure 4.2 and BF soil in Figure 4.3.

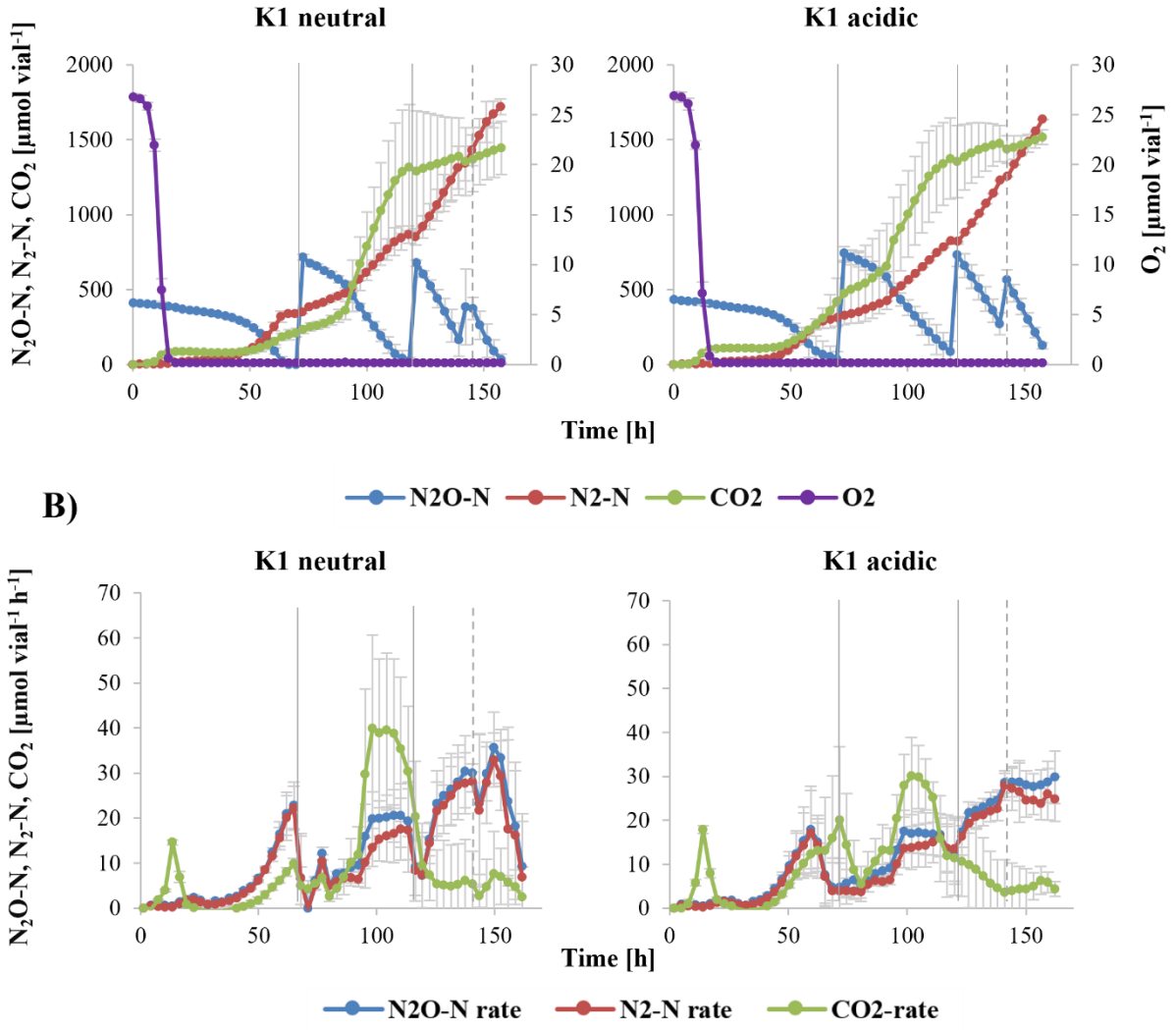

Figure 4. 2 A) Gas kinetics and B) N_2O-N reduction rates and N_2-N and CO_2 production rates for enrichment of African soil K1 using glutamate as the carbon source. Average headspace gas measurements (n=4) are shown for each pH treatment using K1 soil. All vials received an initial dose of 5 mL of N_2O. An addition of 10 mL of N_2O was provided to each vial after approximately 70 h of incubation and after approximately 120 h indicated by a grey, vertical line. An extra addition of 5 mL of N_2O was provided after approximately 140 h indicated by a grey dotted vertical line. One K1 neutral sample (replicate 3) was excluded from the figure as this slurry received doses of N_2O at different time points. Standard deviations are indicated with error bars in both A and B.

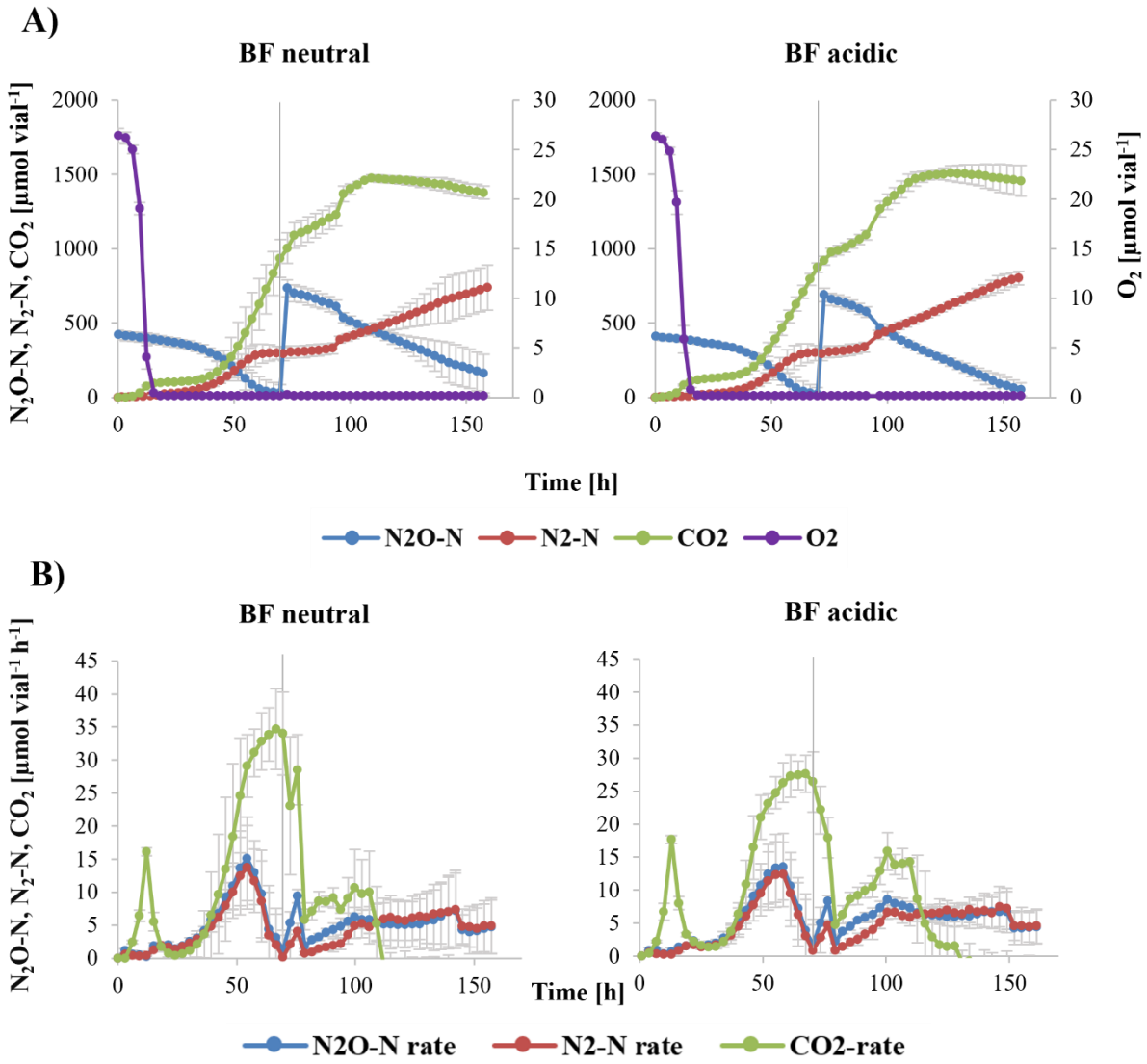

Figure 4. 3 A) Gas kinetics and B) N_2O-N reduction rates and N_2-N and CO_2 production rates for enrichment of African soil BF using glutamate as the carbon source. Average headspace gas measurements (n=5) are shown for each pH treatment using BF soil and the standard deviations are indicated with error bars. All vials received an initial dose of 5 mL of N_2O and an addition of 10 mL of N_2O was provided to each vial after approximately 70 h of incubation indicated by a grey, vertical line.

The gas kinetics of the enrichment slurry vials (Figure 4.2A and Figure 4.3A) show a difference between the two soils where K1 slurries were able to reduce 30 mL N_2O in 160 h, whereas BF slurries only managed a reduction of 15 mL N_2O during this time period. All slurries reduced the O_2 that was given at the beginning. There was little difference in gas kinetics between the initial acid and initial neutral slurries, likely because the pH changed, making them both neutral to slightly alkaline. There were also little differences observed between the replicates in the gas kinetics for BF soil (indicated by small standard deviations), whereas K1 soil replicates had

somewhat more varying gas kinetics. This was the case in the N_2O-N reduction rates and N_2-N and CO_2 production rates as well.

N_2O-N reduction rates and N_2-N production rates were compared between the two soils in Figure 4.2B and Figure 4.3B. The N_2O-N additions are shown as grey vertical lines as either full or dotted depending on the dose given and will be indicated like this further on. The N_2O-N reduction rates and the N_2-N production rates are associated where all the N_2O-N reduced was used for production of N_2-N. BF soil slurries had their fastest N_2O-N reduction rate once the first 5 mL N_2O-N was reduced after 58 h, while the addition of 10 mL N_2O at 73 h caused the reduction rate to slow down. This indicates an inhibition of the N_2OR enzyme when higher concentrations of N_2O are present for the BF soil. Similarly, K1 soil slurries had high N_2O-N reduction rates when the first dose of N_2O was reduced after 62 h, indicating a high initial N_2O reduction rate for both soils. As seen for BF, N_2O-N reduction rates for K1 soil slowed down after 10 mL N_2O was added but increased gradually as the N_2O was removed from the headspace until approximately a production rate of 30 µmol $vial^{-1}$ h^{-1} N_2-N was achieved after 160 h. The increase in N_2O-N reduction rates in K1 soil slurries indicates that more of the functional N_2OR was present and that the N_2O reducing population had grown, thereby suggesting a successful enrichment at neutral pH. By comparing the N_2O-N reduction rates between the soils, it is clear that K1 soil was faster at reducing N_2O than BF, in addition to being less affected by higher doses of N_2O.

The CO_2 production was approximately 1600 µmol $vial^{-1}$ for both soils, where K1 replicates were somewhat more variable than BF (Figure 4.2A and Figure 4.3A). The initial increase in CO_2 after 90 h caused a dilution of the gases in the headspace due to overpressure giving the appearance of a decline in N_2O. As the pH changed during the experiment and ended up at neutral/high it is not possible to quantitatively track the CO_2 measurements as the change of pH affects the solubility of CO_2. The exact time where the pH changed is not known and it is therefore not possible to correct for it. It is still worth noting that an unexpectedly high amount of CO_2 was produced. Another interesting observation is that approximately the same amount of CO_2 was produced in both BF and K1 soil even though they reduced different amounts of N_2O. The production rate of CO_2 in BG soil slurries was high initially and increased further once the initial 5 mL N_2O-N was reduced at 73 h (Figure 4.3B). After 110 h the CO_2 production rate halted and stayed at approximately zero for the rest of the incubation. For K1 soil (Figure 4.2B), the CO_2 production rates showed the same initial peak, but the highest rate was observed after the second dose of N_2O was reduced at 104 h where the CO_2 production rates were at 39

µmol vial^{-1} h^{-1}. The N_2O-N reduction rates and N_2-N and CO_2 production rates for all the replicates are found in Figure A1.

N_2-N production for the soils was at approximately 850 µmol vial^{-1} for BF soil and 1800 µmol vial^{-1} for K1. This corresponds to 425 and 900 µmol vial^{-1} N_2 produced. The amount of N_2O converted to N_2 is expected to result in the oxidation of 7 % and 14 % of the carbon inputted as glutamate (calculations are found in Appendix 6.2.1).

The enrichment with glutamate as the carbon source resulted in a pH rise causing all slurries to end up at neutral/high pH. It was still possible to observe a difference between the two soils, where K1 had higher N_2O reduction rates than BF. A high amount of CO_2 was produced for both soils, but due to the shift in pH it is not possible to quantify this CO_2 throughout the incubation. Due to this pH increase, further enrichments were not performed using this setup.

4.2 Improving the enrichment setup

As the enrichment using glutamate as the carbon source did not keep the pH in the acidic treatments below the desired pH 6.1 during incubation, several tests were performed to find the cause and further improve the method. The threshold of pH 6.1 was based on previous research that demonstrated that N_2OR lacks function below pH 6.1 and keeping pH stable below this threshold was therefore important (Bergaust et al., 2010; Liu et al., 2014).

To improve the enrichment setup, every step was examined in detail. It was hypothesized that the observed pH rise was caused by the glutamate additions. This is because reduction of glutamate produces ammonia (NH_4^+) and causes loss of the anionic glutamic acid.

4.2.1 Finding the reason for the rise in pH

First a test of how fumigated soil affected the pH was done. pH was measured frequently in fumigated and native K1 soil slurries over a period of 1.5 h. This was done aerobically at room temperature and with stirring at 600 rpm. More detailed pH measurements are shown in Table A6. pH did not rise drastically for the soils without any carbon additions, so inoculum of native soil into the fumigated soil were also tested. No rise in pH took place, but a reduction of 0.15 pH units was observed. To further examinate each step of the method, glutamate was given to the slurries. The results are shown in Figure 4.4.

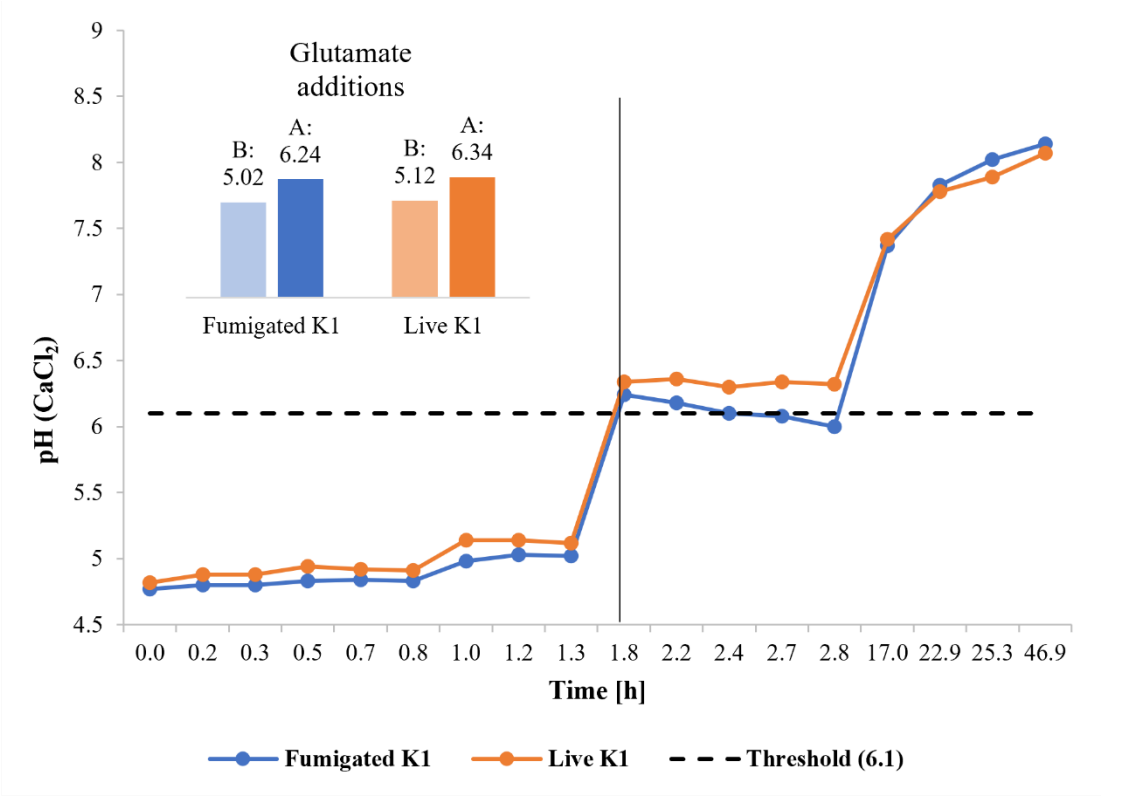

Figure 4. 4. pH measurements in aerobic soil slurries containing fumigated or live (native) K1 soil and MilliQ water before and after glutamate additions. Additions of 30mM 1 M Na-glutamate (pH 7) are indicated with a black vertical line. The box in the left corner shows pH immediately before and after glutamate was added. A indicates pH measured after glutamate was added and B indicates pH before addition of glutamate.

The results in Figure 4.4 showed no significant difference between the fumigated + natural and native K1 soil slurries, but a small rise in pH (~0.3 units) naturally for the soil without using a buffered medium. Addition of the glutamate stock caused pH to rise over 1 pH unit immediately after addition. The glutamate solution was not pH adjusted before use, most likely contributing to the rise. Measurements of pH after 48 h showed that the pH had increased to a value of over 8 for both fumigated and native soil. It was therefore concluded that the metabolism of glutamate and lack of pH adjustment was the reason for the observed pH rise.

4.2.2 Testing if phosphate buffer will keep pH stable

As an effort to buffer this pH rise, phosphate buffered (PB) medium was tested as a replacement for MilliQ water in the soil slurries. Three different PB concentrations were tested; 100 mM, 150 mM and 200 mM at a pH of 5.8 (acidic) and 7.4 (neutral). Native soil was used for these experiments and the slurries were incubated aerobically at room temperature with stirring (600

rpm). The results are shown in Figure 4.5 and more detailed pH measurements are found in table A2, A3 and A4.

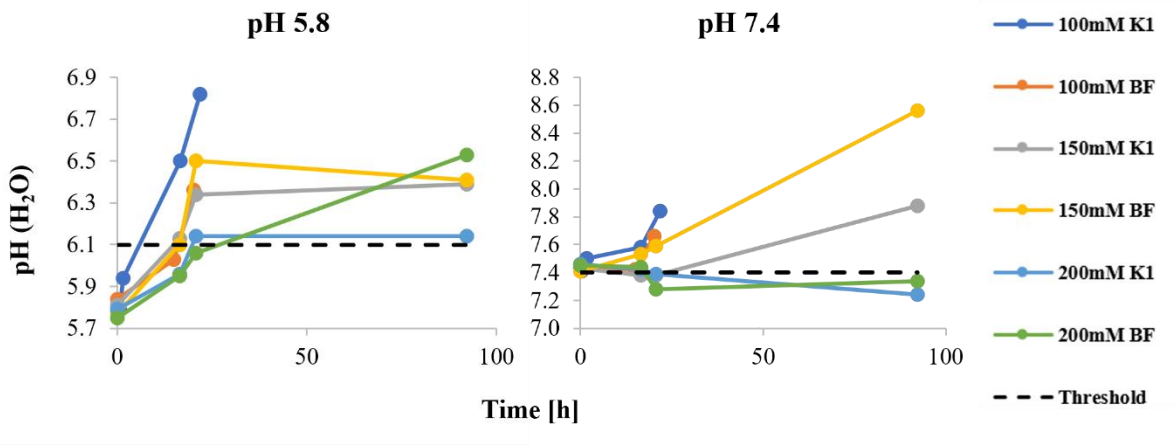

Figure 4. 5. pH measurements of acidic and neutral soil slurries using different phosphate buffer concentrations. Incubations were done aerobically and the slurries with 100 mM PB was incubated for 20 h while the 150 mM and 200 mM PB slurries was measured for 100 h. A threshold indicating the desired pH value is included. For acidic slurries this was at pH 6.1, while the neutral pH measurements had the threshold at 7.4.

None of the buffer concentrations were high enough to keep pH below 6.1 for the acidic slurries (Figure 4.5). For the neutral slurries, pH continued to rise and only a concentration of 200 mM PB was enough to keep pH stable. The two highest buffer concentrations (150 mM, 200 mM) were also tested anaerobically since pH was expected to rise slower without oxygen present due to reduced metabolic activity. Only acidic slurries were tested under anaerobic conditions. The pH measurements are shown in Figure 4.6 and more detailed measurements are shown in table A5.

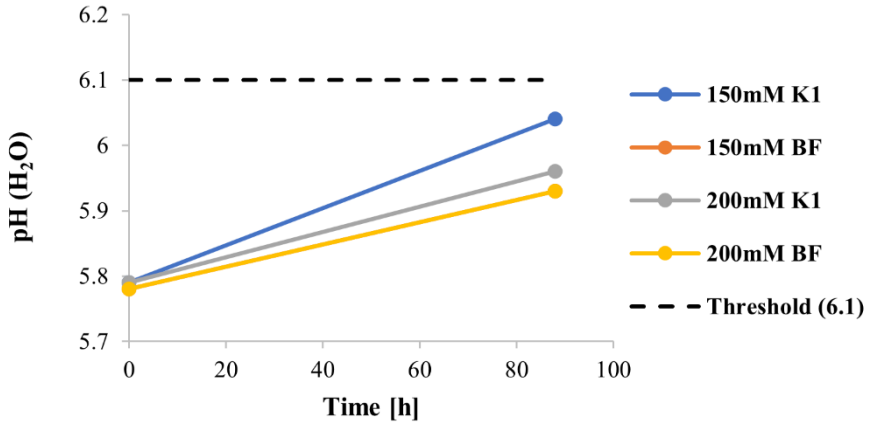

Figure 4. 6. pH measurements for acidic soil slurries with buffer concentrations at 150 mM and 200 mM incubated anaerobically. A threshold was set at pH 6.1. All vials stayed below the threshold for 100 h but was expected to increase further.

While for this short testing period all slurries stayed below the threshold at 6.1, the experiment was expected to run for a longer period, and the pH was expected to continue to rise. It was therefore concluded that a buffered medium was likely not enough to reliably keep the pH stable when glutamate was the carbon source.

4.2.3 Exploring new carbon sources for the enrichment setup

As the pH did not remain acidic even with higher buffer concentrations, different carbon sources had to be explored. This was done using native K1 soil. Tryptic soy broth (TSB) and a hexose mixture (glucose, fructose and xylose) were selected as two possible options and tested on soil slurries that were incubated with 150 mM PB medium, at room temperature and with stirring at 600 rpm. The pH was measured for 40 h. The results are shown in Figure 4.7. and more details are found in table A6.

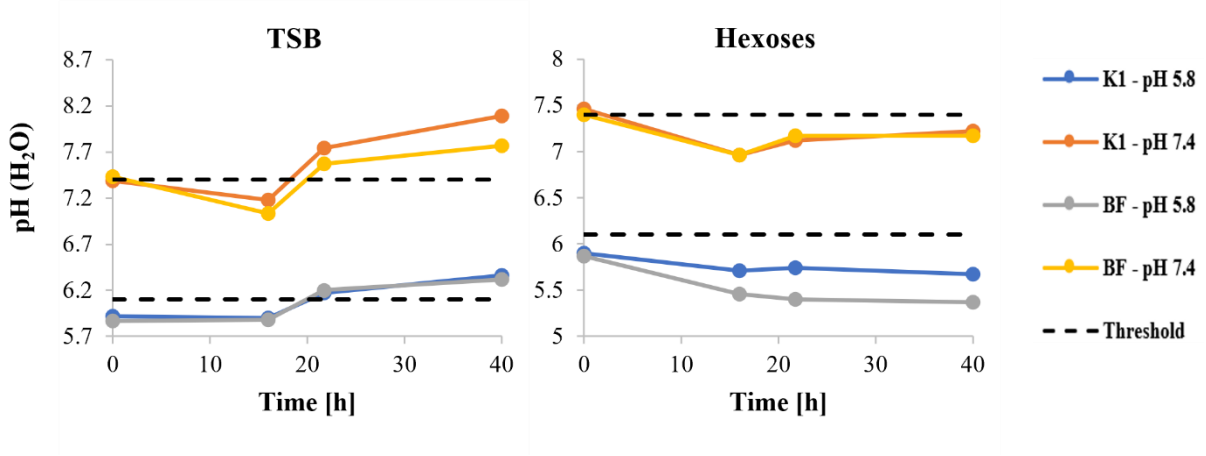

Figure 4. 7. pH measurements of native K1 soil slurries containing 150 mM PB and either TSB or hexose mixture as the carbon source. Thresholds at 6.1 for acidic slurries and 7.4 for neutral slurries are shown. Acidic slurries had an initial pH at 5.8, while neutral slurries started at pH 7.4.

The findings in Figure 4.7 demonstrate that TSB caused the pH to rise above the threshold of the function of N_2OR at pH 6.1, as well as the desired pH value for the neutral slurries at pH 7.4, while the hexose mixture managed to keep pH below the threshold levels for both neutral and acidic pH. Based on this, hexose mix was chosen as the carbon source for further enrichments.

Taken together, the experiments showed that pH increased when the cultures consumed glutamate and that the inclusion of phosphate buffer was not enough to keep pH below 6.1.

Thus, another carbon source had to be used. Testing different carbon sources lead to the decision that a mix of hexoses would be the best choice for keeping pH stable.

4.3 Hexose mixture as carbon source in the enrichment

As the hexose mix turned out to be the best option in regards of maintaining a pH value below 6.1, a new enrichment experiment was set up where hexose mix was used as the carbon source and 150 mM PB was used to buffer the solution. Acidic slurries (initial pH 5.8) were made for both soils with 5 replicates for each soil. The slurries received a He headspace before being given hexose mixture to a final concentration of 30 mM, 0.7 mL O_2 and 5 mL N_2O. N_2O-N reduction rates and N_2-N and CO_2 production rates over a 260 h incubation are shown in Figure 4.8 and associated gas kinetics are found in Figure A2.

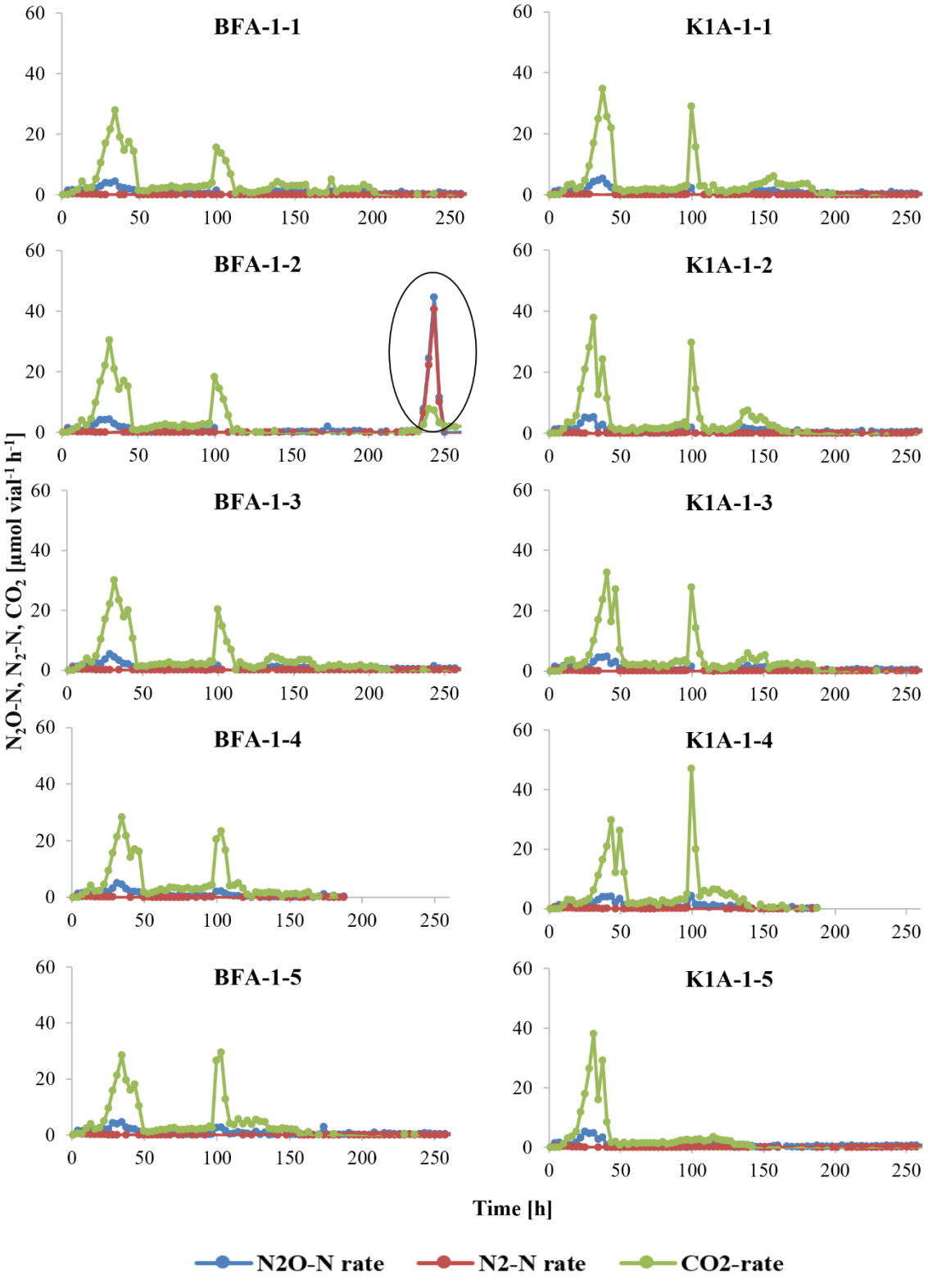

Figure 4. 8. N_2O-N reduction rates and N_2-N and CO_2 production rates for the enrichment using hexoses as the carbon source until 260 h. Five replicates from two soils (BF and K1) were enriched at pH 5.8 in soil slurries. They were initially provided 5 mL of N_2O as well as 0.7 mL of O_2. Reduction of the initial 5 mL N_2O is indicated by a circle.

The rates in Figure 4.8 show that both soils had similar N_2O-N reduction rates and N_2-N production rates which stayed below 5 µmol vial^{-1} h^{-1} for all except BFA-1-2 late during the incubation. After 96 h of incubation, N_2O reduction was expected to be observed based on the first enrichment (section 4.1), but as no reduction had happened a few methods were trialed to stimulate N_2O reduction. BF and K1 slurries 1-3 were given nitrate and more hexose mixture as an attempt to stimulate denitrification and subsequent N_2O reduction. BF replica 4 and 5 and K1 replica 4 received an extra addition of the hexose mixture, while K1 slurry 5 was not given any additions. Nine out of 10 slurries did not reduce any N_2O after 260 h, and BFA-1-2 was the only replicate that managed the reduction after 243 h. A small apparent reduction in N_2O was observed initially for all, but it coincided with a period of high CO_2 production and was assumed to be caused by N_2O dilution by CO_2 production.

The CO_2 production rates for both soils were unexpectedly high, similar to those seen in the glutamate enrichments, and showed two distinctive peaks after 31 h and after 100 h (Figure 4.8). In the first peak the production rate ranged between 30 and 40 µmol vial^{-1} h^{-1} and indicated that substantial amounts of CO_2 were produced early in the incubation. The second peak was observed after additional hexose mixture where the CO_2 production rate ranged between 15 and 50 µmol vial^{-1} h^{-1}. This peak was not seen in the replicate that did not receive additional hexose mixture (K1A-1-5) indicating that the second peak was a consequence of extra carbon additions The CO_2 production in these slurries is shown in more detail in Figure 4.9.

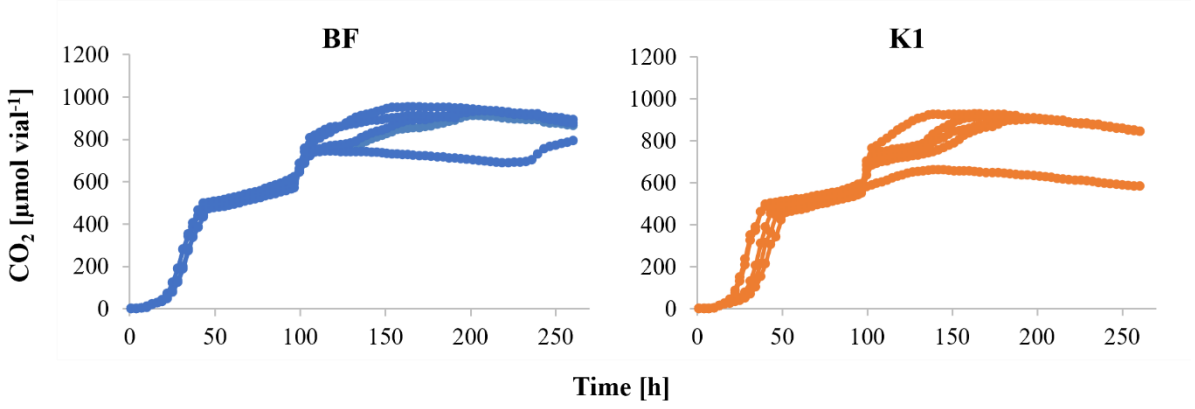

Figure 4. 9. CO_2 production for all replicates in acidic BF and K1 slurries in the first 260 h of incubation with hexose mixture as the carbon source. Both soils produced ~900 µmol CO_2 vial^{-1} for 4 out of 5 replicates.

Interestingly, a high level of CO_2 production occurred in all slurries (Figure 4.9) but without any N_2O reduction taking place (Figure 4.8/gas kinetics in Figure A2). CO_2 production varied between 700 µmol vial^{-1} and 950 µmol vial^{-1} for BF soil and between 580 µmol vial^{-1} and 920

µmol $vial^{-1}$ for K1 soil. This amount of CO_2 was not expected without N_2O reduction and had to be examined further. The unexpectedly high volumes of CO_2 produced might indicate the occurrence of other metabolic processes such as respiration using an alternative unknown terminal electron acceptor or high levels of fermentation. The slurries were stopped for gas measurements for a total of 216 h. 48 h of this was at 23 °C and with stirring at 500 rpm, while the rest was without stirring at 4 °C.

4.4 The effect of iron on soil slurries and CO_2 production

The high CO_2 production seen in both the enrichment experiment with glutamate and hexoses was unexpected and needed to be examined in more detail. As previously mentioned (1.4), African soils are naturally iron rich, and the reduction of Fe(III) to Fe(II) can contribute to CO_2 production. The amount of Fe(II) was therefore interesting to examine in slurries that had been incubated anaerobically with addition of a carbon source (hexose mixture, termed +C) compared to freshly made slurries without addition of carbon as it may indicate an alternative terminal electron acceptor driving respiration of hexoses. The results of Fe(II) quantification are shown in Table 4.1.

Table 4.1. Fe(II) measurements for slurries with carbon additions and incubation for 260 h (+C) and slurries without any carbon additions and incubation.

	BF	BF+C	K1	K1+C
Fe(II) (mg/L)	0.279	2.74	0.143	0.687

The Fe(II) concentrations reveals a clear increase in Fe(II) in slurries that had received carbon and been incubated anaerobically (Table 4.1). For BF soil the Fe(II) concentrations had increased approximately 10x, while K1 had an increase of almost 5x. Based on the increases in Fe(II) in the soil slurries, it was hypothesized that iron could be one contributor to the CO_2 production observed in both enrichment experiments and that this reduction might inhibit N_2O reduction. It would have been ideal to calculate how much of the CO_2 production that could be explained by this Fe(III) reduction, but this was not possible as our measurements were likely a significant undermeasurement due to the difficulty in efficiently extracting Fe(II) from soils.

4.5 Extracting bacterial cells from soil to avoid iron reduction

As an attempt to avoid the iron issue and the unexpected CO_2 production, bacterial cells were extracted from the soils using Nycodenz density gradient centrifugation (Bakken & Lindahl, 1995). The cells, removed from the soil matrix, were then inoculated into a modified Sistrom's medium (Bergaust et al., 2010; Sistrom, 1962) where succinic acid was replaced with 30 mM hexose solution at pH 5.5. The vials were made anoxic through a He headspace before additions of 0.7 mL O_2 and 5 mL N_2O. Measured headspace gas kinetics are shown in Figure 4.10.

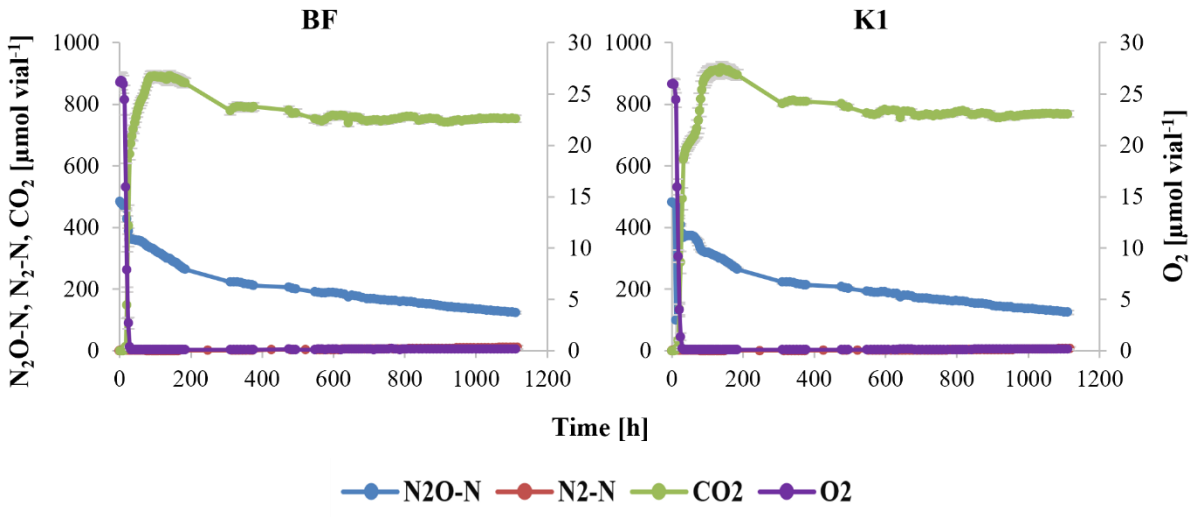

Figure 4. 10. Average (n = 5) gas kinetics for extracted bacterial cells from BF and K1 soil inoculated into a modified Sistrom's medium where succinic acid was replaced with 30 mM hexose mixture at pH 5.5. The headspace was replaced with He and supplemented with 5 mL of N_2O and 0.7 mL of O_2. The standard deviations are indicated by error bars (not visible due to small size).

No N_2 production occurred in the cultures as shown by the gas kinetics in Figure 4.10. There was also no significant difference between the replicates, indicated by small standard deviations which are not visible. The same initial drop in N_2O levels as seen in the enrichment with glutamate and hexoses when CO_2 was produced was observed here as well. The N_2O-N reduction rates and N_2-N and CO_2 production rates for the extracted bacterial cell cultures are shown in Figure 4.11.

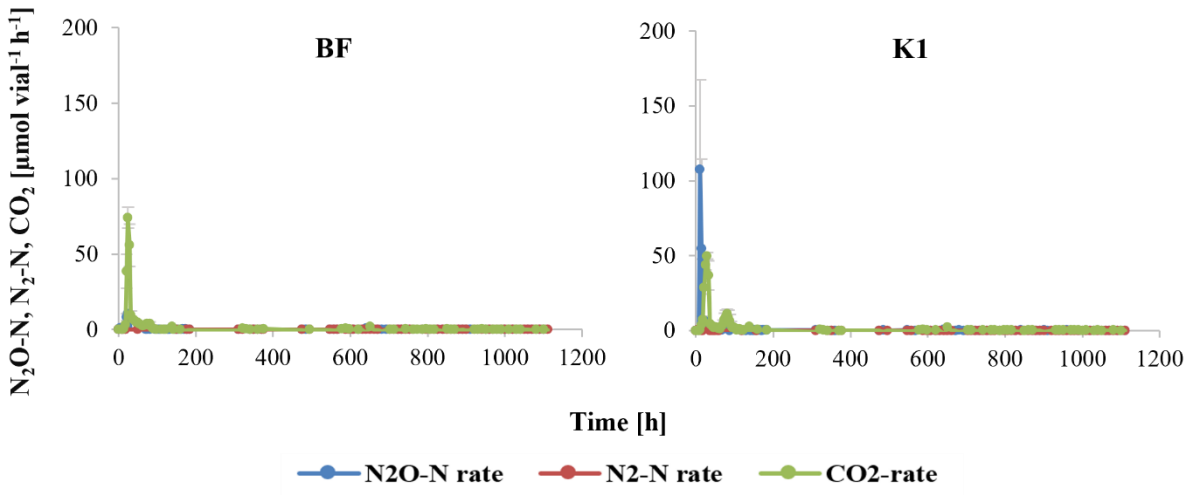

Figure 4. 11. Average (n= 5) N_2O-N reduction and N_2-N and CO_2 production rates for extracted bacterial cell cultures from African soils. Cells were extracted using Nycodenz density gradient and inoculated into a modified Sistrom's medium where succinic acid was replaced with 30 mM hexose mixture at pH 5.5. Standard deviations are indicated by invisible error bars, illustrating small deviations.

The N_2O-N reduction rates and N_2 production rates were approximately zero for all replicates in both soils (Figure 4.11). The N_2O-N reduction rate had a peak at 10 h, but as previously stated, this was caused by dilution of N_2O due to overpressure when CO_2 was produced, and not by reduction of N_2O. The CO_2 production was high initially, after 10 h, for both soils where BF soil produced 74 µmol $vial^{-1}$ h^{-1} CO_2 and K1 produced 50 µmol $vial^{-1}$ h^{-1} CO_2, before it became stable at zero µmol $vial^{-1}$ h^{-1}.

Looking at the CO_2 production, approximately 700-900 µmol $vial^{-1}$ CO_2 was produced and there was little difference between soils regarding CO_2 production. This is similar to the amount CO_2 observed in the soil slurries when hexose mixture was used as the carbon source (section 4.3). These results suggests that it was unlikely that the iron reduction was the main contributor to the high CO_2 production, and that other factors have a larger impact such as fermentation. Extracting bacterial cells was not the solution when it comes to enriching a low pH N_2O reducing bacterial community either, as no N_2O was reduced after such an extended time.

4.6 N_2O reducing soil slurry enrichment using a hexose mixture as the carbon source

As one slurry from the enrichment with hexoses, shown in section 4.3, managed to reduce N_2O, incubation and gas measurements of these enrichment slurries were restarted. The slurries had then been without measurements for 216 h, where 48 h was at 23 ℃ with stirring (500 rpm),

while the rest was at 4 °C without stirring. pH was measured before the experiment was recontinued to make sure the slurries had maintained a low pH (Table A8). All slurries had a pH value below the threshold at 6.1, although BFA-1-1 was right at the border. K1A-1-4 and BFA-1-4 were destructively sampled for iron measurements shown in section 4.4 and was therefore not included further.

4.6.1 Cycle 1 of the enrichment with hexose mixture for BF and K1 soil slurries

The vials in the first cycle of the enrichment with hexose mixture (five replicates for each soil) were incubated between 1250-1700 h and followed with gas measurements. Only two slurries managed to reduce 30 mL of N_2O, while the rest had varying reduction abilities ranging from not being able to reduce any N_2O until a reduction of 25 mL N_2O. Only one slurry did not perform any N_2O reduction across both soils. The fastest slurries in this first cycle were BFA-1-2 and K1A-1-5 that reduced 25 mL N_2O in 1504 h and 30 mL N_2O in 1260 h, respectively. Their gas kinetics and N_2O-N reduction and N_2-N and CO_2 production rates are shown in Figure 4.12, while all the replicates are shown in Figure A6.3.

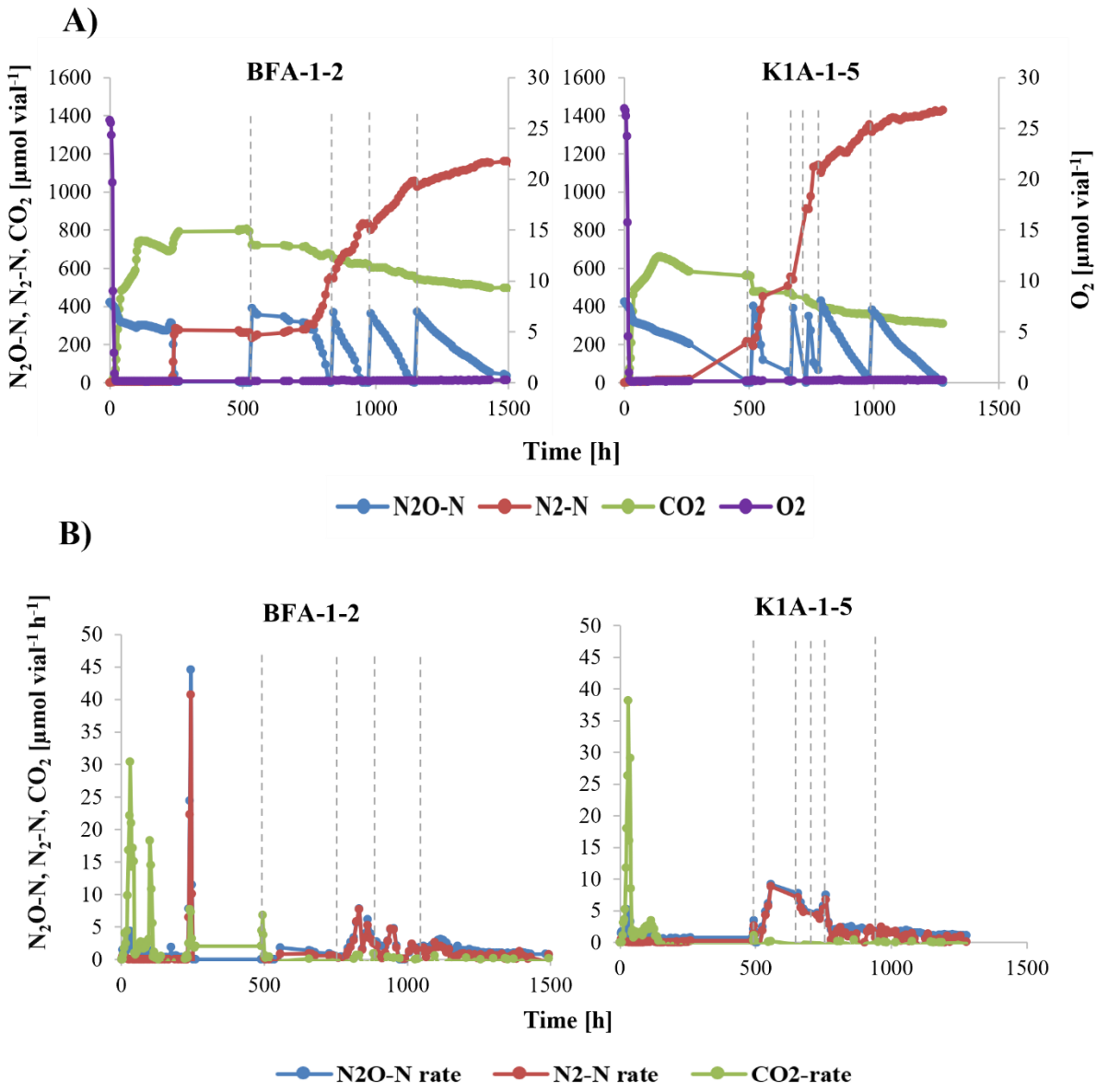

Figure 4. 12. A) Gas kinetics and B) N_2O-N reduction rates and N_2-N and CO_2 production rates in the replicate from each soil that reduced the desired amount N_2O fastest in the enrichment with hexoses cycle 1. All replicates received 5 mL of N_2O initially and grey dotted vertical lines indicate further additions of 5 mL N_2O.

BFA-1-2 and K1A-1-5 was the two slurries from the first cycle that reduced the desired amount of N_2O fastest from each soil (Figure 4.12). As BF soil slurries were slower in reducing N_2O than K1 soil slurries, only 25 mL was reduced by BFA-1-2. BFA-1-2 was the only slurry able to reduce the initial 5 mL N_2O when hexose mixture was first tested (described in 4.3). In addition, K1A-1-5 only received one dose of hexose mixture, whereas BFA-1-2 received both nitrate and additional hexose mixture (section 4.3). Three slurries from each soil had been provided nitrate, which was not optimal as nitrate reduction contributes to pH rise and as acid tolerant N_2O reducers were the target of this enrichment, keeping pH below 6.1 was essential.

After the incubation was ended BFA-1-2 had a pH above the threshold of 6.1, while K1A-1-5 had an end pH of 5.97. Based on this lack of pH control and the nitrate additions, further enrichments using the BF soil were not conducted.

As seen in 4.3, both soils have high CO_2 production rates (30 and 38 µmol CO_2 $vial^{-1}$ h^{-1} for BF and K1, respectively) early in the enrichment before it stabilizes at a low level near nil. For BF, the N_2O-N reduction rate was highest at 243 h where 45 µmol $vial^{-1}$ h^{-1} N_2O was reduced, and the second highest N_2O-N reduction rate was after 832 h where 8 µmol $vial^{-1}$ h^{-1} was reduced. K1A-1-5 had its fastest reduction rate of N_2O-N after 556 h when the second 5 mL addition of N_2O was reduced. At this point almost 9 µmol $vial^{-1}$ N was produced per hour. From 556 h to 758 h the reduction rate stayed between 9 and 7 µmol $vial^{-1}$ h^{-1} before slowing down. At the end of cycle 1 the N_2O reduction rates for both soils were low which may indicate that some nutrient is limiting. As the replicates reduced N_2O at different rates throughout the first cycle of the enrichment, the N_2-N and CO_2 production are compared in Figure 4.13.

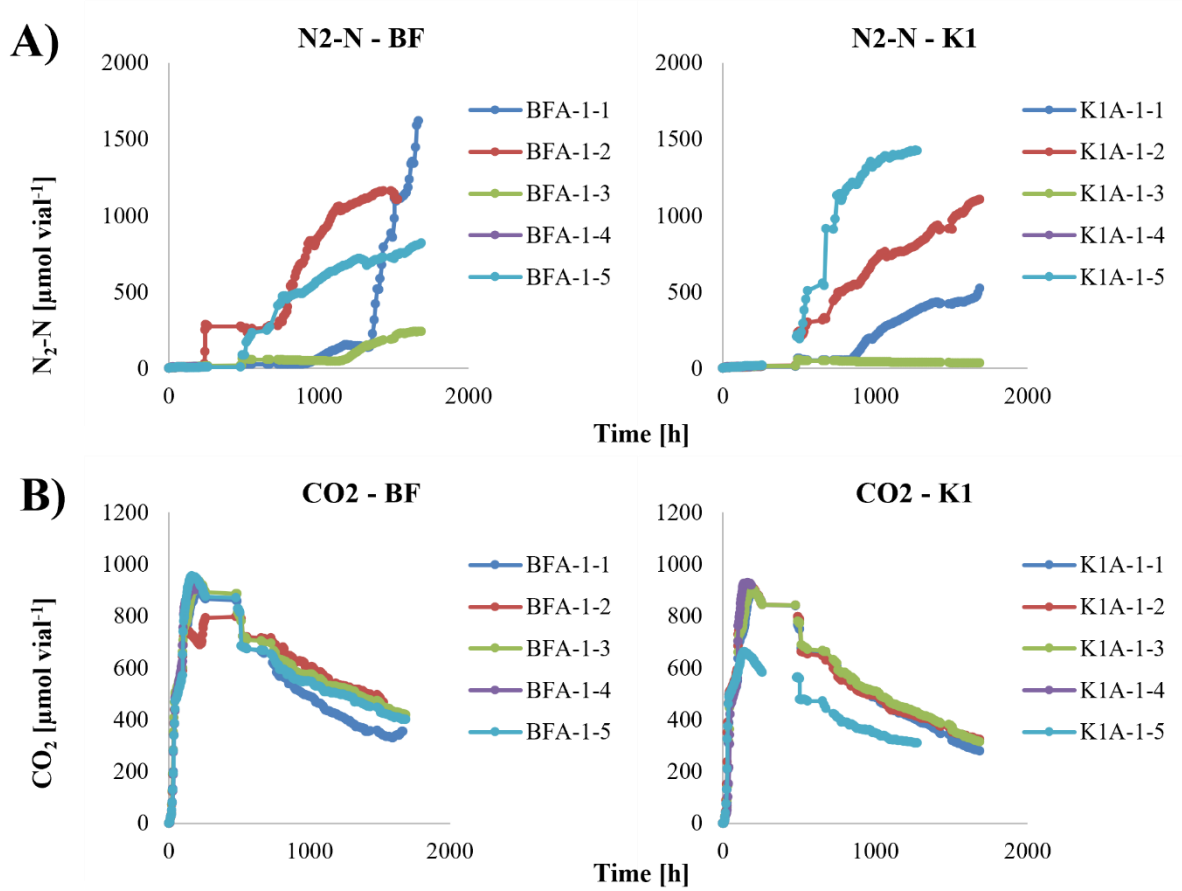

Figure 4. 13. N_2-N and CO_2 production for all cycle 1 slurries. **A)** shows N_2-N production and **B)** shows CO_2 production for all slurries in both BF and K1 soil.

Figure 4.13 shows N_2-N production and CO_2 production in the cycle 1 vials. BFA-1-4 and K1A-1-4 were not continued after 260 h as these were destructively sampled for the iron measurements in section 4.4. In terms of N_2-N production, the two slurries (BFA-1-2, K1A-1-5) shown in Figure 4.12 were the fastest until 1411 h, where BFA-1-1 increased its N_2-N production and N_2O-N reduction and reduced 25 mL in a period of ~350 h. In K1A-1-3 no N_2-N production took place. The CO_2 production was similar in all slurries, regardless of nitrate and hexose mixture additions, and both BF and K1 soil had over 900 µmol $vial^{-1}$ CO_2 produced in the incubation. K1A-1-5 produced 1424 µmol $vial^{-1}$ N_2-N while BFA-1-2 produced 1142 µmol $vial^{-1}$ N_2-N. This corresponds to 712 and 571 µmol N_2 produced per vial and would suggest that 11 % and 8 % of the energy from the hexoses had been used for N_2O reduction and N_2 production. More detailed calculations are shown in Appendix 6.2.

As BFA-1-2 did not manage to keep pH below the threshold of 6.1 a cycle 2 was not started for this soil. K1A-1-5 managed to reduce the total amount of 30 mL of N_2O in addition to keep pH below the threshold and was therefore used as an inoculum for fresh K1 cycle 2 slurries.

4.6.2 The second cycle of the enrichment of low pH N_2O reducers with hexoses as the carbon source

To enrich a larger low pH N_2O reducing community, a second enrichment cycle was started. In this cycle, slurry from the finished cycle 1 K1 replicate shown in Figure 4.12 was used as an inoculum in new K1 slurries (fumigated K1 soils, 150 mM PB, pH 5.8). More hexose mixture was also provided and an initial dose of 5 mL of N_2O. No O_2 was given here as the cells had already produced their desired denitrification enzymes and the transition to anoxia was not needed. As BF soil had a pH above the threshold of 6.1 a cycle 2 was not started for this soil. Five replicates of K1 slurries were prepared. All of them reduced 30 mL of N_2O after 395 h of incubation. The gas kinetics and N_2O-N reduction rates, N_2-N and CO_2 production rates are found in Figure 4.14 and Figure 4.15.

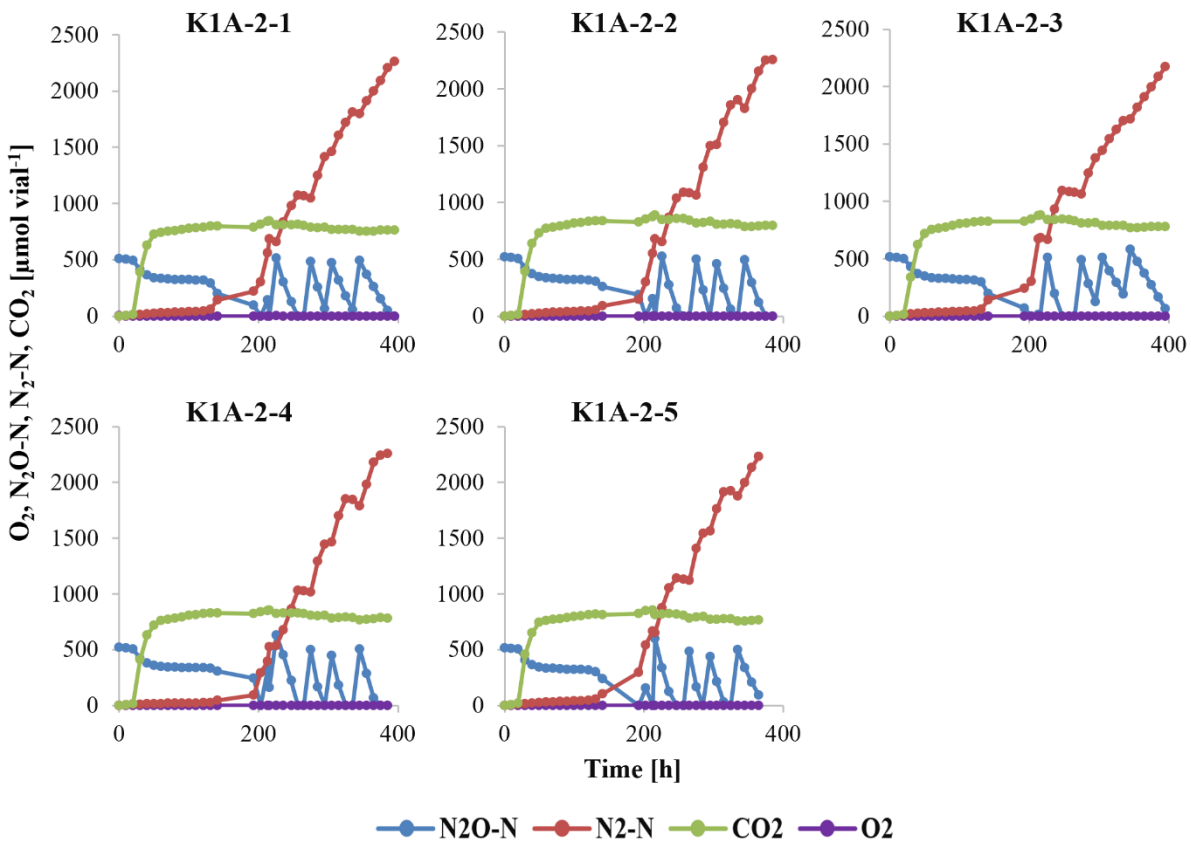

Figure 4. 14. Gas kinetics for all the replicates from the second enrichment cycle for K1 soil with hexoses as the carbon source. The vials were given a He headspace and provided with 0.7 mL O_2 and 5 mL N_2O**.** Dotted vertical lines indicate addition of 5mL N_2O in addition to the initial 5mL dose of N_2O which is not shown here.

The initial 5 mL dose of N_2O was reduced after 230 h, while the next 25 mL was reduced in less than 200 h. This indicates a slow beginning of the reduction, with gradual growth of the N_2O reducing population and following increasing N_2O reduction rates (see Figure 4.15). As previously observed, high amounts of CO_2 were produced initially and caused a small dilution of N_2O in the beginning due to overpressure. From the gas kinetics (Figure 4.14) approximately 800 µmol $vial^{-1}$ CO_2 was produced similar to the amounts CO_2 produced in the cycle 1 slurries (Figure 4.13). Approximately 2300 µmol $vial^{-1}$ N_2-N was produced for the cycle 2 slurries, which corresponds to 1150 µmol $vial^{-1}$ N_2, further suggesting that 17 % of the carbon added had been used for N_2O reduction.

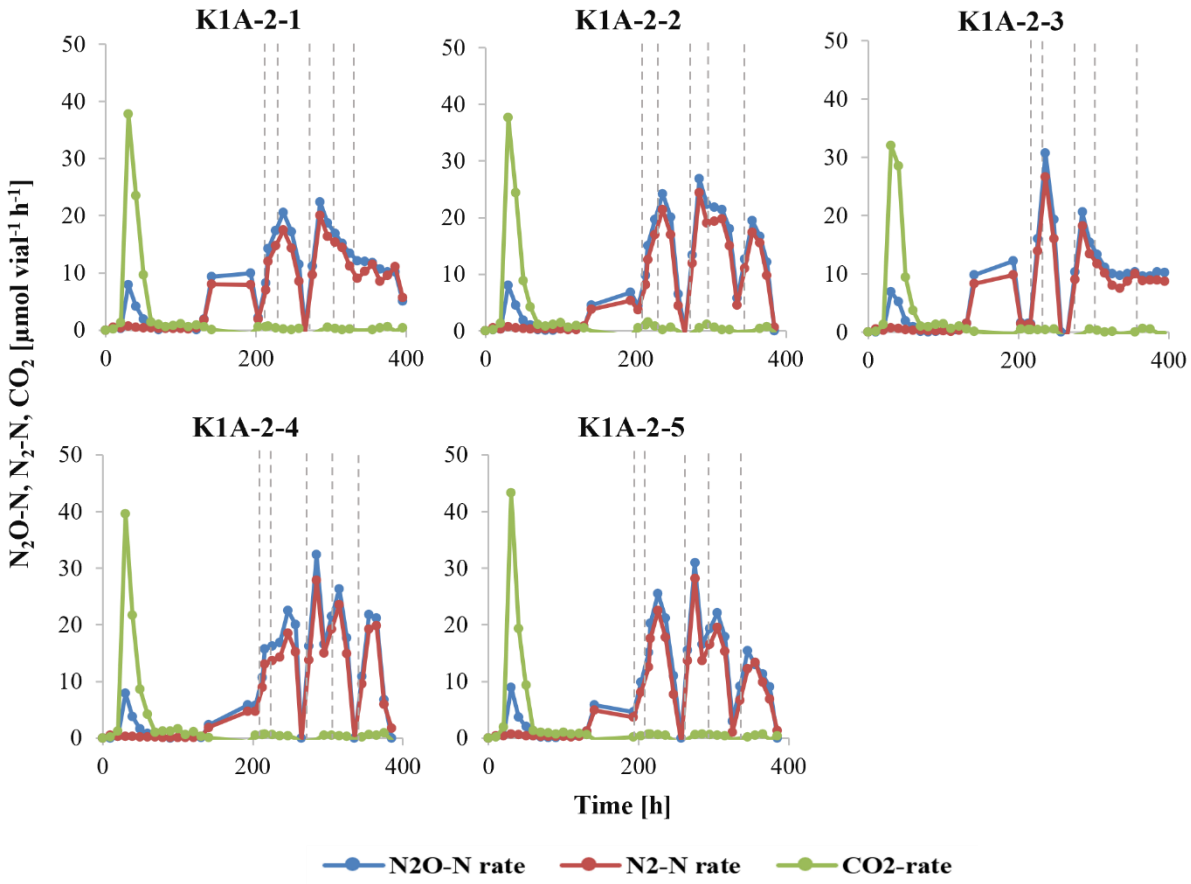

Figure 4. 15. N_2O-N reduction and N_2-N and CO_2 production rates for all replicates from the second enrichment cycle. Dotted vertical lines indicate addition of 5mL N_2O. Initial 5mL N_2O is not shown here.

The rates of N_2O-N reduction and N_2-N and CO_2 production are shown in Figure 4.15. The rates were similar for all replicates, showing good reproducibility. CO_2 was produced at the highest rate after 30 h, where it ranged between 32 µmol vial^{-1} h^{-1} to 43 µmol vial^{-1} h^{-1}, before the production appeared to stop. The N_2O-N reduction and accordingly the N_2-N production started after approximately 200 h and the N_2O-N reduction rates and N_2-N production rates increased throughout the enrichment where the highest N_2O-N reduction rates ranged between 22 and 32 µmol vial^{-1} h^{-1} thereby indicating that a low pH N_2O reducing community was enriched.

pH at the end of cycle 2 ranged between 5.96 to 6.07 amongst the replicates. K1A-2-2 had the lowest pH at 5.96 and was therefore chosen as inoculum for cycle 3.

4.6.3 Third enrichment cycle

A third cycle of the enrichment, set up in the same way as described for cycle 2, was started to further enrich the N_2O reducing population at low pH. All replicates reduced 30 mL of N_2O and stayed below the pH threshold of 6.1, where the measured pH values ranged between 5.99 and 6.05. The gas kinetics for all replicates and N_2O-N reduction rates and N_2-N and CO_2 production rates for all replicates are shown in Figure 4.16 and Figure 4.17.

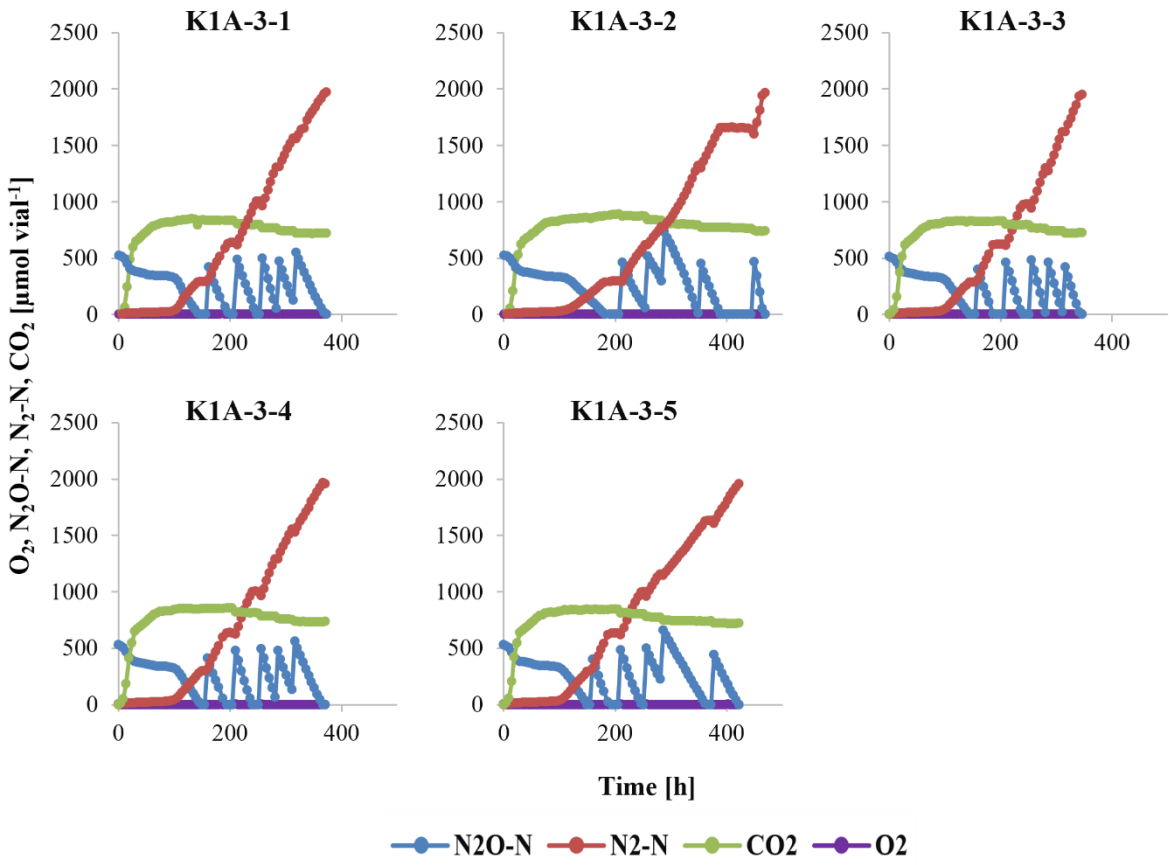

Figure 4. 16. Gas kinetics for all the replicates from the third enrichment cycle. The vials were given a He headspace and provided with 0.7 mL O_2 and 5 mL N_2O initial. Grey dotted vertical lines indicate additions of 5 mL N_2O. The initial dose of 5 mL N_2O is not shown.

The replicates from cycle 3 had highly similar gas kinetics profiles (Figure 4.16) where they all reduced the initial dose of 5 mL N_2O after approximately 150 h and where the next 25 mL of N_2O was fully reduced about 200 – 250 h later. The slurry with the fastest reduction of 30 mL of N_2O was K1A-3-3 which reduced the 30 mL after 346 h, while K1A-3-2 was the slowest replicate and reduced 30 mL of N_2O after 446 h.

The replicates had similar gas kinetic profiles regarding CO_2 production as well where, approximately 800 µmol $vial^{-1}$ were produced in all. N_2-N production was more variable between the replicates and was between 1700 to 1900 µmol N $vial^{-1}$. This corresponds to 850 to 950 µmol N_2 $vial^{-1}$ produced and suggests that 14 - 15 % of the hexose mixture added was used for N_2O reduction. The initial drop in N_2O, due to overpressure, when CO_2 was produced was also observed here.

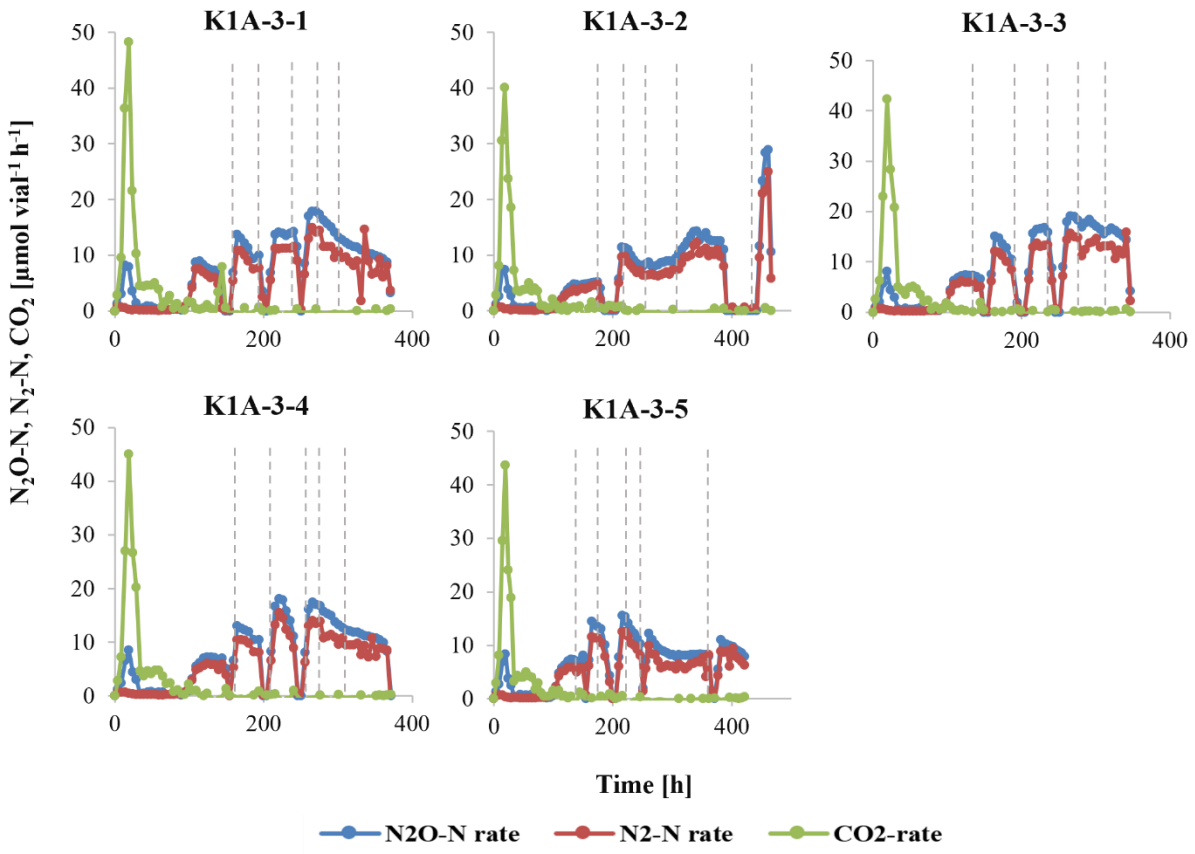

Figure 4. 17. N_2O-N reduction rates and N_2-N and CO_2 production rates for all replicates from the third enrichment cycle. Grey dotted vertical lines indicate additions of 5 mL N_2O. The initial dose of 5 mL N_2O is not shown.

The N_2O-N reduction and N_2-N and CO_2 production rates of the replicates from cycle 3 (Figure 4.17) were similar to the cycle 2 replicates (Figure 4.15) where all had a high initial production rate of CO_2 and N_2O-N reduction rates and N_2-N production rates increased throughout the incubation. The maximum N_2O-N reduction rate in the replicates ranged from14 to 29 µmol $vial^{-1}$ h^{-1}. The increase of reduction rates throughout the enrichment indicates that the low pH N_2O reducing microbes were further enriched.

4.6.4 Comparison of N_2O-N reduction rates and N_2-N and CO_2 production rates between the three cycles

To illustrate the enrichment of the acidic African soil N_2O reducing microbial community the N_2O-N reduction rates and N_2-N and CO_2 production rates from the fastest replicate from each cycle were compared. These replicates were used as the inoculum when starting a new cycle. The rates from all replicates from each cycle can be found in Figure A3.

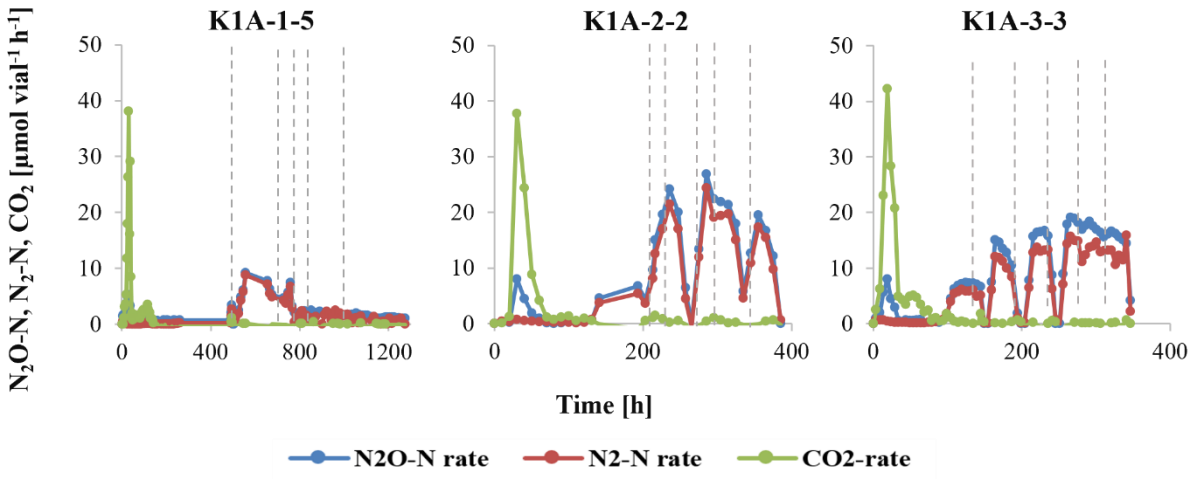

Figure 4. 18. N_2O-N reduction rates and N_2-N and CO_2 production rates for one vial from each cycle of the enrichment with hexoses. The fastest slurry in regards of reducing the total 30 mL of N_2O and thus the replicate that was used as inoculum for the next cycle is shown here**.** Grey dotted vertical lines indicate additions of 5 mL N_2O. The initial dose of 5 mL of N_2O provided to all the slurries is not shown.

Looking at the time each cycle used to reduce 30 mL N_2O it is clear that cycle 2 and 3 were substantially faster than cycle 1 (Figure 4.18). K1A-1-5 used 1260 h for the full reduction, whereas K1A-2-2 and K1A-3-3 reduced the 30 mL of N_2O in 385 h and 346 h, respectively. This was also seen in the N_2O-N reduction and N_2-N production rates, where K1A-1-5 had its highest rate of 9 µmol $vial^{-1}$ h^{-1}, K1A-2-2 reach a maximum rate of 27 µmol $vial^{-1}$ h^{-1}, and K1A-3-3 had its highest reduction and production rate of 19 µmol $vial^{-1}$ h^{-1}. The time used to initialize N_2O reduction by reducing the first 5 mL of N_2O ranged from almost 500 h for cycle 1, 200 h for cycle 2 and 130 h for cycle 3. This faster initial reduction indicates that an N_2O reducing population of bacteria had been enriched during the first cycle and thus subsequent cycles had been inoculated with a higher proportion of N_2O reducing microbes.

The CO_2 production rate was similar throughout the enrichment where it was high at the beginning before it stabilized at zero for all cycles. For cycle 1 the fastest rate was of 38 µmol $vial^{-1}$ h^{-1}, whereas cycle 2 had the highest rate of 38 µmol $vial^{-1}$ h^{-1}, and the third cycle had the

highest CO_2 production rate of 42 µmol $vial^{-1}$ h^{-1}. Small amounts of H_2 were produced in all three enrichment cycles ranging from 20 to 60 µmol $vial^{-1}$ and with a production rate from 2 to 17 µmol $vial^{-1}$ h^{-1}.

Cycle 3 was the last cycle in the acidic N_2O reducing enrichment experiment. The enrichment protocol was successful as low pH N_2O reducers had been enriched using hexose mixture and 150 mM PB in K1 soil, illustrated by the increased N_2O-N reduction rates and N_2-N production rates from cycle 2 and cycle 3.

4.7 Enrichment of K1 soil slurries at neutral pH and with hexoses as the carbon source

As a comparison to the acidic enrichment, a neutral enrichment experiment was started. The experiment had the same setup as before but with neutral pH (pH 7.4) instead of acidic. 30 mM hexose mixture were given to the slurries as well as initial additions of 0.7 mL O_2 and 5 mL N_2O. Due to limited time the first cycle was not finished, but gas kinetics were measured for a time period of 675 h and are shown in Figure 4.19.

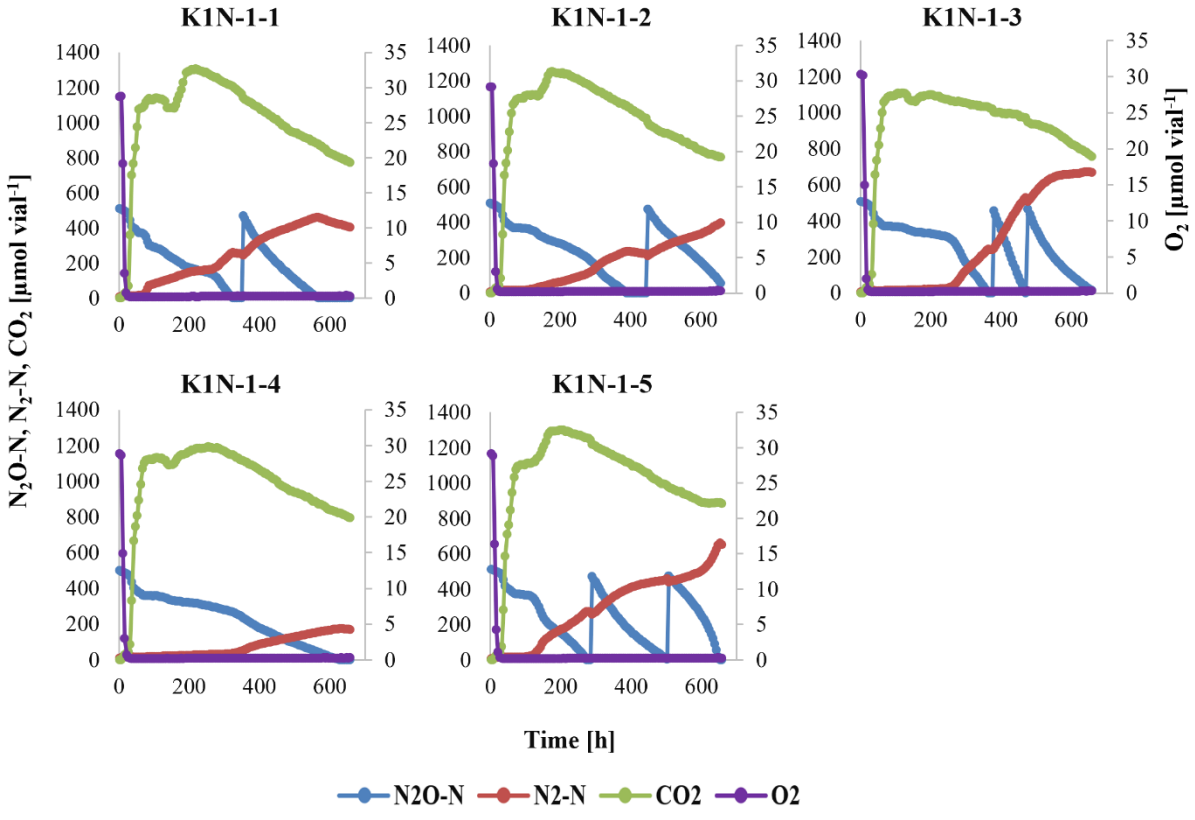

Figure 4.19. Gas kinetics for all the replicates from the neutral enrichment (pH 7.5) using K1 soil slurries. The headspace was replaced with He and the slurries received 5 mL of N_2O and 0.7 mL of O_2.

Gas kinetics for the neutral enrichment of K1 soil are shown in Figure 4.19. All replicates reduced the initial O_2 given after 32 h. Differences in the N_2O reduction capability from each replicate was seen, where K1N-1-5 and K1N-1-3 reduced 15 mL N_2O, K1N-1-1 and K1N-1-2 reduced 10 mL, and K1N-1-4 only reduced 5 mL N_2O. All the replicates showed high amounts of CO_2 produced ranging between 1074 to almost 1300 µmol $vial^{-1}$. The characteristic drop in N_2O when CO_2 production occurs was also present here. The rates of N_2O-N reduction and N_2-N and CO_2 production are shown in the figure below (Figure 4.20).

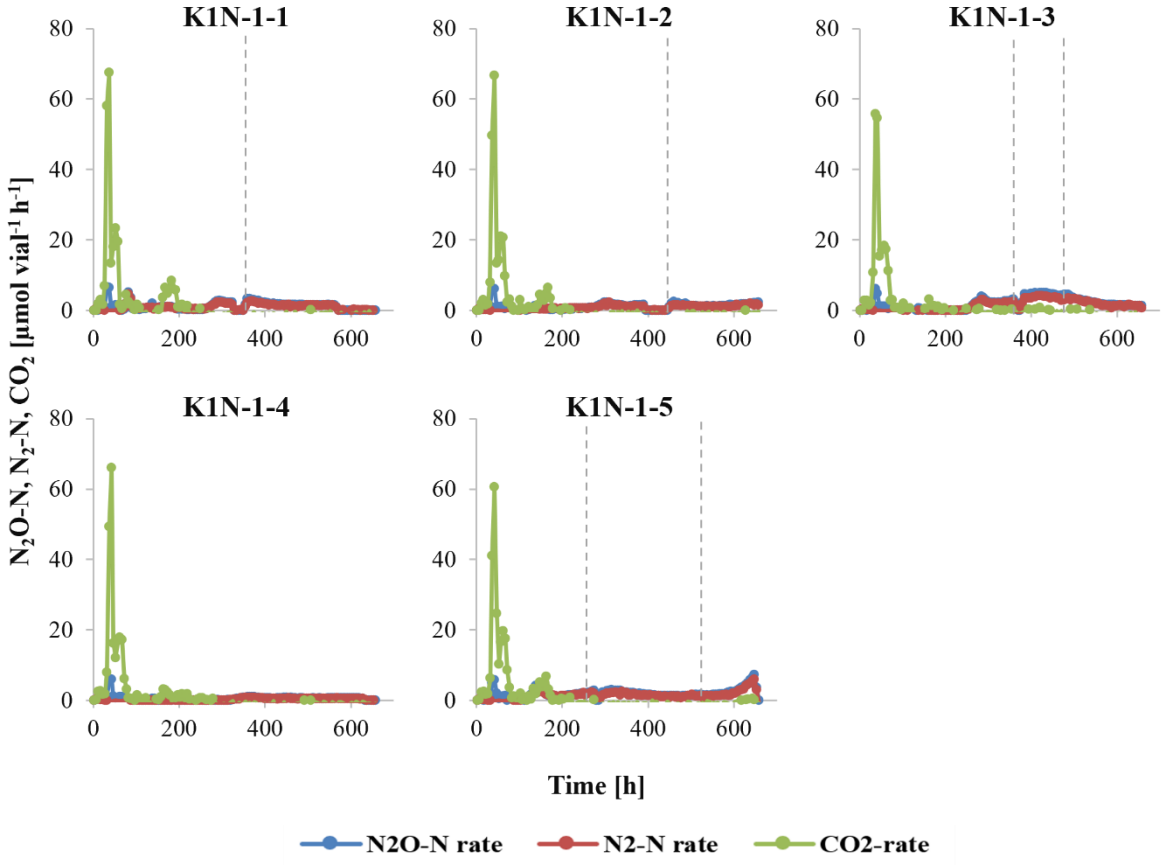

Figure 4. 20. N_2O-N reduction rate and N_2-N and CO_2 production rates for the replicates from the neutral enrichment using K1 soil slurries. Grey vertical dotted lines indicate additions of 5 mL N_2O, in addition to the initial 5 mL not illustrated.

N_2O-N reduction rates and associated N_2-N production rates were low for all slurries in the neutral enrichment. K1N-1-3 and K1N-1-5 had some increasing rates after 400 h and 600 h, respectively where 4 and 7 µmol N_2O-N $vial^{-1}$ h^{-1} was reduced. The CO_2 production rates were high initially, between 55 and 68 µmol $vial^{-1}$ h^{-1}, before the production rates were lowered and dropped down to zero. As time was limited in this experiment, the incubation had to be stopped

before all 30 mL N_2O was given, and these results are presented mainly for the comparison of rates between the acidic and neutral enrichment.

4.8 Isolation of low pH N_2O reducing microbes from the African soils

One of the aims of this study was to isolate organisms capable of N_2O reduction at low pH from acidic African soils. To achieve this selective isolation was performed. Dilutions of the slurry materials ($1:10^7$, $1:10^6$ and $1:10^5$ for native K1 soil and $1:10^5$, $1:10^4$ and $1:10^3$ for K1 cycle 1 and 2 slurries) were transferred to 1/10 or undiluted modified TSA plates, pH adjusted to pH 5.8 with the pH indicator dye bromocresol purple, and kept in a chamber with N_2O as the sole terminal inorganic electron acceptor where they were grown anaerobically. Once growth had taken place, colonies were picked based on differences in morphology in order to increase the chances of isolating a diversity of organisms. The cells from the initial colonies were re-streaked twice for purity and incubated aerobically on fresh 1/10 or full-strength modified TSA plates. The dye in the medium aimed at avoiding fermenting organisms, as seen by color change in the indicator dye due to mixed acid fermentation and contaminations. The picked colonies were so inoculated into liquid medium (1/10 or undiluted TSB) at pH 7.0 and 5.8 and incubated in vials that had been made anoxic with a He headspace and provided with 0.7 mL O_2 and 1 mL N_2O. The cultures were incubated for one week after which gas measurements were performed using the robotized system described above (Endpoint analyses).

4.8.1 Endpoint analyses of the isolates

The first round of isolation was from slurries made with unenriched native soils (named K1-L and BF-L) while the second round was using enrichment slurries from cycle 1 after 5 mL of N_2O was reduced (named K1-1 and BF-1). 16 isolates from K1 soil were grown in TSB, as well as BF-L-1 and BF-L-2. BF-1-3 to BF-1-16 were grown in a 1/10 dilution of TSB. For the first and second round of isolation using K1 soil and the first round with BF soil, gas composition was measured continuously for 42 h, while the rest was measured at endpoint. Amongst the 32 isolates, only one was able to reduce N_2O, and only at neutral pH. This isolate was designated K1-1-11 and its gas kinetics measurements are shown in Figure 4.21. This isolate was not able to reduce N_2O at acidic pH. The rest of the isolates did not reduce N_2O but several showed an of CO_2 production of over 1000 µmol $vial^{-1}$, which suggests that fermenting organisms had

been isolated instead of N_2O reducers. Some of the isolates produced trace amounts of NO indicating other denitrifying enzymes, but since N_2OR was the enzyme of interest in this study, these were not investigated further.

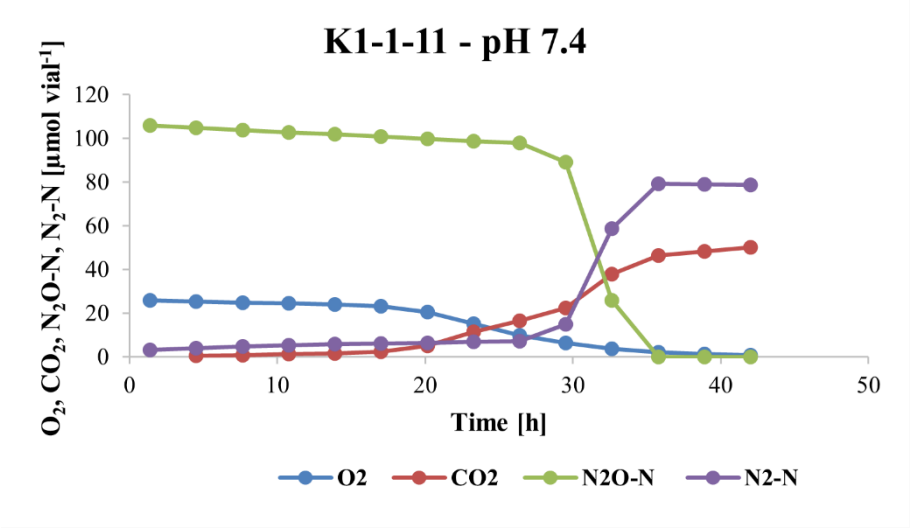

Figure 4. 21. Gas kinetics for isolate K1-1-11 at neutral pH. Out of fourteen isolates from the second incubation, this was the only slurry that reduced the provided 1 mL of N_2O.

A third round of isolation was also done using K1 slurries from the end of cycle 2. This was performed as described above using isolation on anaerobic plates, growth on aerobic plates to ensure a pure culture of the organism, incubation in liquid culture in gas tight vials and analysis of the headspace after a week of anaerobic incubation to check for the conversion of N_2O to N_2. No N_2O reduction took place for any of these isolates after a week of incubation. Like the two first isolation endpoint analyses, high amounts of CO_2 were produced, indicating fermentation. These findings imply the presence of fermenting organisms in high abundances and that they grew well on TSB, probably by fermenting the glucose contained in this medium (Sigma-Aldrich, USA).

4.8.2 Sequencing of the 16S rRNA gene from African soil isolates

To taxonomically classify our isolates, Sanger sequencing of the 16S rRNA gene was performed by Eurofins Genomic on the 14 K1 isolates from the second enrichment cycle isolated in the third round of isolation. The sequencing was done on PCR products of the full 16S rRNA gene in the forward and reverse direction using 27F and 1492R primers. A consensus sequence was made from the forward and reverse sequences, which was analyzed with nucleotide BLAST against the reference RNA sequence database to find closely related species.

The isolation sequences, together with the best hits from BLAST, were then used to make a phylogenetic tree (Figure 4.22).

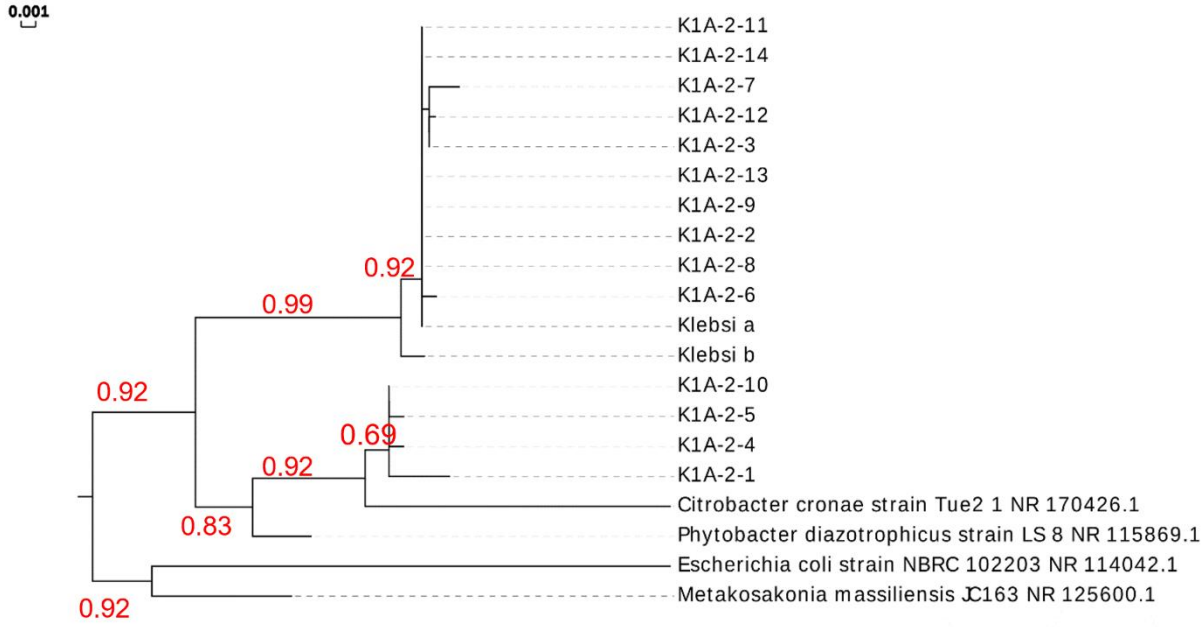

Figure 4. 22. **Phylogenetic tree based on isolates detected by Sanger sequencing of the 16S rRNA gene and their closest matches from nucleotide BLAST**. Sequences were first run through nucleotide BLAST against the reference RNA sequence database and the best reference hits are included in the tree. Confidence scores are shown in red (analogous to bootstrapping values). Klebsi a: *Klebsiella quasipneumoniae subsp. similipneumoniae strain 07A044 (NR_134063.1*. Klebsi b: *Klebsiella pneumoniae subsp. rhinoscleromatis strain R-70 (NR_037084.1)*

The phylogenetic tree shown in Figure 4.22 illustrates the relationship between the different isolates from K1 cycle 2. The isolates are grouped into two clades. One clade is closely related to *Klebsiella* and includes 10 of the isolates. The other group is related to *Citrobacter* and *Phytobacter* and includes 4 of the isolates.

Using the software SINA along with the SILVA database (Pruesse et al., 2012) all the isolates from K1 cycle 2 were classified with the least common ancestor (LCA) method. The results showed that the isolates were all classified as the family *Enterobacteriaceae*. More details are found in Table A9. None of these *Enterobacteriaceae* species are known N_2O reducers and none were able to reduce N_2O in these experiments.

4.9 16S rRNA gene amplicon sequencing of soil slurries

16S rRNA gene amplicon sequencing was performed using native soil slurries and slurries from each of the K1 acidic enrichment cycles. This was done by extracting and purifying DNA, sequencing the DNA using Illumina MiSeq sequencing (performed by Novogene) and analyzing the sequences using the GHAP pipeline (https://researchdata.edu.au/greenfield-hybrid-analysis-pipeline-ghap/981523). 2507 OTUs were found where 5 species clearly dominated the enriched slurries (Table 4.2). The genomes of the closest relatives (identified by BLAST searching the OTU representative sequences) were examined for the presence of *nosZ* by annotation of the genomes using BlastKOALA, and the presence of *nosZ* in similar species was researched in the literature.

**Table 4. 2. Relative abundance of the top 5 most abundant OTUs from the African soil K1 with taxonomy determined by SINA. ***Jones et al. (2011), ** Kruse et al. (2017) K1-L indicates the native K1 soil and K1A-1 to K1A-3 indicates the three enrichment cycles with hexoses.

Closest match	Match%	K1-L	K1A-1	K1A-2	K1A-3	*nosZ* present in closest match	*nosZ* present in similar species in literature
Bacillus bataviensis	99.1	3.14	5.62	51.57	39.57	No	Yes*
Klebsiella sp.	100	0.72	2.20	14.55	37.55	No	No
Enterobacter kobei	98.6	0.67	67.05	3.99	0.63	-	No
Desulfitobacterium metallireducens	96.7	0.47	9.10	15.23	11.93	No	Yes**
Desulfitobacterium metallireducens	95.1	0.30	6.21	10.33	7.76	No	Yes**

The relative abundances of the closest related species are shown above (Table 4.2). The community in native K1 soil had high diversity and evenness with no dominating organism. In cycle 1, an OTU belonging to the genus *Enterobacter* was dominating, while for cycle 2 and 3, *Bacillus* and *Klebsiella* were the OTUs present in the highest abundances. As the matches are not 100 %, the OTUs are likely not these species but relatives within the same or a closely

related genus. Two different *desulfitobacterium* OTUs are also found at high abundances within the enriched slurries.

Taken together, these results indicate that low pH N_2O reducing bacteria was enriched in K1 soil slurries with hexoses and 150 mM where pH was kept stable below 6.1 over three enrichment cycles. Isolations attempts were unsuccessful, but the 16S rRNA gene amplicon sequencing showed interesting results, demonstrating an enriched microbial community dominated by 5 species where three are found within genera known to harbor the *nosZ* gene.

5. Discussion

5.1 Designing an enrichment protocol to select for low pH N_2O reducers

In this study we aimed to develop a protocol to enrich and isolate low pH N_2O reducers from acidic African soils. When designing and developing an enrichment protocol several important choices need to be made. These include the soils used, if they should be enriched as intact soil, in soil slurries or as extracted bacteria cells, the use of buffering, what electron donor and acceptors to provide as well as how they should be provided.

The first choice was regarding the soil used for the enrichment experiments. Two acidic African soils (Bush Fallow, BF and Kpaliga 1, K1) were used in this study, both from cropping fields in north Ghana. Since the soils are from the same area, it is expected that they have been exposed to similar weathering and acidification over the years. The reasoning for using two similar soils is to potentially confirm the findings made. Still, the soils have been exposed to different cropping and agricultural management and so some differences between them would not be unexpected. Using soils from Africa was interesting as the soils are substantially different from European soils and are therefore expected to contain different soil microbial communities. In addition, no research has been conducted on low pH N_2O reducers from this area and finding evidence of such organisms would be of great interest. It would also be interesting to see if the same species are dominating in European soil and African soils, and if not, which species that dominate in these African soils.

The choice of how to enrich the microbes was also important and several options, using intact soils, soil series or extracted bacteria, was considered. The use of intact soils was advantageous since it would include the total bacterial community and thus provide a broad specter of bacteria, in addition to being the closest imitation of real environmental settings. Still, there are difficulties with this method as controlling contributing factors, such as pH and carbon content, would be problematic due to spatial variability on the microscale. Another option would be to use a Nycodenz cell extraction, or a similar process, but as only a small proportion of the microbial community would be extracted (Frostegård et al., 1999) this could lead to the potential exclusion or loss of low pH N_2O reducers and were therefore not an ideal method. Enriching soil slurries was therefore the best option as it would allow for pH control, providing

carbon evenly and include a broad specter of microbes. The soils used in this enrichment was fumigated to lower the starting biomass before being inoculated with native soil (or finished enrichment slurries) to allow for enrichment of the desired microbes.

The choice of electron donor was essential in this enrichment process since different microbes utilize different energy sources. Thus, to ensure the enrichment of a diverse N_2O reducing bacterial population it was important to choose a donor that is available to a broad range of microbes. An electron donor is needed to supply the organisms with energy and make sure that the microbes do not starve so that they are able to grow and maintain cellular functions. Important considerations when deciding the electron donors was their effect on pH, which organisms that were capable of utilizing it for respiration and which metabolism these organisms harbor e.g., fermenting organisms or respiring microbes. Potential carbon sources explored in this enrichment were glutamate, TSB and a mix of hexoses. A small amount of yeast extract was provided to together with the carbon source to ensure that the microbes had all the nutrients needed.

Another important choice was the decision of whether to provide the slurries with only N_2O or to include other nitrogen oxides. Providing nitrogen oxides as NO_3^- and NO_2^- could be advantageous as it would lead to increased enrichment rates, whereas providing only N_2O would be beneficial for enhancing the chances of enriching denitrifiers capable of the N_2O reducing step of denitrification. As full-fledged denitrifiers are in majority compared to only N_2O reducing bacteria and could potentially outgrow the sole N_2O reducers in the presence of other nitrogen electron acceptors, it was decided to only give N_2O. Giving only N_2O as an electron acceptor should not exclude full-fledged denitrifiers as low oxygen levels are the main trigger of the maturation of N_2OR and thus, the enzyme should be matured and functional with or without other nitrogen oxides present. Another argument for the decision to provide only N_2O was that the reduction of nitrite causes pH to rise, and as keeping pH stable and below 6.1 was of significance in this study it was important to minimize factors contributing to a pH rise.

All the first cycle slurries in all enrichment experiments and the isolation received 1 % oxygen to ensure a gentle transition from oxic to anoxic conditions. Observations performed by Højberg et al. (1997) showed that a smooth transition to an anoxic environment was important to ensure enough energy for enzyme maturation, thus avoiding being trapped in anoxia. This was also observed by Lycus et al. (2017) where a great number of truncated and complete denitrifiers

were isolated using a protocol that started under fully oxic conditions, followed by a gentle transition to anoxic respiration.

The next choice was how to provide the electron acceptors. As mentioned above, all the first cycle vials and isolation vials received oxygen to ensure a smooth transition between aerobic and anaerobic respiration. In addition, all enrichment vials received 5 mL of N_2O at the beginning of each incubation to initiate N_2O reduction. The desired volume to be reduced was 30 mL of N_2O which was supposed to be given to each replicate through several smaller doses. It was important not to provide too high amounts of N_2O, as toxic effects have been observed in previous studies of denitrifiers (Sullivan et al., 2013).

Another important choice was whether or not to include a buffer in the soil slurries. Providing the slurries with a buffer would lead to a control of the pH but too high concentrations of PB have been observed to hamper cellular metabolism for some microbes (Å. Frostegård. pers comm). In the initial enrichment experiment buffering was not used as it was hoped that the intrinsically low pH of the soils would not rise too high. Once the initial experiments showed that pH did rise above the threshold of 6.1 during the enrichments, the use of buffering was explored an option to mitigate this effect. Phosphate buffer was chosen as it is a buffer that is not consumed as a carbon source, it is not chelating, and it is tolerated by bacteria in general and covers a rather wide pH range. However, it does not provide any buffering below pH 5.7 and is therefore on the limit of our requirements.

5.2 Initial attempts of enriching low pH tolerant N_2O reducing bacteria in soil slurries

5.2.1 Main findings from the initial enrichment

The initial enrichment was with soil slurries at both neutral (pH 7.5) and acidic (pH 5.6) pH for both soils, using glutamate as the electron donor and without buffering of the slurries. pH rose above 7 for all replicates before 73 h, and there was therefore little difference in the gas kinetic profiles between the initial acidic and initial neutral slurries for each soil (Figure 4.2A, Figure 4.3A). As one of the aims in this study was to enrich low pH N_2O reducers, maintaining acidic environments was essential and, since the pH rose to neutral when the organisms consumed glutamate, no further enrichment was performed using this setup.

A difference between the two soils was observed where K1 soil slurries reduced N_2O faster than BF soil slurries. This is evident as the K1 soil slurries reduced 30 mL of N_2O in 260 h,

while BF slurries only reduced 15 mL of N_2O during the same period (Figure 4.2A, Figure 4.3A). The N_2O-N reduction rates further confirmed this where K1 soil slurries had twice as high reduction rates as the BF soils slurries (Figure 4.2B, Figure 4.3B), thus implying that even though the soils were exposed to similar conditions they had some differences in their N_2O reduction ability, possibly because the population of N_2O reducers was lower in the BF soil.

A noteworthy observation in the first enrichment was the potential toxicity of high concentrations of N_2O. This was observed for both soils by declining N_2O-N reduction rates after 10 mL of N_2O was given to the vials after an initial incubation of 73 h (Figure 4.2B, Figure 4.3B). After this point, the N_2O-N reduction rates for BF soil stayed below 7 μmol $vial^{-1}$ h^{-1} throughout the enrichment without any further increase. The K1 soil was less affected by the higher concentrations of N_2O and managed to continue the reduction until the total 30 mL of N_2O was reduced. The decline in rates implies a toxicity of amounts above 10 mL of N_2O for these soil microbes. Toxic effects of high concentrations of N_2O have been observed in previous studies for denitrifiers such as *Pseudomonas stutzeri* (Conthe et al., 2018) and *Paracoccus denitrificans* in which concentrations as low as ~0.1 mmol/L were reported to interfere with the vitamin B12 metabolism to the microbes (Sullivan et al., 2013).

Another reason for the declining N_2O reduction rates could be that the carbon source was limiting. This is however unlikely. Assuming a growth yield of fast-growing bacteria to be 0.4 (g biomass-C g^{-1} substrate C consumed) (Pirt, 1965) about 40 % of the carbon was assimilated by the cell for growth and maintenance. In the enrichment experiments, we chose to add 5x excess carbon of that needed to reduce 30 mL of N_2O (calculations are found in Appendix 6.2) When the calculations of the amount of carbon needed were performed, the 40 % of the carbon used for growth and cell maintenance was not included. Yet, the carbon source provided to the bacteria was still 3x in excess of what would be needed for reduction of the 30 mL of N_2O.

5.2.2 pH rise caused by the reduction of glutamate

The initial choice of electron donor for the enrichments was glutamate. Glutamate was chosen as the electron donor since it nearly is a universally available carbon source and can be utilized by most heterotrophic bacteria. This means a potential broad specter of microbes could be enriched and isolated if glutamate was provided as the electron donor. In addition, glutamate has a C/N ratio of 5 which is close to the C/N ratio of 4, found for most soil bacteria (Bakken, 1985).

One problem we encountered during this initial enrichment was the increase of pH to a neutral level (Figure 4.4). This increase was thought to be a result of the addition of glutamate to the soil slurries due to the uptake of protons along with glutamate and excretion of ammonia which reacts with water to $NH_4^+ + OH^-$. Since $P_{ka} = 9.3$ for the equilibrium, > 99 % of the released NH_3 will react to NH_4^+ if pH < 7 (Figure 5.1).

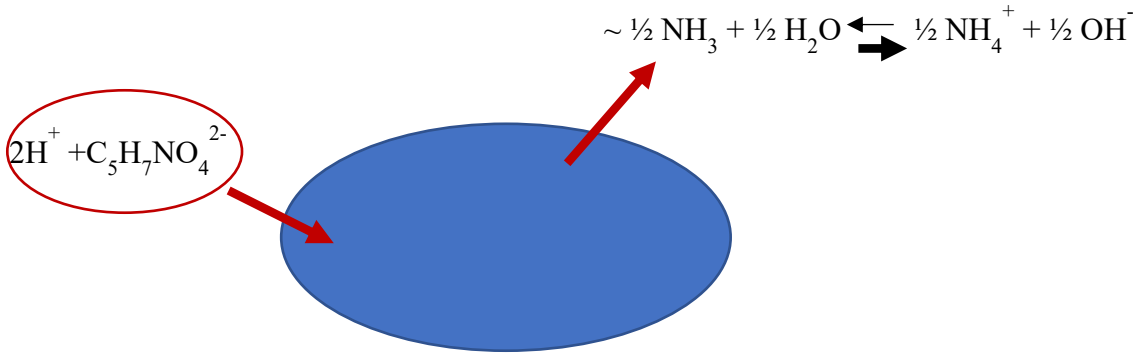

Figure 5. 1. Schematic illustration of how glutamate consumption by the bacteria affects the surrounding pH.

The immediate increase in pH observed when the enrichment setup was tested (Figure 4.4) was probably caused by the lack of pH adjustment of the glutamate stock solutions. This absence of pH adjustment was an issue throughout the enrichment experiments, where none of the carbon stock solutions was pH adjusted to acidic or neutral pH and is expected to influence the start pH of the slurries. Regardless, pH continued to increase over two units after 48 h, and so the consumption of glutamate is considered the main contributor to the pH rise. In the enrichment with hexoses and 150 mM PB pH was kept stable even though the vials were given a hexose solution at a higher pH (6.47), thus confirming that consumption of glutamate was the reason for the increased pH observed in the initial enrichment.

5.2.3 Adaptations of the enrichment protocol after initial enrichment attempts

To keep pH below 6.1 in the acidic treatments, which is the threshold level found to severely hamper N_2OR maturation by Bergaust et al. (2010), three different concentrations of PB (100 mM, 150 mM and 200 mM) were tested in soil slurries amended with glutamate (Figure 4.5, Figure 4.6). While none of these PB concentrations were enough to stabilize the pH < 6.1 in the acidic slurries during oxic incubation, pH remained below 6.1 during the anoxic incubation when 150 and 200 mM PB were used. Based on the first enrichment it was expected, however,

that the incubation time would be longer than 100 h and it was therefore decided that buffering the slurries was not enough while using glutamate as the carbon source. As we aimed to enrich and isolate as wide a range of microbes as possible, higher PB concentrations were not tested since excessive concentrations of phosphate may be toxic to several microbes (Å. Frostegård. Pers. comm).

As glutamate consumption turned out to cause a rise in pH, two other carbon sources were tested, TSB and a mix of hexoses. TSB was a good candidate as it contains mostly amino acids and peptides which are almost universally available for microbes and was used with great success in the isolation of denitrifying bacteria performed by Lycus et al. (2017). A mix of hexoses was another, possibly better, option as it was expected to keep pH stable and is known to be a common carbon source in soils through the breakdown of cellulose (Gunina & Kuzyakov, 2015). Although glucose is used by most bacteria, we chose a mixture of hexoses, including glucose, to capture as diverse a set of isolates as possible. A disadvantage of using hexoses is that fermenting organisms use sugars as their energy source (Müller, 2001) so that the chances of enriching and isolation of fermenters would be high. Still, the mixture of hexoses was given, since TSB caused an increase in pH above the threshold of 6.1 after 22 h, whereas the slurries given hexose mixture kept the pH stable below 6.1 through an incubation of 40 h (Figure 4.7). In this experiment, N_2O reducers in acidic environments were of interest and it was therefore essential to keep the pH stable below 6.1 and hexose mixture was therefore chosen as the carbon source in further enrichment experiments even if it potentially excludes some microbes.

Based on the first enrichment with glutamate for K1 soil (that ended up at neutral pH) (Figure 4.2) and the neutral enrichment using hexoses (Figure 4.19) it is possible to compare the two carbon sources, where the enriched slurries with glutamate had a substantially faster reduction of 30 mL of N_2O compared to those with hexoses as the carbon source. In the neutral enrichment with hexoses, 15 mL of N_2O was reduced after incubation of 657 h. This was almost 6x slower than in the glutamate enrichment (with neutral pH) where the reduction was complete in 115 h. The preference for glutamate as the carbon source is further strengthened by looking at the N_2O-N reduction rates and N_2-N production rates where the reduction rates in K1 soil with glutamate reached a level of 30 - 35 µmol N_2-N $vial^{-1}$ h^{-1} while the reduction rates for K1 soil with hexoses reached a level of 7 µmol N_2-N $vial^{-1}$ h^{-1} at the highest. Based on this it can be argued that glutamate was more accessible as an energy source to the microbes in these soils than hexoses. It could therefore be that some of the N_2O reducers were not able to access the sugars, but could

access the glutamate, causing the hexose enrichment to enrich only a subset of the N_2O reducers. Another possibility could be that the N_2O reducers were using a downstream metabolite of the hexoses and were thus slowed by waiting for the upstream breakdown to be performed by other organisms. A third option could be that the nitrogen found in glutamate played a role in the faster reduction rates as no other nitrogen compounds were provided in the enrichment with hexoses. Regardless, keeping an acidic stable environment was crucial in these experiments and faster reduction times were not important, thus hexoses were the more appropriate choice here. This shows the importance of improving the enrichment setup used, as the carbon source greatly affects the pH in the media as well as the reduction time of N_2O.

Another improvement of the enrichment protocol was changing the size of the doses of N_2O that was be given. As previously discussed, additions of 10 mL of N_2O seemed to hamper the oxidation of vitamin B12 and thus have toxic consequences for the microbes. The following enrichments were therefore only provided with doses of 5 mL of N_2O and no such toxicity was observed.

5.3 Unexpectedly high CO_2 production: Iron reduction and/or fermenting organisms?

Throughout the initial enrichment (with glutamate) and the second enrichment (with hexoses), unexpectedly high amounts of CO_2 were produced based on the stoichiometry of the reduction of glutamate and hexoses (glucose) (Equation 1 and 2, respectively). The reduction of N_2O to N_2 requires 2 electrons and so 9 mol of N_2 should be produced by the breakdown of glutamate and 12 mol of N_2 should be produced by the breakdown of glucose if all the electrons were used for reduction of N_2O. In both cases this equals the production of almost twice the amount of (1.8 for glutamate and 2 for glucose) N_2 produced compared to the of CO_2 produced. This was not the case in these experiments where none of the enrichments showed gas kinetic profiles where N_2 production was higher than CO_2 production.

$$C_5H_9NO_4 + 6H_2O \rightarrow 5CO_2 + NH_4^+ + 17H^+ + 18e^- \text{ (Equation 1)}$$

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^- \text{ (Equation 2)}$$

The CO_2 production from the enrichment with glutamate is an approximately value as the pH rose and thus affected the solubility of CO_2 and thereby the amount of CO_2 found in the gas headspace. It is still interesting that the same amount of CO_2 was produced for both BF soil and K1 soil, even though they received different amounts of N_2O and therefore were expected to produce different amounts of CO_2. This further indicate that other factors play an important part in the CO_2 production observed.

In addition, this CO_2 production took place before any N_2O reduction occurred, seemingly inhibiting the reduction of N_2O for a period of time. These observations led to the assumption that CO_2 was produced by another factor than N_2O respiration. Although it was most likely caused by fermentation, another hypothesis was that it was derived from iron reduction.

Interestingly, the CO_2 production rates in all experiments was high initially before slowing down and staying at low levels or at approximately zero throughout the incubation periods. This was also unexpected as N_2O was continuously reduced and thus, CO_2 was expected to be continuously produced. This is strange and the reason for this is uncertain, but might be caused by some solubility or adsorption which is not observed in the headspace.

5.3.1 Reduction of Fe(III) to Fe(II)

One potential contributor to the high CO_2 observed in both the enrichment with glutamate and hexoses was the reduction of Fe(III) to Fe(II) and thus the production of CO_2. Dubinsky et al. (2010) estimated that reduction of Fe(III) could account for more than a third, and up to 50 %, of the CO_2 production, observed under anoxic incubation in tropical forest soil communities such as the African soils used in these experiments. These results suggest that iron reducers in such soil may compete with denitrifiers for electron donors and that reduction of Fe(III) might be a primary form of microbial respiration in these ecosystems consisting of highly weathered soils containing high concentrations of Fe(III). Based on these observations it can be argued that iron reducers in such soils may compete effectively with denitrifiers for electron donors and consequently contribute to the CO_2 production observed.

In the present study, it was found that the anoxically incubated slurries of both soils that had received carbon (hexoses) had higher concentrations of Fe(II) than freshly made slurries (10 times higher for the BF soil and 5 times higher for the K1 soil, Table 4.1). These findings indicate that Fe(III) reduction occurred, thereby potentially contributing to the production of

CO_2. Ginn et al. (2017) also showed interesting results where the Fe(II) production in tropical forest soils had increasing rates with increasing numbers of anoxic spells, indicating rapid growth of iron reducing bacteria. Fe(II) is difficult to measure in complex media so the quantification of Fe(II) reported in Table 4.1 is therefore likely an underestimation (Bosch et al., 2010; Ettwig et al., 2016), which strengthens the hypothesis that iron reduction contributed to the CO_2 production observed. Another reason for the likely underestimation is that abiotic oxidation of Fe(II) to Fe(III) may happen once the vials were opened before measurements. To get more accurate measurements in this study anaerobic conditions should be kept and the Fe(II) concentrations should have been extracted in HCl as performed by Ginn et al. (2017) instead of 0.01 M $CaCl_2$ used in the present study. The role of iron reduction in the African soils used in these experiments remains uncertain due to the difficulty of quantifying the concentrations of Fe(II) and more detailed investigations would be of interest.

It is a common notion in microbiology that aerobic respiration (of O_2), anaerobic respiration using other electron acceptors, and fermentation, will take place orderly, determined by the thermodynamics of the reactions (Jørgensen, 2000). Thus, reduction of Fe(III) to Fe(II) should not take place before reduction of N_2O to N_2. However, it is a possibility as studies have been reported where the redox tower does not apply in nature (Chen et al., 2017). In the present study the Fe(II) concentrations increased during incubation with hexoses (Table 4.1) suggesting that there was some competition for electrons that may have affected the N_2O reduction. If this was the case it could explain the slow start in N_2O reduction where organisms carrying N_2OR had to reach a certain level to be able to compete with Fe(III) for the electrons and thus begin to reduce N_2O.

The observation of increased concentrations of Fe(II) in soil slurries with hexoses is interesting and the results indicate that N_2O reduction could be hampered by Fe(III) reduction. This could mean that different soils need specified agricultural treatments depending on their mineral compositions, such as iron content, when developing methods to combat N_2O emissions. As previous research aimed at isolating N_2O reducing microbes has mainly been performed on European soil and due to a lack of research focus in countries which have these iron-rich soils, this iron issue might have been overlooked. This is still speculative and needs to be investigated further before any conclusions can be made.

5.3.2 Fermenting organisms

Another explanation for the unexpected CO_2 production may be the presence of fermenting organisms. This hypothesis is supported by the enrichment using extracted soil bacterial cells (Figure 4.10, Figure 4.11), where high amounts of CO_2 production were observed in the enrichment cultures of extracted soil bacteria even though iron was removed through the extraction process and no N_2O reduction took place. If Fe(III) reduction was the main contributor to the CO_2 production, it was expected that the amount of CO_2 would be lower in the extracted bacterial cell enrichment than in the enrichments with hexoses. This was not the case. The CO_2 production in the cultures of extracted soil bacteria ranged from 700 – 900 µmol $vial^{-1}$, which was the same range as observed in all three cycles of the enrichment with hexoses (700 – 950 µmol $vial^{-1}$).

Interestingly, the H_2 production was low in all the hexoses enrichments ranging from 20 to 60 µmol $vial^{-1}$ produced and with production rates ranging between zero and 17 µmol $vial^{-1}$ h^{-1}. This was unexpected as this gaseous product usually are a major fermentation product. An explanation for this could be that the majority of the microbes in the enrichments had a fermenting pathway in which CO_2 was the main product. Research performed by Solomon et al. (1994) showed that *Klebsiella* species had a higher production rate of CO_2 than of H_2 when excess amounts of glycerol were present. As excess amounts of glucose were provided to our slurries, which may be reduced to glycerol, this was likely the case in these experiments as well. As the Sanger sequencing of the 16S rRNA gene and 16S rRNA gene amplicon sequencing showed great abundance of *Klebsiella* species in the K1 soil (Figure 4.22 and Table 4.2) this hypothesis is further strengthened.

5.3.3 Unsuccessful enrichment of N_2O reducing microbes in cultures of extracted soil bacteria

In the extracted bacterial cells experiment no N_2O reduction occurred during the incubation of 1050 h. The reason for this is not clear. Generally, about 10 – 20 % of the total bacterial soil community is obtained when extracting bacterial cells with Nycodenz (Frostegård et al., 1999). Possibly, the populations of N_2O reducing bacteria were small in these soils. If so, the abundance of extracted bacteria may have been too small to overcome the potential fermenting population in the soils. Other possible explanations could be that the extraction was inefficient, or that the cells did not survive in the Sistrom's medium used. Regardless the reason, no N_2O was reduced after a long incubation period, and it was concluded that this was not a successful

enrichment. As CO_2 was produced in large amounts, this experiment further supports the hypothesis that fermenting organisms are present in high abundances in these soils and thus contribute to producing CO_2.

5.4 A successful enrichment of N_2O reducers in acidic African K1 soil (Enrichment with hexoses as the carbon source)

As a mix of hexoses in buffered medium was successful in keeping the pH below 6.1 (described in chapter 4.2.3, Figure 4.7) this was chosen for a final experiment aiming to enrich N_2O reducing bacteria in acidified soil slurries by passing them through several enrichment cycles. In the first cycle for both soils, the reduction of N_2O was slow and after 260 h only one slurry had reduced the initial 5 mL of N_2O (Figure 4.8). This was unexpected based on the enrichment with glutamate where both soils used 50 h to reduce the initial 5 mL and where K1 soil used a total of 160 h to reduce the desired amount of 30 mL of N_2O (Figure 4.2A). As an attempt to initiate N_2O reduction in the first cycle of the enrichment with hexoses, six of the slurries (three from each soil) received nitrate and additional hexose mixture after 96 h. This was not ideal as the reduction of nitrite in denitrification increases pH. The one slurry that managed to reduce 30 mL of N_2O after 1260 h (K1A-1-5, Figure 4.12A) had not received any nitrate additions and kept the pH acidic throughout the incubation period. From what is known about regulatory biology of denitrification, N_2O reduction is in some organisms constitutively expressed (low transcription) in the presence of low concentrations of O_2, while others initiate transcription when O_2 concentrations approach zero. In some organisms, NO, is an additional trigger of *nosZ* transcription (Spiro, 2012). As the three K1 slurries that did receive nitrate reduced zero to 10 mL of N_2O, it seems like the N_2O reduction was induced without the need for NO.

In the first cycle of the enrichment with hexoses, the amount of N_2O-N reduced, and thus the N_2-N produced, was highly variable (Figure 4.13A). One replicate (number four) from each soil was destructively sampled for Fe(II) measurements and was therefore not continued after 260 h. For K1 soil the amount of N_2O reduced varied from none to 30 mL, whereas for the BF soil all replicates reduced at least 5 mL of N_2O, and 30 mL was reduced at the most. One of the replicates (BFA-1-1) took long to begin reduction where the initial 5 mL was reduced after 1301 h, before the next 25 mL was reduced from 1301 h to 1646 h. This sudden and fast reduction might indicate that hotspots where the bacteria could perform the reduction occurred, bunt this is unlikely in a slurry and a more probable reason could be that some inhibitory

substance was removed. Based on the varying amounts of N_2O reduced it can be argued that the two soils contain a small population of N_2O reducers where different amounts have been inoculated into different replicates through chance and thus leading to varying reduction capabilities.

The N_2-N production rates corresponded to the N_2O-N reduction rates in all replicates which implies that all the N_2O given have been reduced to N_2. The reduction of N_2O to N_2 is carried out by the enzyme N_2OR, and it is presumed that the N_2O reduction rate equals the number of cells expressing *nosZ* genes present. Thus, an increasing N_2O reduction rate indicates an increased amount of N_2OR and thus a growing population of microbes able to reduce N_2O.

The lack of N_2O reduction observed initially in this experiment was hypothesized to be caused by factors such as 1) no transcription of the N_2OR enzyme, 2) no maturation of the N_2OR enzyme or 3) a low population of N_2O reducing organisms which are not competitive for hexoses. The first reason was not likely as N_2O reduction was observed in one slurry after 260 h (Figure 4.8), and in all but one slurry after a longer incubation period (Figure A2). The second reason was more probable, as it is known that low pH hampers or inhibits the maturation of the N_2OR enzyme (Bergaust et al., 2010). A long maturation time may be the reason for the slow start in N_2O reduction, but as the reduction takes place throughout the incubation the hypothesis that no maturation of the enzyme occurs is rejected. The second reason is still possible as the maturation of N_2OR could be slow due to the low pH or that maturation happened as a result of an unknown repair mechanism. The third option is also a potential reason. Only a few acid tolerant N_2O reducers have been found until now, suggesting that the low pH N_2O reducing population is small. This, in turn, suggests that if fermenting organisms are present in large amounts, they will outgrow or compete with the N_2O reducing population. Fermenting organisms use sugars as their energy source, reducing the sugars to fatty acids that might be the energy source utilized by the N_2O reducing microbes. It is also a possibility that the competition between the fermenting organisms and the N_2O reducing organisms initially is in favor of the fermenting organisms before the N_2O reducing organisms take over.

After a long incubation time, ranging from 1260 – 1700 h, one K1 soil slurry reduced the total amount of 30 mL of N_2O, and one BF soil slurry reduced 25 mL of N_2O (K1A-1-5, BFA-1-2, Figure 4.12). The BF replicate had a pH above the threshold of 6.1 and a second cycle was therefore not started for this soil. The K1 replicate kept pH stable below the threshold and was therefore chosen as an inoculum for the second cycle in the enrichment. In the second and third cycle, all replicates reduced 30 mL of N_2O in under 395 and 446 h (Figure 4.14, Figure 4.16),

respectively, while keeping pH stable, indicating a successful enrichment of the acidic N_2O reducing microbial community in the African K1 soil. By comparing of the enriched organisms (through 16S rRNA gene amplicon sequencing) we aim at getting a clearer picture of who the low pH N_2O reducers are.

5.4.1 Development of N_2O reduction kinetics throughout the sequential enrichment cycles

N_2O reducing organisms from African K1 soil were successfully enriched over three cycles while keeping the pH acidic. The enrichment of the N_2O reducing community in K1 soil slurries is clearly visualized by the time the reduction of 30 mL of N_2O took in each cycle. In the first cycle, K1A-1-5 used 1260 h to reduce the 30 mL, while the fastest replicate (K1A-2-2) in the second cycle used 385 h on the reduction and the fastest replicate (K1A-3-3) from the third cycle used 346 h (Figure 4. 18). Based on these timepoints it can be argued that the acidic N_2O reducing community had been enriched throughout the cycles. Since there was little difference between the second and third cycles no further enrichment was performed for the K1 soil.

Another interesting observation is the reduction time of the initial 5 mL of N_2O given to all replicates. For K1A-1-5 the 5 mL of N_2O was reduced after 516 h, while for K1A-2-2 it was reduced after 203 h whereas K1A-3-3 used 144 h on the reduction. This strongly suggests that a higher population of N_2O reducers were inoculated between each cycle, which further confirms that the population of low pH N_2O reducers had increased in the enriched slurries. This increase in the initial reduction may also indicate that the growing N_2O reducing population outcompete the fermenting population more rapidly than when no N_2O reducing population has been enriched.

Comparison of the N_2O-N reduction and N_2-N production rates further supports this (Figure 4.18). K1A-1-5 had its highest N_2O-N reduction rate of 9 µmol $vial^{-1}$ h^{-1}, whereas K1A-2-2 and K1A-3-3 had their highest reduction rates of 27 and 19 µmol $vial^{-1}$ h^{-1}, respectively. This shows that N_2O is reduced at increasingly high rates throughout the cycles, where the replicates in the second cycle had the fastest reduction rates. The slower rates in the third cycle compared to the second cycle further supports the decision of ending the enrichment after three cycles instead of five, as this may indicate that the population reached a maximum in the second cycle. In the first cycle, the N_2O-N reduction rates and N_2-N production rates slow down towards the end of the enrichment. This may indicate that the microbes are experiencing limitations in some

nutrients after a long incubation period. Due to the excess amounts of hexose mixture provided to the slurries, this nutrient is most likely not carbon. These slower reduction rates towards the end are observed in a smaller scale for the second cycle, and not in the third cycle. The long incubation time in the first cycle could be the explanation for why this is mainly observed in the first cycle.

5.4.2 Comparison of N_2O reduction kinetics between acidic and neutral enrichments with hexoses

An enrichment with neutral pH using hexoses as the carbon source was also started. This was aimed at answering the question of whether all the N_2O reducers in these acidic soils were acid tolerant or if it was just a small subset that were capable of N_2O reduction at low pH. Due to time limitations the first cycle of the neutral enrichment was not completed which makes the comparison restricted. It is also difficult to compare the first neutral cycle to the first acidic cycle as acidic cycle 1 was left without measurements for 260 h, and the time points of reduction might be different without that period.

Based on the incubation that was performed it was seen that the neutral enrichment had similar maximum N_2O-N reduction rates as the acid cycle 1 enrichment (7 µmol $vial^{-1}$ h^{-1} and 9 µmol $vial^{-1}$ h^{-1}, respectively) (Figure 4.20, Figure 4.12). This similarity between the acidic and neutral enrichments is further confirmed when comparing the time the slurries used to reduce 15 mL of N_2O. K1N-1-3 and K1N-1-5 both used approximately 675 h to reduce 15 mL of N_2O, whereas K1A-1-5 from the acidic enrichment used 676 h to reduce 15 mL N_2O. The lack of difference between the acidic and neutral enrichments was somewhat unexpected as the general view is that most microbes thrive at neutral pH, and it was therefore anticipated that the neutral enrichment would have higher reduction rates than the acidic. This suggests that amongst the N_2O reducers, a large proportion are likely to be acid tolerant N_2O reducers. One possible explanation for the slow reduction rates in the neutral soil slurries is that bacteria have a narrow pH range for optimal growth which is similar to the soil pH (Bååth, 1996; Fernández-Calviño & Bååth, 2010). Thus, since the African soils are naturally acidic with a pH measured in soil slurries to 5.8 the acidic soil slurries provided more ideal conditions for the metabolic reactions to take place than the neutral slurries. If faster reduction rates had been observed in the neutral slurries, it would be conceivable that the N_2O reduction took place in microsites with a locally higher pH or that the N_2O reducers in the soils were living off an alternative metabolism under neutral condition so that the N_2O reductase was largely unused. As this was not the case here,

it is believed that acid tolerant N_2O reducers adapted to these African soils do exist and that the enrichments harbored bacteria that were able to express functional NosZ enzymes at acidic pH.

5.5 Isolates from the African soils at low and neutral pH

No bacteria able to reduce N_2O at acidic pH were isolated in this study. One isolate, K1-1-11, managed to reduce 1 mL of N_2O at pH 7.0, but not at pH 5.8 (Figure 4.21). High amounts of CO_2 were produced by all the isolates which suggest that fermenting organisms had been isolated rather than N_2O reducers.

The setup for isolation was based on experiments performed by Lycus et al. (2017) which led to the successful isolation of one organism from acidic soil that reduced N_2O at low pH in pure culture. In the study performed by Lycus et al. (2017), microbes were grown aerobically on 1/10 TSA plates pH adjusted to 5.7 prior to being grown aerobically and anaerobically in 1/10 TSB medium before endpoint analyses were performed. In the present study, the organisms were grown on either or both 1/10 and full-strength modified TSB/A pH adjusted to 5.8 in order to capture as many microbes as possible before endpoint analyses were performed to examine if N_2O was reduced by these isolates or not. In contrast to Lycus et al. (2017), this study aimed to enrich and isolate only N_2OR containing organisms and not different truncated denitrifiers. In addition, African soils were used which are expected to contain different microbial communities than Norwegian soils, thus aiming to isolate new acidic tolerant N_2OR containing bacteria. Another difference was the enrichment before the isolation process to enhance the N_2O reducing community and thereby improving our chances of isolating microbes able to produce functional NosZ enzymes at pH below 6.1.

Fourteen of the isolates from K1 soil were classified by Sanger sequencing of the near full length 16S rRNA gene before closely related species were identified using nucleotide BLAST against the reference RNA sequences database. The isolates were grouped into two clades where one clade was closely related to *Klebsiella* and the other clade was closely related to *Citrobacter* and *Phytobacter* (Figure 4.22). *Klebsiella* was the dominating clade where ten of the fourteen isolates were classified. *Klebsiella*, *Citrobacter,* and *Phytobacter* are all found within the family *Enterobacteriaceae* and further analyses showed that all the isolates were found within that family. The species within this family are known to ferment glucose (Borman et al., 1944; Drancourt et al., 2001; Werkman & Gillen, 1932) found in both our hexoses mixture

and the TSB medium. These findings strengthen the hypothesis that fermenting organisms are present in abundance in the enriched slurries and contribute to the dominating CO_2 production.

As no acidic tolerant N_2O reducing organisms were isolated in this study, TSB and TSA were probably not the optimal growth medium for these soil microbes. The isolation process was performed without any previous knowledge about the microbes present, and it is therefore not unexpected that no N_2O reducing bacteria were detected. Further isolation work should implement the hexose mixture used in the enrichment as it is already known that the microbes may utilize this as an energy source and perform N_2O reduction. Another improvement for further isolation experiments would be to increase the incubation period throughout the isolation steps as the gas kinetics from the enrichment has shown that these are slow-growing organisms that need weeks rather than days to reduce N_2O. Based on the knowledge obtained in this study modifications in the isolation protocol should be made to better exclude the fermenting species and select the N_2O reducers.

5.6 Five dominating species in the enriched K1 soil

The 16S rRNA gene amplicon results found in Table 4.2 present the five species that dominated the K1 soil after three rounds of enrichment. These species were related to *Bacillus bataviensis*, *Klebsiella sp*., *Enterobacter kobei*, and *Desulfitobacterium metallireducens*. In the first cycle, the dominating species related to *Enterobacter kobei* had a relative abundance of 67.05 %. This species was not present in high abundances in the second and third cycle indicating the exclusion of this species when higher amounts of N_2O reducers were present. The species related to *Enterobacter kobei* and the *Klebsiella sp.* are most likely the same species isolated and classified in section 5.6 and Figure 4.22 and thus, are known not to reduce N_2O.

In the second and third cycle, the most abundant species were related to *Bacillus bataviensis* and *Desulfitobacterium metallireducen*. Based on the match percentage, the species present in the K1 soil are not *Bacillus bataviensis* and *Desultifobacterium metallireducens* but closely related species. *Bacillus bataviensis* is not known to harbor the *nosZ* gene, but the gene has been identified in other *Bacillus* species. Jones et al. (2011) detected the *nosZ* gene in 14 *Bacillus* isolates from Swedish soil where N_2 was the dominant end product at pH 7. Studies by Cheneby et al. (2004) also reported findings of denitrifying *Bacillus* strains at neutral pH in French soil communities. The enrichment of *Bacillus* species in addition to the findings mentioned above, strongly suggests that *Bacillus* strains are important members of the

denitrifying soil community and that some species from the genus are capable of N_2O reduction at low pH. Similar results were obtained for *Desulfitobacterium metallireducense*. This species has no known *nosZ* gene, but several other *Desultifobacterium* species harbor the gene. Kruse et al. (2017) found that eleven out of twelve analyzed *Desulfitobacterium* species encoded the NosZ protein and that the *nosZ* gene sequences were similar to those of a known functional N_2O reductase identified by Liu et al. (2008), further suggesting that these *Desulfitobacterium* species are functional N_2O reducers.

Interestingly, both *Bacillus* and *Desulfitobacterium* are Gram-positive bacteria. There is currently limited knowledge present on the denitrification abilities and features in Gram-positive bacteria, but studies of *Bacillus* species have shown that this bacterium carries all the denitrification enzymes where the denitrification process is equivalent to Gram-negative organisms except for the contribution of membrane-bound or associated proteins instead of free floating periplasmic ones (Heylen & Keltjens, 2012; Mania et al., 2014). Still, obtaining a greater understanding of the denitrification pathway for these organisms would be of immense interest.

Another interesting and noteworthy observation is that no *Rhodanobacter* species were enriched in this soil. All studies so far have suggested that *Rhodanobacter* is the only organism capable of low pH N_2O reduction and that these species are dominating in denitrifying communities in low pH environments (Green et al., 2012; Lycus et al., 2017; Van Den Heuvel et al., 2010). The result of this study provides the first evidence that other low pH N_2O reducing organisms exist and thus, several organisms could be used as potential N_2O sinks in acidic soils thereby contribution to the mitigation of N_2O emissions. Finding several organisms capable of N_2O reduction in acidic environments is also of interest as they could give greater insight into how the N_2OR enzyme is regulated under such conditions.

In the native soil, a variety of species were present in low abundance. This was expected as soils contain a great variety of microbes. As the five dominating species were found in low abundances in the native soil, it confirmed that they had been enriched throughout the cycles. This further verifies the successful enrichment of acidic N_2O reducers.

5.7 Further work and conclusions

In this study, a successful enrichment protocol has been developed using hexoses as the carbon source where microbes capable of reducing N_2O below pH 6.1 have been enriched in K1 soil

over three cycles. Further work is needed to effectively isolate the microbes capable of this reduction, but from the 16S rRNA gene amplicon results, it is observed that genera with species that are known to harbor the *nosZ* gene are present and have been enriched, and thus strongly suggests an enriched N_2O reducing microbial community at a pH below 6.1. The results strongly indicate the existence of low pH N_2O reducers and that these microbes are likely species within the genus *Bacillus* or *Desulfitobacterium* that have a functional N_2OR under acidic conditions. These findings are of great interest as they allow for deeper investigations into the microbes capable of N_2O reduction at low pH and thus, more information about how the N_2OR enzyme functions for these microbes under acidic conditions. This is of immense importance because pH is a main controller of N_2O emissions globally (Bergaust et al., 2010), which make a very significant contribution to anthropogenic global warming. If low pH N_2O reducers could be harnessed to combat this issue it could provide an important method for reducing these global emissions.

Based on the predicted low pH N_2O reducers identified by 16S rRNA gene amplicon sequencing, modifications of the isolation protocol should be made to further select the desired microbes. Such modifications involve providing the isolates with hexose mixture as the electron donor, incubation of the cultures for a longer time as well as the application of antibiotics selective against Gram-negative but not Gram-positive organisms in the agar as the enriched species containing a *nosZ* gene are both Gram-positive while the undesired fermenting organisms were Gram-negative. In addition, metagenomics targeting the *nosZ* gene and proteomics aiming for the N_2OR enzyme should be executed to confirm or refute the presence of the gene or enzyme produced by these organisms in the enrichment material. It would also be of great interest to enrich the low pH N_2O reducing community in BF soils and thereby compare the microbial composition of the two soils.

Microbes capable of only N_2O reduction and lacking the other denitrification steps are of particular interest as such organisms could function as co-inoculants with plant growth-promoting bacteria into the soils and thus work as sinks for N_2O emissions. Still, getting greater knowledge on how the process and the enzyme works could lead us one step closer to mitigating emissions of N_2O.

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6. Appendix

6.1 pH measurements

Several pH measurements were performed during this study and all measurements are shown in detail here.

Table A1. pH measurements from fumigated and native K1 soil. After 1.8 h glutamate was given to a final concentration of 30 mM (yellow) and caused the pH to rise over 1 unit. These measurements are done in 0.01M $CaCl_2$ aerobically.

Time (h)	Fumigated K1	Native K1
0.0	4.77	4.82
0.2	4.80	4.88
0.3	4.80	4.88
0.5	4.83	4.94
0.7	4.84	4.92
0.8	4.83	4.91
1.0	4.98	5.14
1.2	5.03	5.14
1.3	5.02	5.12
1.8	6.24	6.34
2.2	6.18	6.36
2.4	6.10	6.30
2.7	6.08	6.34
2.8	6.00	6.32
17.0	7.37	7.42
22.9	7.83	7.78
25.3	8.02	7.89
46.9	8.14	8.07

Table A2. Aerobic pH measurements of soil slurries with 100 mM PB and 30 mM glutamic acid + yeast extract.

	K1		BF	
Time (h)	pH 7.4	pH 5.8	pH 7.4	pH 5.8
0.0	7.43	5.78		
0.3	7.41	5.79		
0.7	7.42	5.79		
1.7	7.50	5.94	7.43	5.84
16.6	7.58	6.50	7.42	6.03
21.8	7.84	6.82	7.66	6.36

Table A3. pH measurements of soil slurries with 150 mM PB and 30 mM glutamic acid and yeast extract incubated aerobically.

	K1		BF	
Time (h)	**pH 7.4**	**pH 5.8**	**pH 7.4**	**pH 5.8**
0.0	7.43	5.81	7.41	5.77
16.5	7.38	6.13	7.53	6.10
20.8	7.38	6.34	7.59	6.50
92.3	7.88	6.39	8.56	6.41

Table A4. pH measurements of soil slurries with 200 mM PB and 30 mM glutamic acid yeast extract. These measurements were done aerobically.

	K1		BF	
Time (h)	**pH 7.4**	**pH 5.8**	**pH 7.4**	**pH 5.8**
0.0	7.46	5.79	7.45	5.75
16.5	7.42	5.96	7.44	5.95
20.8	7.39	6.14	7.28	6.06
92.3	7.24	6.14	7.34	6.53

Table A1 to A4 were all measured aerobically, at room temperature and with 600rpm.

Table A5. pH measurements of acidic soil slurries with 150 mM or 200 mM PB and 30 mM glutamic acid + yeast extract. These measurements were done anaerobically, at 22 ℃ and 650 rpm.

	K1		BF	
Time (h)	**150mM**	**200mM**	**150mM**	**200mM**
0.0	5.79	5.78	5.79	5.78
88.0	6.04	5.93	5.96	5.93

Table A6. pH measurements of soil slurries in 150 mM PB and either TSB or hexose mixture as the carbon source. These measurements were done aerobically, at room temperature and 600 rpm.

	K1				BF			
	TSB		**Hexose mix**		**TSB**		**Hexose mix**	
Time (h)	**pH 7.4**	**pH 5.8**	**pH 7.4**	**pH 5.8**	**pH 7.4**	**pH 5.8**	**pH 7.4**	**pH 5.8**
0.0	7.39	5.92	7.46	5.90	7.43	5.87	7.40	5.87
16.0	7.18	5.90	6.96	5.71	7.03	5.88	6.96	5.46
21.7	7.74	6.17	7.12	5.74	7.57	6.20	7.17	5.40
40	8.09	6.36	7.22	5.67	7.77	6.32	7.17	5.37

Table A7. pH measurements of soil slurries with 150 mM PB and no carbon additions. These measurements were done aerobically, at room temperature and 600rpm.

	K1		BF	
Time (h)	**pH 7.4**	**pH 5.8**	**pH 7.4**	**pH 5.8**
0.0	7.34	5.76	7.34	5.72
16.0	7.37	5.81	7.34	5.76
21.7	7.43	5.86	7.43	5.84
40	7.34	5.78	7.37	5.73

Table A8. pH measurements of the first part of the enrichment with hexose mixture as the carbon source. These were taken after the slurries had been standing at 4 ℃ without stirring for 1 week.

Name	PH
K1A-1-1	6,02
K1A-1-2	5,98
K1A-1-3	5,98
K1A-1-5	5,97
BFA-1-1	6,09
BFA-1-2	5,70
BFA-1-3	5,90
BFA-1-5	6,01

6. 2 Calculations

Using the ideal gas law to find out how many moles 30 mL N_2O corresponds to

T = 20 ℃ = 293.15 K

V = 30 mL = 30 * 10^{-3} L

P = 1 atm

R = 0.08205 L atm mol^{-1} K^{-1}

$$n = \frac{pV}{RT} = \frac{1\ atm * 30 * 10^{-3}\ L}{0.08205\ L * atm * mol^{-1} * K^{-1} * 293.15\ K} = 0.001247 = 1.25\ mmol$$

6.2.1 Glutamate as the carbon source

$$C_5H_9NO_4 + 6H_2O \rightarrow 5CO_2 + NH_4^+ + 17H^+ + 18e^-$$

Reduction of N_2O to N_2 needs 2 e^- → 9mol N_2O/mol glutamate.

1.25 mmol N_2O/9 mol glutamate → 139 µmol glutamate is needed to reduce 30 mL N_2O

As we wanted 5x excess carbon we added 700 µmol glutamate (~700 µL from a 1 M stock).

If 700 µmol glutamate is respired, it will reduce 9*700 µmol N_2O → 6300 µmol N_2O

Example from figure 4.2:

- 1800 µmol $vial^{-1}$ N_2-N was produced for K1 soil. This corresponds to 900 µmol $vial^{-1}$ N_2 produced and results in the oxidation of 14 % of the carbon given as glutamate (900 µmol/6300 µmol = 0.1428 = 14 %) if all the electrons were used for N_2 production.

6.2.2 Hexose mixture as the carbon source

This calculation is based on glucose to simplify it.

$$C_6H_{12}O_6 + 6H_2O \rightarrow 6CO_2 + 24H^+ + 24e^-$$

Reduction of N_2O to N_2 needs 2 e- → 12 mol N_2O/mol hexoses.

1.25 mmol N_2O/12 mol hexoses → 117 µmol hexose mix is needed to reduce 30mL N_2O.

As we wanted 5x excess carbon we added 560 µmol hexose mixture (~560 µL from a 1 M stock)

If 560 µmol hexoses is respired, it will reduce 12*560 µmol N_2O → 6720 µmol N_2O

Example from figure 4.13:

- 1424 µmol vial^{-1} N_2-N was produced for K1 soil, corresponding to 712 µmol vial^{-1} N_2. This suggests that 11 % of the energy given in the form of hexoses had been used for N_2O reduction and N_2 production (712 µmol/6720 µmol = 0.106 = 11 %) if all the electrons were used for N_2 production.

6.3 Isolates

Table A9. Table showing the results from Sanger sequencing together with the closest match from nucleotide BLAST and classified using the database SILVA.

	BLAST		SILVA	
Sample	**Percent identity**	**Best hit**	**Percent identity**	**LCA tax**
K1A-2-1	97,19%	*Phytobacter diazotrophicus (taxID: 395631)*	98,21%	*Enterobacteriaceae;*
K1A-2-2	98,52%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	99,32%	*Enterobacteriaceae; Klebsiella;*
K1A-2-3	99,16%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	98,8%	*Enterobacteriaceae; Klebsiella;*
K1A-2-4	98,31%	*Phytobacter diazotrophicus (taxID: 395631)*	98,4%	*Enterobacteriaceae;*
K1A-2-5	99,48%	*Phytobacter diazotrophicus (taxID: 395631)*	98,21%	*Enterobacteriaceae;*
K1A-2-6	99,37%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	99,32%	*Enterobacteriaceae; Klebsiella;*
K1A-2-7	97,75%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	98,51%	*Enterobacteriaceae; Klebsiella;*
K1A-2-8	99,63%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	99,51%	*Enterobacteriaceae; Klebsiella;*
K1A-2-9	99,16%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	99,32%	*Enterobacteriaceae; Klebsiella;*
K1A-2-10	97,57%	*Phytobacter diazotrophicus (taxID: 395631)*	98,29%	*Enterobacteriaceae;*
K1A-2-11	99,37%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	99,32%	*Enterobacteriaceae; Klebsiella;*
K1A-2-12	99,30%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	99,32%	*Enterobacteriaceae; Klebsiella;*
K1A-2-13	97,96%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	99,62%	*Enterobacteriaceae; Klebsiella;*
K1A-2-14	99,72%	*Klebsiella quasipneumoniae subsp. Similipneumoniae (taxID: 1463164)*	99,70%	*Enterobacteriaceae; Klebsiella;*

6.4 Figures

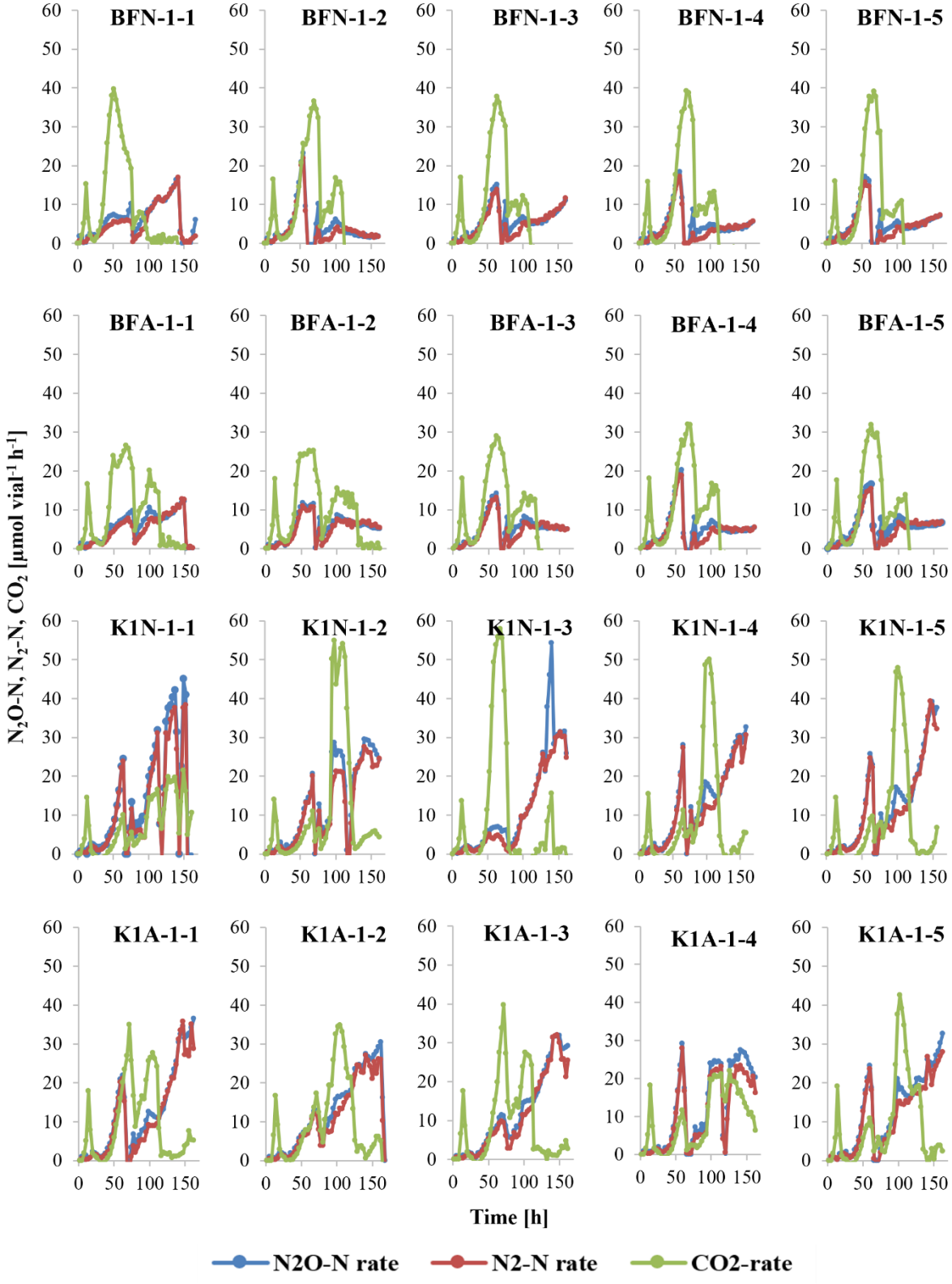


Figure A1. N_2O-N reduction rates and N_2-N and CO_2 production rates for all replicates from the enrichment with glutamate.

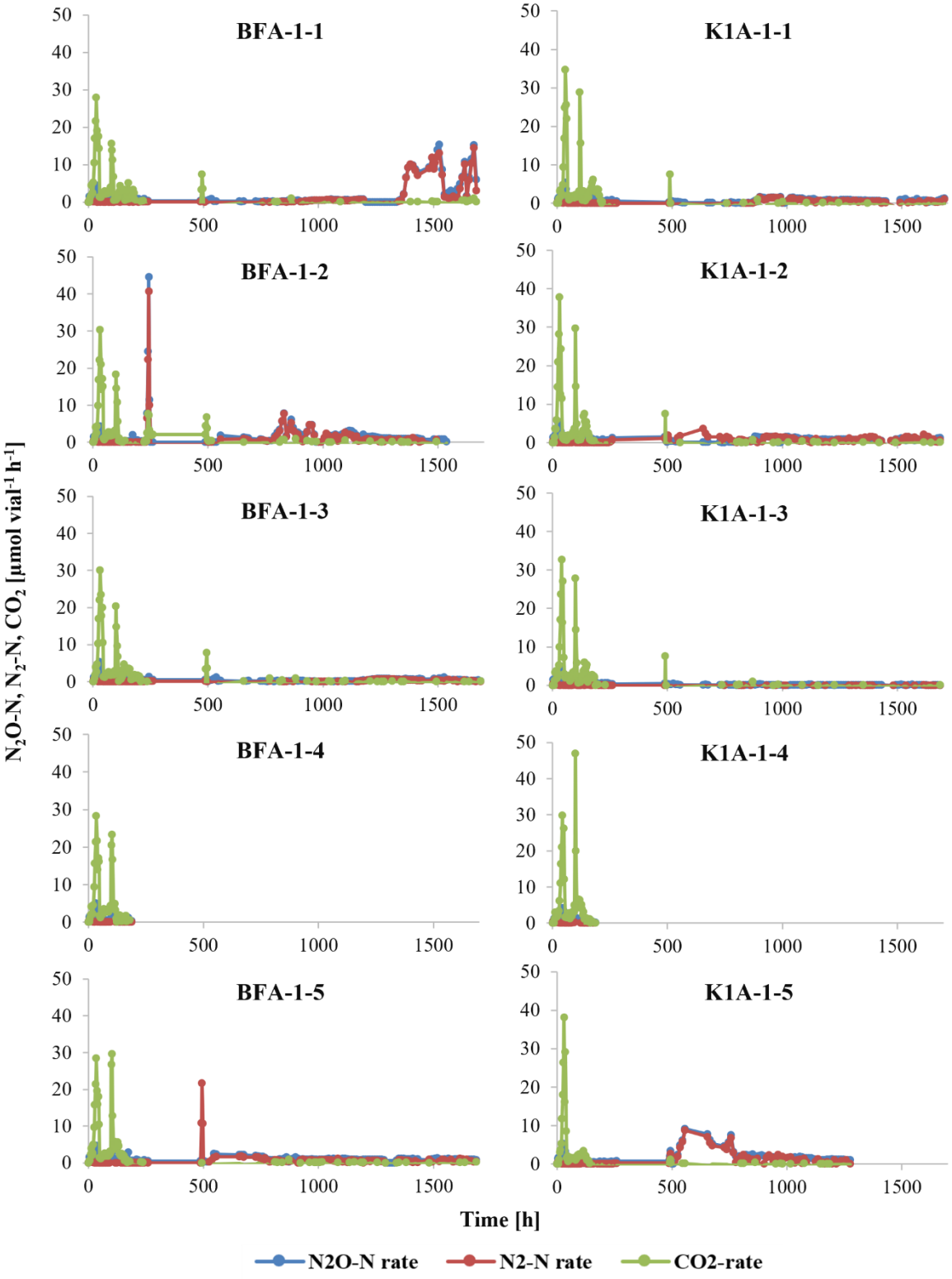


Figure A2. N_2O-N reduction rates and N_2-N and CO_2 production rates for the enrichment with hexoses cycle 1.

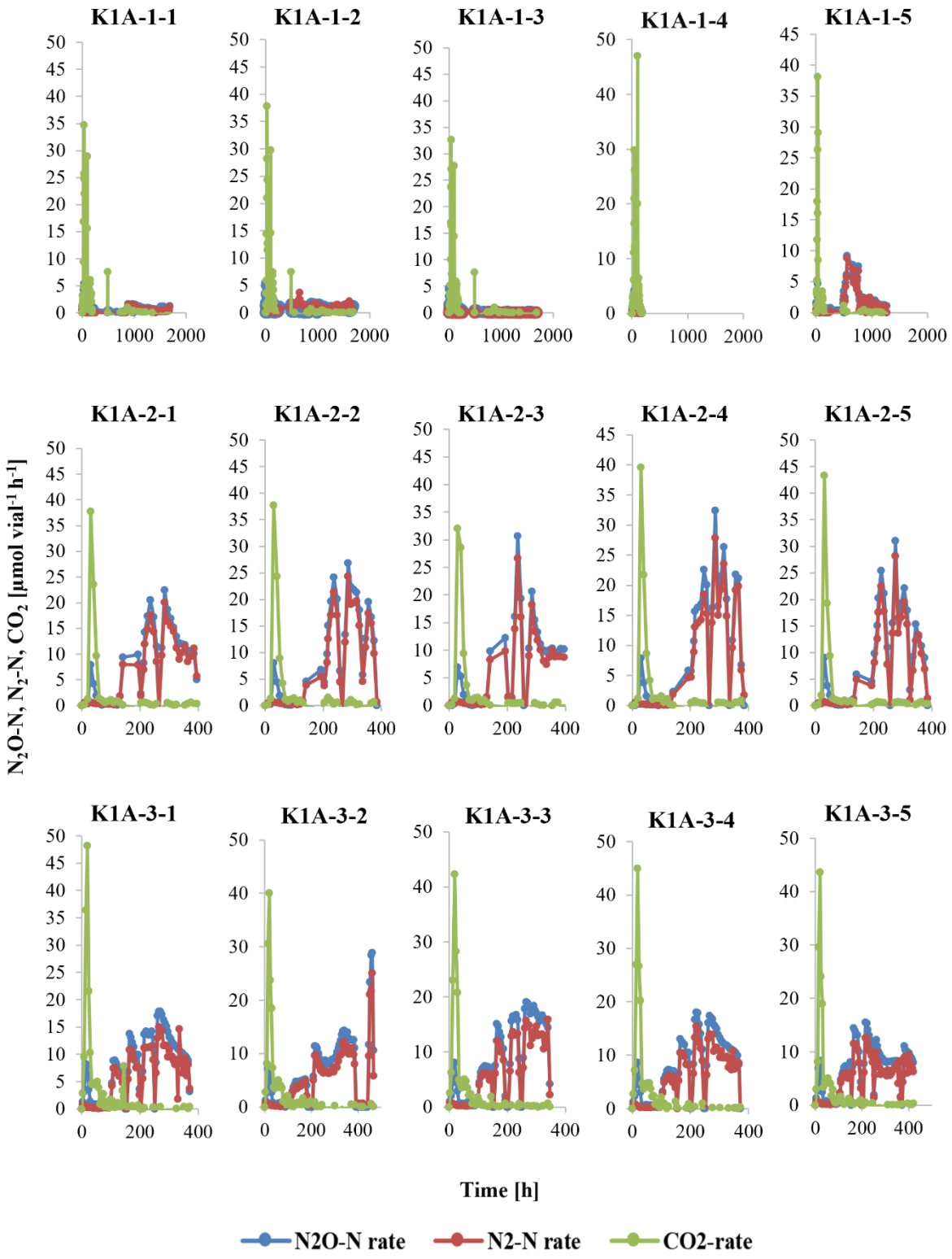
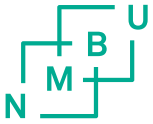


Figure A3. N_2O-N reduction rates and N_2-N and CO_2 production rates for all the replicates from K1 soil in the three cycles of the enrichment with hexoses.



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