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Denitrification role in N_2O Emission

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Sammendrag

Denne masteroppgaven fokuserer på denitrifisering av N2O, som er en viktig del av nitrogenforurensning og en global miljøutfordring. Nitrogen er et avgjørende element som regulerer artsmangfoldet i ulike økosystemer. Mens molekylært nitrogen (N2) utgjør flertallet av atmosfærisk nitrogen, er nitrogendioksid (N_2O) en betydelig form for nitrogenforurensning.

Nitrifisering og denitrifisering, drevet av mikroorganismer, bidrar til N_2O-produksjon i økosystemer. Denitrifisering, som forekommer under oksygenbegrensede forhold, innebærer konvertering av nitrat til N_2 gjennom mellomtrinnene nitritt, nitrogengass og nitrogendioksid. Spesifikke enzymer letter hvert trinn i denitrifiseringsprosessen, slik at denitrifiserende organismer kan respirere og generere energi i fravær av oksygen. Effektiviteten av denitrifisering og reduksjon av N_2O påvirkes av regulatoriske fenotyper som vises av denitrifiserende organismer, der tilstedeværelsen av genet NosZ spiller en avgjørende rolle.

Miljøfaktorer som jordens pH-verdi, karbon- og nitrogenkonsentrasjoner, temperatur og fuktighetsnivå har betydelig innvirkning på N_2O-utslipp. Å forstå disse faktorene og optimalisere denitrifiseringsprosesser er avgjørende for å redusere N_2O-utslipp og dets miljøpåvirkning. Den genetiske mangfoldigheten og metabolske preferanser hos denitrifiserende bakterier bidrar også til variasjoner i N_2O-utslipp, noe som understreker behovet for omfattende strategier for å håndtere og redusere N_2O-utslipp i ulike økosystemer.

Abstract

This master thesis focuses on the denitrification of N_2O, an important aspect of nitrogen pollution and a global environmental concern. Nitrogen is a vital element that regulates species diversity in various ecosystems. While molecular nitrogen gas (N_2) constitutes the majority of atmospheric nitrogen, nitrous oxide (N_2O) is a significant form of nitrogen pollution. Nitrification and denitrification, driven by microorganisms, contribute to N_2O production in ecosystems.

Denitrification, occurring under oxygen-limited conditions, involves the conversion of nitrate to N2 through intermediate steps of nitrite, nitric oxide, and nitrous oxide. Specific enzymes facilitate each step of the denitrification process, allowing denitrifying organisms to respire and generate energy in the absence of oxygen. Denitrification efficiency and N_2O reduction are influenced by regulatory phenotypes exhibited by denitrifying organisms, with the presence of the NosZ gene playing a crucial role.

Environmental factors such as soil pH, carbon and nitrogen concentrations, temperature, and moisture levels significantly impact N_2O emissions. Understanding these factors and optimizing denitrification processes are vital for mitigating N_2O emissions and their environmental impacts. Genetic diversity and metabolic preferences of denitrifying bacteria further contribute to variations in N_2O emissions, emphasizing the need for comprehensive strategies to manage and reduce N_2O emissions in different ecosystems.

List of abbreviations

BNR	Biological nutrient removal
C	Carbon
cNor	c-dependent nitric oxide reductase
CRP	Cyclic-AMP receptor proteins
Cyt	Cytochrome
DRP	Denitrification Regulatory Phenotype
FNR	Fumarate and nitrate reduction regulator
IPCC	Intergovernmental Panel on Climate Change
MAGs	Metagenome-assembled genomes
MGD	Molybdenum cofactor center
N	Nitrogen
N_2	Nitrogen gas
N_2O	Nitrous Oxide
N_2OR	N_2O reductase
Nar/Nap	Nitrate reductases
Nir	Nitrite reductases
NirS	Cytochrome cd1 nitrite reductase
NO	Nitric Oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
Nor	Nitric oxide reductases
NosZ	Nitrous oxide reductase
NosZI and NosZII	Nitrous oxide reductases
Nox	Nitrogen oxides
NRB	Nitrous oxide respiring bacteria
OTUs	Operational taxonomic units
P. Denitrificans	Paracoccus denitrificans
PO	Progressive onset
ppbv	Parts per billion by volume
RCO	Rapid, complete onset
SOC	Soil organic carbon
TAT	Twin-arginine translocation
WSC	Water-soluble carbon
Zur	Zinc uptake regulator

Table of Content

Master Thesis' Setup

This master thesis is divided into six chapters. The start includes a short introduction to the master thesis.

Chapter 2 gives a detailed description of the background and theory of nitrous oxide, denitrification, and phenotype variants. N_2O emissions and factors affecting N_2O emissions are presented in chapter 3 with focus on pH and temperature of the soil. In chapter 4 future perspectives are presented. The last chapter, chapter 5, summarizes and concludes the master thesis.

1 Introduction

In this master thesis the focus will be on denitrification of N_2O emission.

Nitrogen (N) is a crucial element that regulates species diversity in marine, terrestrial, and freshwater ecosystems (Marcarelli et al., 2022). It serves as a primary nutrient, essential for the formation of proteins, DNA, and chlorophyll. In the atmosphere, nitrogen is predominantly found as molecular nitrogen gas (N_2), making up approximately 78% of the atmospheric composition (Yu et al., 2019).

Nitrous oxide (N_2O) is a significant form of nitrogen pollution and a global environmental issue (Davidson et al., 2014). Understanding its behavior and underlying factors is crucial for effectively mitigating N_2O emissions.

Nitrification and denitrification, regulated by microorganisms, drive N_2O production in ecosystems. Nitrification oxidizes ammonium to nitrate, generating N_2O. Denitrification reduces nitrate in low oxygen conditions, producing various nitrogen gases, including N_2O (Huang et al., 2022).

Denitrification is a reductive process that converts nitrate to N_2 under oxygen-limiting conditions. It involves the sequential formation of nitrite, nitric oxide, and nitrous oxide. Specific enzymes, such as nitrate reductases, nitrite reductases, nitric oxide reductases, and nitrous oxide reductases, facilitate each step of the process. Denitrifying organisms utilize these enzymes to respire and generate energy in the absence of oxygen. The expression of denitrification-related genes is triggered by oxygen depletion and inhibited by high oxygen concentrations. Nitrate reductases, such as Nar and Nap, catalyze the reduction of nitrate to nitrite. Nitrite reductases, including NirK and NirS, convert nitrite to nitric oxide. Nitric oxide reductases (cNor and qNor) further reduce nitric oxide to nitrous oxide. Finally, nitrous oxide reductases (NosZI and NosZII) convert nitrous oxide to nitrogen gas. These enzymes play a crucial role in the denitrification pathway, enabling organisms to utilize nitrogen oxides as terminal electron acceptors.

Denitrifying organisms exhibit varying regulatory phenotypes, influencing their capacity to serve as sinks for nitrous oxide during denitrification. The presence of the NosZ gene in denitrifying bacteria is crucial for efficient denitrification and N_2O reduction. Different regulatory phenotypes affect the accumulation of denitrification intermediates, such as nitric oxide and nitrite, impacting denitrification efficiency. Strict control of downstream NOx reductases is necessary to prevent toxic intermediate accumulation. N_2O emissions contribute to global warming and stratospheric ozone depletion, with agriculture being a major source.

Understanding and optimizing denitrification processes are essential for mitigating N_2O emissions and their environmental impacts.

Environmental factors, such as soil pH, carbon and nitrogen concentrations, and temperature, have a significant impact on nitrous oxide emissions. Soil pH influences the ratio of N_2O to dinitrogen produced during denitrification, with low pH inhibiting N_2O reduction. Temperature and moisture levels affect microbial activity involved in nitrification and denitrification, leading to variations in N_2O production. Organic carbon concentration in soil influences denitrification rates, as higher levels provide more substrates for denitrifiers. The genetic diversity and metabolic preferences of denitrifying bacteria also play a role in N_2O emissions. Understanding these factors is crucial for predicting and managing N_2O emissions in different ecosystems and developing mitigation strategies.

2 Theory and Background

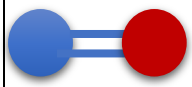
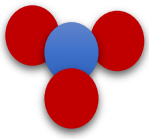
Nitrogen (N) holds great significance as a biologically vital element known for its intricate nature. Its influence extends to the regulation of species diversity in marine, terrestrial, and freshwater ecosystems (Marcarelli et al., 2022). Nitrogen plays a fundamental role as a primary nutrient, crucial for the formation of various molecules such as proteins, deoxyribonucleic acid (DNA), and chlorophyll. Within the atmosphere, nitrogen primarily exists as molecular nitrogen gas (N_2), constituting around 78% of the atmospheric gas composition (Yu et al., 2019).

Nevertheless, N_2, in its molecular nitrogen gas form, is not readily available to numerous organisms, rendering it a scarce resource that constrains biological productivity across diverse ecosystems (Aryal et al., 2022).

The work by Aryal et al. (2022) emphasizes the importance of industrial N synthesis to sustain the growing human population. However, this process also carries significant implications for both the environment and the human health. The release of NH_3, NO_x, and N_2O emissions during various stages of food production, processing, consumption, industrial activities, and transportation has resulted in a range of environmental issues. These issues include challenges related to air and water quality, soil health, biodiversity, and the stability of the global climate system. It is crucial to recognize that N, in its different chemical forms present in the atmosphere, air, soil, and water, plays a critical role in all aspects of climate change.

The table below shows the different states of nitrogen and their respective molecular structure.

Table 1: Different states of nitrogen and their respective structures.

N	N_2	NO	N_2O	NO2-	NO3-
Nitrogen	Nitrogen gas	Nitric Oxide	Nitrous Oxide	Nitrite	Nitrate
					

2.1 Nitrous Oxide (N_2O)

According to Davidson et al. (2014), nitrous oxide (N_2O) stands as a significant form of N pollution, and it has been identified as one of the three global environmental issues. Once an N atom enters a reactive state, it can trigger a cascade of environmental problems as it moves through terrestrial and aquatic ecosystems and eventually reaches the atmosphere. To effectively mitigate N_2O emissions, a thorough understanding of its behavior and underlying factors is essential.

2.2 Nitrification and denitrification

Nitrification and denitrification are the two primary biochemical processes, predominantly regulated by microorganisms, responsible for N_2O production in terrestrial and aquatic ecosystems. Nitrification occurs under aerobic conditions and involves the oxidation of ammonium to form nitrate. During this process, N_2O is generated as a by-product. On the other hand, denitrification encompasses various forms, such as heterotrophic denitrification, nitrifier denitrification, chemodenitrification (abiotic), and codenitrification (biotic). Denitrification involves the reduction of oxidized forms of nitrogen, such as nitrate, leading to the production of various gaseous nitrogen species, including N_2O. Importantly, denitrification occurs under low oxygen conditions. These processes collectively contribute to the production of N_2O and play a crucial role in the nitrogen cycle within ecosystems (Huang et al., 2022).

2.3 Denitrification

Numerous organisms possess the ability to sustain their respiratory metabolism even in the absence of oxygen. Among the anaerobic dissimilatory pathways, denitrification is considered one of the most energetically advantageous processes (Bakken et al., 2012; Torregrosa-Crespo et al., 2020). Denitrification enables organisms to derive energy by utilizing alternative electron acceptors, such as nitrate, nitrite, or nitric oxide, instead of oxygen. This metabolic flexibility allows them to thrive in environments with low oxygen availability and effectively carry out the denitrification process, which is characterized by the reduction of nitrogen compounds. Denitrification serves as an energetically favorable pathway for these organisms, enabling them to generate energy in anaerobic conditions.

Denitrification is a dissimilatory process carried out by microorganisms, involving the stepwise reduction of nitrate and nitrite to gaseous compounds such as nitric oxide (NO), nitrous oxide, and dinitrogen (Knowles, 1982; Zumft, 1997). This process leads to the loss of nitrogen from natural ecosystems and agricultural fields and plays a role in nitrogen removal during

wastewater treatment (Bouwman et al., 2002). Additionally, denitrification can facilitate the anaerobic degradation of organic pollutants. Notably, one of the end products of denitrification is N_2O (Wei et al., 2015). Through denitrification, microorganisms contribute to the cycling of nitrogen compounds, affecting ecosystem nutrient dynamics and the environmental fate of nitrogen-containing compounds.

Nitrous oxide is produced as an inorganic metabolite by prokaryotic cells during the process of denitrification (Walter et al., 2007). As part of the denitrification pathway, prokaryotic organisms metabolize nitrogen compounds, such as nitrate and nitrite, and generate N_2O as a byproduct. This metabolic activity is primarily carried out by certain groups of bacteria and archaea that possess the necessary enzymes to facilitate the reduction of nitrogen compounds, ultimately leading to the formation of N_2O. The production of N_2O during denitrification contributes to its release into the environment, where it plays a significant role as a greenhouse gas and ozone-depleting substance.

Denitrifiers are organisms that possess the capability to convert nitrate (NO_3^-) or nitrite (NO_2^-) into nitric oxide or nitrous oxide through the process of denitrification. While denitrification is commonly associated with prokaryotic organisms, particularly bacteria and archaea, it is important to note that certain fungi are also capable of denitrification (Zumft, 1997). This highlights the widespread distribution of denitrification as a metabolic trait across different organisms and demonstrates the diverse range of species involved in nitrogen cycling processes. Many denitrifiers exhibit genetic diversity, with variations in their denitrification pathways. Some denitrifying organisms possess truncated denitrification pathways, lacking or not expressing the complete set of enzymes necessary for the complete denitrification process. This can result in the formation of nitrite as well as the release of nitric oxide and nitrous oxide into the atmosphere (Castellano et al., 2017).

Notably, certain bacteria that carry a functional N_2O reductase but lack other denitrification genes can act as sinks for N_2O. On the other hand, there are denitrifying bacteria that lack the nosZ gene and therefore emit N_2O (Philippot et al., 2011). These variations in denitrification capabilities among different organisms contribute to the complex dynamics of N_2O production and consumption in various environmental systems.

Cultivation-based research has identified certain genera, namely Pseudomonas, Ralstonia, Alcaligenes, Paracoccus, Rhodobacter, Rubrivivax, Thauera, Burkholderia, Bacillus, and Streptomyces, as the prevailing denitrifying microorganisms in diverse environments. These genera have been consistently observed to play significant roles in the denitrification process across different ecosystems (Sara et al., 2007)

In marine environments, denitrification is primarily carried out by specific species such as Shewanella baltica and Marinobacter spp., as highlighted in the study conducted by Sara et al. (2007). These marine denitrifiers possess adaptations that enable them to thrive in the unique conditions of marine ecosystems, making them key contributors to the nitrogen cycling dynamics in these habitats. The identification and understanding of the dominant denitrifying genera in various environments provide valuable insights into the ecological functioning and potential impacts of denitrification processes in different ecological contexts.

Denitrifying microorganisms are frequently found in various natural settings, including soil, marine and freshwater sediment, as well as wastewater treatment systems (Sara et al., 2007).

Denitrifiers play a crucial role in the production of N_2O, making them the primary group responsible for its generation (Walter et al., 2007). Among denitrifiers, only prokaryotes have the capability to convert N_2O into N_2. This conversion from N_2O to N_2 represents the final step in the complete process of nitrate denitrification and functions as an independent form of respiration. Within the denitrifying cell, N_2O is produced through the activity of respiratory NO reductase. The reduction of N_2O to di-nitrogen is specifically carried out by the N_2O reductase enzyme encoded by the nosZ gene (Walter et al., 2007).

This part can be summarized as follows:

Denitrification is a metabolic process that allows organisms to generate energy without oxygen by using alternative electron acceptors. It involves the reduction of nitrogen compounds, such as nitrate and nitrite, to produce gases like nitric oxide, nitrous oxide, and dinitrogen. Denitrification is widespread among bacteria, archaea, and fungi, enabling them to thrive in low oxygen environments. It plays a role in nitrogen loss from ecosystems and wastewater treatment, while also contributing to the production of greenhouse gas N_2O. Denitrifiers, including various genera and marine species, are found in diverse environments like soil, sediment, and wastewater systems. They exhibit genetic diversity and different denitrification pathways, impacting the dynamics of N_2O production and consumption. Prokaryotes are capable of converting N_2O to N_2, representing the final step in denitrification through the nosZ gene and N_2O reductase enzyme.

2.3.1 Denitrification enzymes

Under oxygen-limiting conditions, denitrification is a reductive process that involves the conversion of nitrate to N_2 through the intermediate formation of nitrite, nitric oxide, and nitrous oxide (Castellano et al., 2017). This process is a form of respiration and occurs in four sequential

stages: the reduction of NO_3^- to NO_2^-, the reduction of NO_2^- to NO, the reduction of NO to N_2O, and the reduction of N_2O to N_2. Each step in this metabolic pathway is facilitated by specific reductase enzymes (Figure 1) (Rob et al., 2007).

These enzymes include nitrate reductases (Nar/Nap), nitrite reductases (NirK/NirS), nitric oxide reductases (cNor/qNor), and nitrous oxide reductases (NosZI and NosZII), which are encoded by the narG/napA, nirK/nirS, c-norC/q-norC, and nosZ clades I (nosZI) and II (nosZII) genes, respectively (Zumft, 1997; Castellano et al., 2017). Through the activity of these enzymes, denitrifying organisms are able to sustain respiratory metabolism by utilizing nitrogen oxides (NO_x) as terminal electron acceptors in the absence of oxygen. The synthesis of the denitrification proteome, which includes NAR, NIR, NOR, and N_2OR enzymes along with other associated proteins, is triggered by oxygen depletion and inhibited by high oxygen concentrations (Bakken et al., 2012).

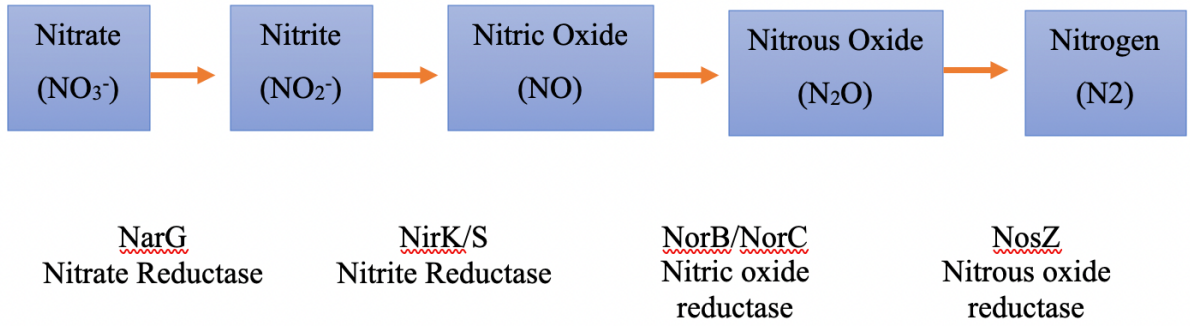


Figure 1: Complete denitrification pathway, enzymes and their respective reductase genes

2.3.2 Nitrate Reductase (Nar)

The initial step of denitrification involves the reduction of NO_3^- to NO_2^- by the enzyme nitrate reductase (Nar). Nitrate reductases are a group of molybdenum-dependent enzymes and can be classified into four major categories: eukNR, Nas, Nap, and Nar (Watkins et al., 2014). For the purpose of this discussion, the focus will be on two types of nitrate reductases: the dissimilatory membrane-bound Nar and the periplasmic nitrate reductase (Nap).

Nar nitrate reductases are enzymes that are embedded in the cell membrane, with the catalytic subunit positioned towards the cytoplasm. These enzymes play a crucial role in catalyzing the reduction of nitrate to nitrite, enabling the generation of energy within the cell. Nar nitrate reductases are typically composed of multiple subunits encoded by the narGHJI operon, with narGHI responsible for encoding the subunits of the enzyme. The expression of the narGHJI

operon is regulated in response to environmental conditions, specifically in the presence of high nitrate concentrations and low oxygen levels (Mauffrey et al., 2015).

The NarGHI enzyme complex is composed of three subunits and acts as a trimeric structure. NarG serves as the active subunit, while NarH is a soluble subunit that connects NarG with NarI, which is a subunit embedded in the cell membrane (Phillippot, 2002).

The Nar complex receives electrons from quinol, typically ubiquinol, in denitrifying organisms, and is involved in respiratory electron transfer. The oxidation of quinol takes place at the periplasmic side of NarI, leading to the release of H+ ions and the movement of two electrons from the low-potential heme bl to the high-potential heme bh within the enzyme. This charge separation contributes to the generation of an electrochemical gradient of H+ ions across the membrane, making the enzyme electrogenic (Richardson et al., 2007).

The subunits of the nitrate reductase complex, NarGHI, work in coordination with a NarK transporter, which facilitates the import of nitrate and the export of nitrite to the periplasmic side of the cell. The reduction of nitrate by NarGHI involves the transfer of electrons from ubiquinol through NarI and NarH to the catalytic subunit NarG, which contains the molybdenum cofactor. Finally, electrons are transferred from NarH to the molybdenum cofactor in NarG (Bruce, 2015).

In the nitrate reductase complex NarGHI of E. coli, the reduction of nitrate involves the participation of ubiquinol (UQH2) as an electron donor. Ubiquinol transfers electrons to the heme b molecule in NarI, initiating a cascade of electron transfer events. The electrons then pass through iron-sulfur centers present in NarH and NarG, ultimately reaching the molybdenum cofactor center (MGD) located in NarG. This electron transfer process generates a proton motive force, with the reduction of nitrate occurring at the cytoplasmic side (N-side) and the release of protons taking place at the periplasmic side (P-side). This proton motive force contributes to the generation of cellular energy (Bruce, 2015).

The NarGHI complex facilitates the transfer of two electrons through its electron transfer pathway to the Mo-bisMGD site, where nitrate is reduced by consuming two cytoplasmic protons. Since the catalytic subunit of Nar is located in the cytoplasm, nitrate needs to be transported into the cell. This is accomplished by two transporters. The proton-nitrate symporter NarK1 is activated during the initial stages of nitrate respiration, and the protons co-transported with nitrate are consumed during the nitrate reduction process. As nitrite accumulates, NarK1

is replaced by NarK2, which functions as a nitrate/nitrite antiporter, maintaining the steady state. This membrane process, coupled with formate oxidation, generates the proton motive force (PMF) necessary for ATP synthesis, contributing to cellular energy production (González et al., 2006).

Periplasmic nitrate reductase (Nap) is composed of two subunits, NapA and NapB, and functions as a heterodimer. The catalytic subunit, NapA, contains a [4Fe-4S] cluster and a molybdopterin cofactor, while NapB is a bi-heme c-type cytochrome (Rajta et al., 2020). In the Nap complex, electrons derived from quinol molecules are typically transferred through one or two cytochrome c-containing proteins, NapC and NapB, before reaching the catalytic subunit, NapA (Richardson et al., 2007). NapC, a tetra-heme c-type protein, is membrane-anchored and facilitates the transfer of electrons from the quinol pool to periplasmic reductases, enabling the reduction of nitrate (González et al., 2006).

2.3.3 Nitrite Reductase (Nir)

The second step of denitrification involves the action of nitrite reductase (Nir), which converts nitrite to nitric oxide, resulting in the conversion of dissolved nitrogen into gaseous nitrogen. There are two main types of Nir enzymes: copper-containing Nir encoded by the NirK gene and cytochrome cd1-Nir, a heme protein encoded by the NirS gene (Decleyre et al., 2016).

Cytochrome cd1-Nir enzymes are homodimeric proteins, while Cu-Nir enzymes are trimeric. Typically, these two types of enzymes are not found together within the same bacterium; denitrifiers possessing cytochrome cd1-Nir lack Cu-Nir (Cabello et al., 2009).

However, some studies have reported the presence of both NirK and NirS enzymes in the same organism. For example, Lea et al. (2018) detected simultaneous expression of both nirS and nirK in Pseudomonas stutzeri strain JM300. Similarly, Bradyrhizobium oligotrophicum S58, a nitrogen-fixing oligotrophic bacterium, carries a set of genes for complete denitrification, including both nirK and nirS genes (Sanchez et al., 2018).

Nitrite transport across the cell membrane is facilitated by transmembrane transporters known as NarK proteins, with the NarK2 subtype specifically involved in nitrate/nitrite antiporting. After nitrate reduction, the resulting nitrite is exported from the cell through the plasma membrane via the action of the NarK2 transport protein. Additionally, it is worth noting that cytochrome (Cyt) plays a role in this process (Khan et al., 2012).

2.3.4 Nitric Oxide Reductase (Nor)

Nitric oxide reductase (NOR) is an integral membrane enzyme that also plays a crucial role in the reduction of nitric oxide to nitrous oxide. NOR belongs to the heme-copper oxidase superfamily and serves as a defense mechanism against the harmful effects of NO, which can act as a potent cytotoxin affecting various cell types (Margareta, 2017). The detoxification of NO is achieved by converting it into the less toxic nitrous oxide (Margareta, 2023).

NOR is a copper-containing enzyme that exists in two subtypes, namely cNor and qNor. The cNor subtype receives electrons from cytochrome c, while qNor has an N-terminal extension that enables it to utilize quinol as an electron donor (Shapleigh, 2013). Electron transfer occurs from soluble electron donors, utilizing the heme c of NorC, to the binuclear center of NorB, where the reduction of NO takes place (Ian et al., 2002 and de Vries et al., 2007). Additionally, there is a third type of NOR known as qCuANOR, which exhibits bifunctionality by employing both menahydroquinone and a specific c-type cytochrome as electron donors (de Vries et al., 2007).

2.3.5 Nitrous Oxide Reductase (Nos)

The final step in denitrification involves the reduction of nitrous oxide to dinitrogen by the enzyme nitrous oxide reductase (Nos). In most denitrifying bacteria, Nos is a homodimeric copper-containing enzyme (NosZ) that is localized in the periplasmic space (Durand et al., 2021). Each monomer of NosZ possesses two copper centers: CuA and CuZ. CuA forms the active site of the enzyme, capable of accepting and transferring a single electron, while CuZ is a tetranuclear [4Cu:2S] cluster that binds and activates N_2O during the catalytic process.

The NosZ gene is part of the Nos gene cluster (NosRZDFYL), where the preceding gene, NosR, encodes a polytopic membrane protein that serves as an electron donor for N_2O reduction (Zhang et al., 2019). Together, the NosZ enzyme and its associated gene cluster play a crucial role in the final step of denitrification, converting N_2O into N_2.

The electrons required for the reduction of N_2O in the Nos enzyme are sourced from the quinone pool and are transferred to Nos either through the cytochrome bc1 complex and small soluble periplasmic proteins or via specific membrane-associated electron transfer proteins unique to Nos (Spiro, 2012).

It is worth noting that in Gram-positive bacteria such as B. azotoformans and Thiobacillus denitrificans, Nos is membrane-bound. In certain bacteria like P. denitrificans, it has been

demonstrated that electron transfer to Nos occurs through cytochrome c (Durand et al., 2021). Notably, Nos is the only known enzyme responsible for catalyzing the reduction of N_2O (Jonassen et al., 2022).

The functional gene NosZ, which is responsible for N_2O reductase activity, can be classified into two distinct clades: clade I and clade II. In clade I organisms, the NosZ gene is consistently found adjacent to a NosR gene, which is believed to be an expression regulator involved in electron transport to the N_2O reductase. However, the NosR gene is absent in the genomic context of NosZII organisms (Sanford et al., 2012; Hallin et al., 2018).

The translocation mechanism for the NosZ protein in clade I organisms appears to be the twin-arginine translocation (TAT) pathway, while NosZII organisms are associated with the Sec translocation pathway, where protein folding occurs in the periplasmic space (Semedo et al., 2020).

Several studies have investigated the physiological differences between clade I and clade II bacteria in relation to their N_2O reduction capabilities and responses to oxygen. Yoon et al. (2016) observed that clade II bacteria, such as Dechloromonas aromatica and Anaeromyxobacter dehalogenans, exhibited higher affinities for N_2O but lower maximum reduction rates compared to clade I bacteria like Stutzerimonas stutzeri (previously known as Pseudomonas stutzeri) and Shewanella loihica. On the other hand, Suenaga et al. (2018) found that the N2O reduction kinetics were not sufficient to distinguish between clade I bacteria (S. stutzeri and Paracoccus denitrificans) and clade II bacteria (Azospira spp.) in their study.

Despite these findings, the behavior of clade I and clade II N_2O reducers in the presence of oxygen remains unclear and requires further investigation.

The movement of nitrogenous oxides and electrons during denitrification can be visualized as a flow, as shown in Figure 2. Substrates and products containing nitrogen are depicted in blue, while quinone electron carriers are represented by green hexagons. Proteins involved in denitrification are shown in dark gray boxes, with metal cofactors of denitrification enzymes displayed in smaller text. Dashed arrows indicate diffusion, connecting consecutive steps in the denitrification pathway, from NO3 transport to N2 release. DH denotes NADH dehydrogenase (complex I), and Cyt bc1 represents cytochrome bc1 (complex III), which are shared components of the aerobic electron transport chain (Vaccaro et al., 2015).

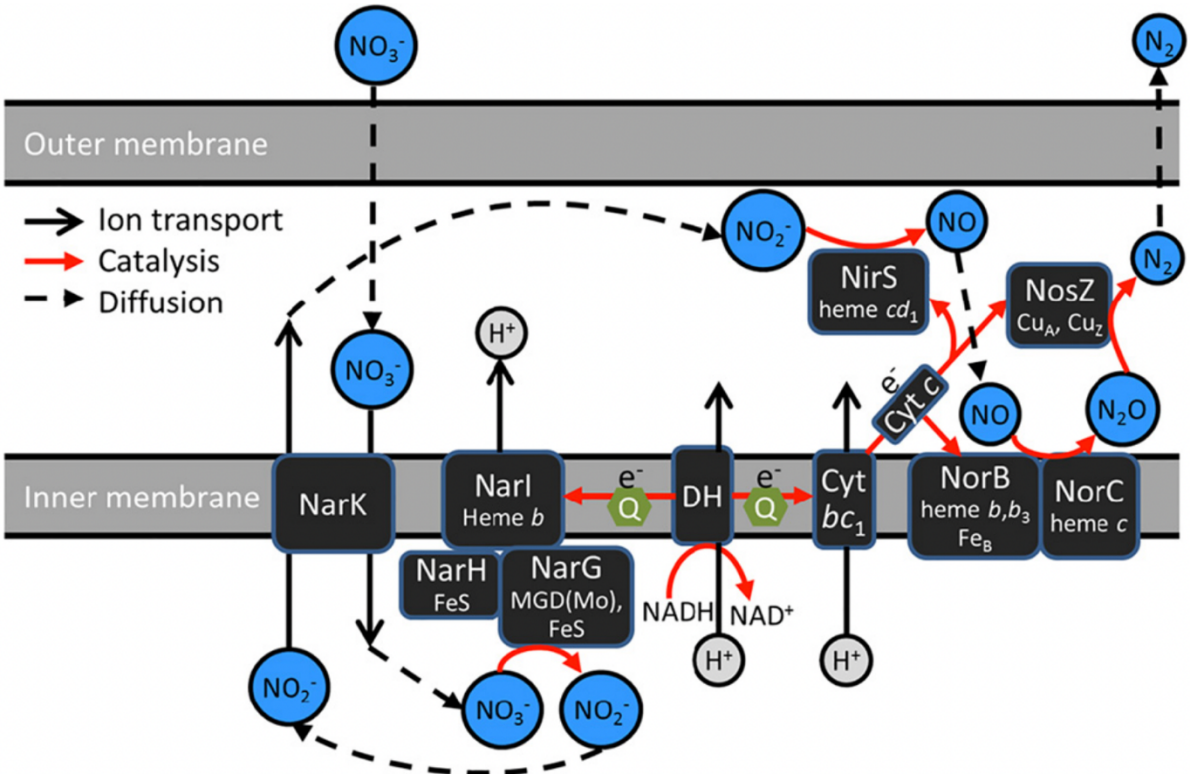


Figure 2: The figure above shows the flow of the nitrogenous oxides and electrons through denitrification as drawn by Vaccaro et al. (2015).

Table 2 below show the denitrification reactions and their respective enzymes and genes.

Table 2: Enzymes and genes involved in denitrification reactions from Bhatia and Phatania (2019)

Denitrification reactions	Enzymes	Genes
$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2$	Nitrate reductase	*Nap*A, *Nap*B, *Nar*GHI
$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$	Nitrite reductase	*NirS*, *Nir*K
$2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$	Nitric oxide reductase	*Nor*B, *Nor*C
$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O$	Nitrous oxide reductase	*Nos*Z

2.3.6 Transcriptional regulation

Indeed, the coordinated expression and activity of the different reductases involved in denitrification are crucial for the prevention of toxic concentrations of nitrite and nitric oxide (Bergaust et al., 2008). The regulation of the genes and operons encoding these reductases is controlled by various transcriptional regulators, which respond to both intra- and extracellular signals. These signals can include oxygen levels, NO concentrations, nitrate availability, and the activity of the electron transport chain (Bakken et al., 2012).

The regulation of denitrification is influenced by the energetic efficiency of oxygen respiration compared to denitrification. Oxygen respiration yields higher ATP and growth compared to denitrification, making it energetically favorable. Consequently, the denitrification enzymes are downregulated in the presence of oxygen to conserve energy. Strict regulation of the downstream NOx reductases is also essential to prevent the accumulation of toxic denitrification intermediates, particularly NO and, to a lesser extent, NO_2^-. The pH of the environment can also influence the toxicity of these intermediates (Bergaust et al., 2012; Gaimster et al., 2018) – this will be further discussed in chapter 3.3.1.

Overall, the precise regulation of denitrification enzymes and the balanced expression of reductases are necessary for efficient energy metabolism and to prevent the accumulation of toxic byproducts.

Certain organisms respond to the imminent lack of oxygen by synthesizing specific enzymes involved in denitrification to sustain respiratory metabolism. These enzymes encompass the membrane-bound cytoplasmic nitrate reductase (Nar), cytochrome cd1 nitrite reductase (NirS), cytochrome c-dependent nitric oxide reductase (cNor), and nitrous oxide reductase (NosZ). The transcription of the genes encoding these reductases, namely NarG, NirS, NorBC and nosZ, is regulated by specific proteins. Among these regulators, FNR-type proteins such as FnrP, NarR, and NNR play a role in controlling the expression of these genes (Hassan et. al., 2016).

FNR (fumarate and nitrate reduction regulator), that responds to changes in oxygen and nitrate availability, proteins are a significant subgroup of bacterial transcriptional regulators known as cyclic-AMP receptor proteins (CRP). It can activate the transcription of genes involved in denitrification, including NarG, under anoxic conditions.

NarR is another FNR-type protein that is specifically involved in regulating the expression of the narG gene. It responds to nitrate and nitrite levels and helps activate the transcription of narG in the presence of these compounds (Crack et al., 2016). FnrP contains a 4Fe-4S cluster that allows it to sense oxygen levels, while NarR serves as a nitrate sensor, regulating the expression of nitrate reductase.

NNR (NarL/NarP-type regulator), on the other hand, utilizes a heme group to detect nitric oxide and control the expression of nitrite, nitric oxide, and nitrous oxide reductases (Crack et al., 2016). Further NNR is a transcription factor that regulates the expression of genes involved in denitrification, including nirS, norBC, and nosZ. NNR responds to nitric oxide and other signaling molecules, and it can activate or repress the transcription of these genes depending on the environmental conditions. It should be noted that these sensors remain inactive during aerobic growth conditions (Hassan et al., 2016). Additionally, the NarXL proteins function as a two-component sensor regulator system, responding to the presence of nitrate and/or nitrite (Spiro, 2012).

The regulation of these reductase genes by FnrP, NarR, and NNR helps coordinate the expression of the denitrification pathway in response to changing oxygen and nitrate levels, ensuring the appropriate functioning of these enzymes for efficient energy metabolism and the prevention of toxic byproduct accumulation (Spiro, 2012).

The build-up of nitric oxide intermediates can have harmful effects on cellular function. To combat the resulting nitrosative stress, bacteria employ a transcriptional regulator called DnrF, which belongs to the CRP/FNR-type family of regulators. This family of transcription factors, including CRP (cAMP receptor protein) and FNR, is widely used by many bacteria to respond to nitrosative stress. These regulators exhibit great flexibility and can function as either transcriptional activators or repressors, depending on the specific regulatory context (Ebert et al., 2017).

The CRP/FNR superfamily encompasses a group of proteins involved in regulating the expression of genes that encode respiratory nitric oxide reductases. Within this superfamily, proteins such as NNR/NnrR and DNR (dissimilatory nitrate respiration regulator) are specifically responsible for activating transcription in response to the presence of NO (Spiro, 2012).

A well-known example of the CRP/FNR regulators is the global transcription factor FNR in Escherichia coli, which senses oxygen levels through a sensitive [4Fe-4S] cluster. Another Fe-S cluster-containing regulator, NsrR in Bacillus subtilis, plays a significant role in the detoxification of nitric oxide. In the presence of an intact Fe-S cluster, NsrR represses the expression of the hmp gene under anaerobic conditions. However, the accumulation of NO causes the inactivation of NsrR by destabilizing the Fe-S cluster, resulting in the derepression of hmp transcription (Ebert et al., 2017).

These proteins play a crucial role in coordinating the cellular response to NO and ensuring the appropriate expression of genes involved in NO detoxification or utilization.

Pseudomonas aeruginosa, a bacterium distinct from E.coli and Bacillus subtilis, possesses a CRP/FNR-like regulator known as DNR. DNR has been found to play a crucial role in the precise and hierarchical regulation of denitrification genes, such as nirS, norCB, and nosR. Similarly, in Pseudomonas stutzeri, a related species, a regulator called DnrD is involved in nitric oxide signaling and the transcriptional control of denitrification genes (Ebert et al., 2017). These findings highlight the diversity of CRP/FNR-like regulators and their significance in regulating denitrification pathways in different bacterial species.

Paracoccus denitrificans (P. denitrificans) has been extensively studied as a model organism for understanding the gene regulation of bacterial denitrification. It possesses all four functional gene clusters involved in denitrification: Nar, Nir, Nor, and Nos, as explained in the subsections above. This genetic capability enables P. denitrificans to perform the complete reduction of nitrate to dinitrogen under micro-oxic and anoxic conditions. The comprehensive study of P. denitrificans has provided valuable insights into the molecular mechanisms and regulatory processes underlying the denitrification pathway (Bergaust et al., 2012).

In P. denitrificans, the regulation of denitrification involves the participation of three FNR-like proteins: FnrP, NNR, and NarR. FnrP and NNR act as sensors for oxygen and nitric oxide levels, respectively, while NarR senses nitrate and nitrite. When oxygen becomes depleted, FnrP is activated and triggers the transcription of the Nar operon in conjunction with NarR. Both FnrP and NarR undergo negative autoregulation to maintain a balance in their concentrations. Following the presence of NO, NNR becomes activated and induces the expression of genes involved in nitrite, nitric oxide, and nitrous oxide reduction. The expression

of the nos gene is co-regulated by both FnrP and NnrR (van Spanning et al., 2007; Bergaust et al., 2012).

Additionally, the expression of Nir is repressed by NirI, while NosR represses the expression of Nos. Furthermore, a recently identified regulator called DenR (also known as sRNA-29) inhibits the expression of Nir, Nor, and Nos, likely through an indirect interaction with a GntR-type regulator (Durand et al., 2021). The complex interplay of these regulatory factors ensures the precise control of denitrification gene expression in P. denitrificans.

NirI and NosR are proteins that play important roles in the transcriptional regulation of nitrite reductase and N_2O reductase genes, respectively, in certain organisms. In the case of Ps. stutzeri, the nosR gene is located adjacent to and upstream of the NosZ structural gene. This arrangement suggests that NosR functions as a regulatory factor necessary for the transcription of NosZ (Spiro, 2012).

FNR, NNR, and NarR are key regulators involved in sensing and responding to oxygen and denitrification intermediates. Additionally, copper plays a significant role in the regulation of denitrification, particularly in the activity of the NosZ enzyme. Research has demonstrated that the downregulation of NosZ expression in copper-limited environments can result in a net emission of N_2O (Gaimster et al., 2018).

A study examining the impact of zinc depletion on the transcriptome of P. denitrificans revealed notable findings. Specifically, it was observed that genes responsible for nitric oxide reductase (NorCB) and nitrite reductase (NirS) showed increased expression levels under zinc-depleted conditions. Surprisingly, the NosC gene, associated with nitrous oxide reductase, also displayed a nearly ten-fold upregulation. Furthermore, the data suggested a direct regulation of the NorCB genes by the zinc uptake regulator (Zur) (Gaimster et al., 2018).

A less explored mechanism for gene regulation in P. denitrificans and denitrifying bacteria is through the use of small RNAs (sRNAs). These sRNAs are a recently discovered group of regulatory RNAs that are typically 40-500 nucleotides long. They have the ability to downregulate the expression of specific target mRNA molecules by either inhibiting translation or promoting mRNA degradation, achieved through complementary base pairing (Gaimster et al., 2018).

According to Bakken et al. (2012), P. denitrificans exhibits an excellent denitrification phenotype at pH 7, showing efficient reduction of nitrogen oxides (NOx) all the way to N2, while minimizing the emissions of both nitric oxide and nitrous oxide. This indicates that P. denitrificans is highly proficient in converting NOx intermediates to harmless nitrogen gas under these pH conditions.

The ability of P. denitrificans to efficiently reduce NOx to N2 is a desirable characteristic as it helps mitigate the release of greenhouse gases and harmful nitrogen compounds into the environment. This performance highlights the effectiveness of P. denitrificans in carrying out denitrification and its potential as a beneficial organism in bioremediation and wastewater treatment processes.

Interestingly, P. denitrificans exhibits an early expression of the N_2O reductase (N_2OR) compared to the nitrite reductase and nitric oxide reductase enzymes. This suggests that N2OR is expressed before the other denitrification enzymes, possibly to ensure efficient reduction of N2O in response to oxygen depletion. Furthermore, it is observed that only a subset of cells are capable of expressing NIR and NOR in a timely manner before all the oxygen is depleted, highlighting the heterogeneity in gene expression within the population.

These findings emphasize the intricate and coordinated regulation of denitrification genes in P. denitrificans, involving the interplay of multiple transcriptional regulators and the dynamic expression patterns of different enzymes.

<u>This part can be summarized as follows:</u>

Denitrification is a metabolic process involved in the reduction of nitrate to dinitrogen. The regulation of denitrification genes is controlled by various transcriptional regulators that respond to intra- and extracellular signals. FNR-type proteins, such as FnrP, NarR, and NNR, act as sensors for oxygen, nitrate, and nitric oxide, respectively, and play important roles in the regulation of denitrification genes.
Environmental factors like oxygen availability, pH and copper levels as explained in future chapters. The presence of small regulatory RNAs also influence the regulation of denitrification. Precise regulation of denitrification enzymes and the balanced expression of reductases are necessary for efficient energy metabolism and to prevent the accumulation of

toxic intermediates. Understanding the regulatory mechanisms underlying denitrification provides valuable insights into this important metabolic pathway.

2.4 Phenotype variants

While denitrifying organisms may possess similar regulatory proteins, their regulatory phenotypes can vary significantly (Lycus et al., 2017). The denitrification phenotype of microbial communities plays a crucial role in greenhouse gas emissions. One important aspect of the denitrification phenotype is its capacity to serve as a sink for nitrous oxide.

The variation in the NosZ gene can have an impact on the rates and outcomes of denitrification. A significant phenotype associated with the reduction of nitrous oxide is the presence and activity of denitrifying bacteria that possess the NosZ gene. Some denitrifying bacteria have the ability to produce the complete range of denitrification products, including N_2O, while others can effectively convert N_2O to N_2 through the function of the NosZ gene. The presence of denitrifiers carrying the NosZ gene is thus important for enhancing the overall efficiency of denitrification and reducing N_2O emissions.

2.4.1 Accumulation of Intermediates

A microbial community that exhibits low activity of NOS compared to other denitrification reductases such as NAR, NIR, and NOR, is likely to be a significant source of N_2O emissions. On the other hand, a community with high relative NOS activity is expected to emit less N_2O and may even act as a net sink for N_2O generated during nitrification. This phenomenon has been observed in microcosm experiments involving Leca-particle biofilms, as reported in Mao et al. (2008) and discussed by Conthe et al. (2019).

Duffner et al. (2022) have referred to extensive research conducted to investigate the causes of incomplete denitrification and the transient accumulation of denitrification intermediates in heterotrophic denitrifiers carried out by Liu et al. (2013) and Lycus et al. (2017). These studies suggest that multiple factors contribute to this phenomenon. One factor is the absence of genes encoding the denitrification reductases, which are essential enzymes for complete denitrification. Additionally, transcriptional regulation and post-translational processes influenced by environmental conditions are known to play significant roles in the regulation of denitrification. The studies have shed light on these complex processes and their impact on the efficiency of denitrification.

Denitrifiers that lack the NosZ gene are known to produce predominantly N_2O as a denitrification byproduct. On the other hand, organisms that possess only the NosZ gene are capable of consuming N_2O, acting as net consumers of this greenhouse gas. The complete set of denitrification reductases, including NIR, NAR, NOR, and NOS reductases, is found in full-fledged denitrifiers. The genetic regulation of these reductases plays a crucial role in determining the accumulation of nitrite and the emissions of nitric oxide and N_2O. The study conducted by Hassan et al. (2016) provides insights into the genetic regulation of these reductases and their impact on the production and consumption of NO_2 and N_2O during denitrification.

To prevent the accumulation of toxic denitrification intermediates such as nitric oxide and, to a lesser extent, nitrite, a strict regulation of the downstream NOx reductases is crucial. The activity and expression of these reductases need to be tightly controlled to maintain optimal levels of NO and NO_2^-. The regulation may vary depending on the pH conditions. Furthermore, during denitrification, another important intermediate produced is nitrous oxide. The article conducted by Gaimster et al. (2018) further highlights the significance of regulating the NOx reductases to prevent the accumulation of toxic intermediates and the production of N_2O during denitrification.

The production of denitrification intermediates and the conversion to N_2 gas are influenced by various factors such as the physico-chemical conditions of the growth environment, enzyme kinetics, and the differential gene expression patterns among denitrifying species. Different genera of bacteria have been specifically studied and isolated from diverse environments to investigate the denitrification rates and their capacity to express denitrification genes. These studies aim to understand the mechanisms and variations in denitrification processes across different microbial communities. The research conducted by Suri et al. (2021) likely provides valuable insights into the rates of denitrification and the gene expression patterns of denitrifying bacteria in various environmental settings.

The assembly of the denitrification machinery in bacteria incurs an energetic cost. Additionally, the production and consumption of the toxic intermediates NO_2^- and NO need to be tightly coordinated to prevent their accumulation. This synchronization of processes requires precise regulatory control mechanisms to ensure the efficient and safe operation of denitrification. According to the research conducted by Lycus et al. (2017) likely delves into the intricate regulatory networks that govern denitrification and highlight the importance of maintaining a

balance between the production and consumption of these harmful intermediates. Understanding these regulatory mechanisms is crucial for optimizing denitrification processes and minimizing the potential negative impacts associated with the accumulation of toxic compounds.

Research by Bergaust et al. (2008) and Lycus et al. (2017) have shown that the accumulation of nitric oxide during denitrification can have a significant impact on the process. NO accumulation can lead to the cessation of denitrification even before all nitrate or nitrite is fully reduced. This suggests that the presence of NO can act as a regulatory signal, triggering a halt in denitrification to prevent further accumulation of this toxic intermediate. The regulatory mechanisms that respond to NO levels and modulate denitrification activity are likely essential for maintaining a balanced and controlled denitrification process.

According to Lycus et al. (2017), there is significant variation among denitrifying bacteria in how they regulate denitrification in response to the transition from aerobic to anaerobic respiration. This variation in regulatory phenotypes highlights the complexity of the denitrification process. While denitrification provides an advantage for bacteria in utilizing alternative electron acceptors under anaerobic conditions, it also incurs an energetic cost.

In addition to the energetic cost, Lycus et al. (2017) mention the production and consumption of toxic intermediates such as nitrite and nitric oxide must be carefully coordinated to prevent their accumulation. Strict regulatory control is necessary to synchronize the production and consumption of these compounds, thereby avoiding their toxic effects. The regulatory network involved in denitrification is intricate and has been extensively studied in only a few model organisms. These studies have revealed the involvement of numerous transcriptional regulator enzymes and ancillary factors, indicating the complexity and importance of regulatory mechanisms in denitrification.

In a recent investigation conducted by Mania et al. (2020), strains belonging to the Bradyrhizobium genus were examined to assess their denitrification characteristics. While most of the strains displayed the ability to perform multiple denitrification steps, only half of them exhibited N_2O reduction capabilities. Interestingly, all the N_2O-reducing strains consistently demonstrated a strong preference for N_2O reduction over NO_3^- reduction when subjected to oxygen-depleted conditions. This preference was attributed to a competitive process involving the electron pathways leading to the Nap and Nos enzymes.

Their quantitative analysis of denitrification gene transcripts revealed that the transcript levels of the Nap gene were consistently five to eight times lower than those of the Nos gene. Consequently, it could not be ruled out that the observed phenomenon was a result of the relatively low abundance of the Nap enzyme compared to the Nos enzyme.

This study's findings provide valuable insights into the denitrification phenotype of Bradyrhizobium strains, emphasizing the significance of understanding enzyme abundance and regulation in determining the specific denitrification pathway and the production of N_2O.

Lycus et al. (2017) and Liu et al. (2013) have further highlighted the presence of distinct regulatory phenotypes among denitrifiers capable of complete denitrification. In the study conducted by Liu et al., (2013) eight Thauera strains were investigated, and they were classified into two distinct Denitrification Regulatory Phenotype (DRP) types. Four strains exhibited a rapid, complete onset (RCO) of denitrification genes without nitrite accumulation, while the remaining strains demonstrated a progressive onset (PO) of various denitrification genes along with nitrite accumulation. The PO group exhibited delayed transcription of NirS until all available nitrate was consumed. This research sheds light on the diverse regulatory mechanisms employed by denitrifying bacteria, emphasizing the importance of understanding their regulatory phenotypes to comprehend the denitrification process more comprehensively.

The strains characterized by RCO phenotype initiate denitrification as soon as oxygen is depleted, producing NO, N_2O, and N_2. These strains do not accumulate nitrite because it is rapidly converted into NO. On the other hand, strains with PO phenotype first reduce nitrate to nitrite, leading to the accumulation of nitrite before eventually converting it into NO. In RCO strains, the early transcription of NirS and NosZ occurs even in the presence of oxygen. However, in PO strains, the transcription of NirS and NosZ is delayed until nitrate is reduced to nitrite. These observations suggest that RCO organisms have a greater capacity to act as potent sinks for N_2O. They are effective at reducing N_2O at an early stage of denitrification compared to PO organisms (Liu et al., 2013).

3 N_2O Emission

The advent of the Industrial Revolution and the extensive use of fertilizers in agriculture have considerably augmented the availability of nitrogen in terrestrial and aquatic ecosystems. Consequently, this has led to a notable rise in atmospheric nitrous oxide levels, as noted by Zhang et al. (2020) and Yalan et al. (2023). By the year 2022, the concentration of N_2O reached 335.3 parts per billion by volume (ppbv), surpassing the preindustrial concentration by approximately 24% (Yalan et al., 2023).

Figure 3 below, by Thomson et al., (2012) shows the distribution of global nitrous oxide emissions from different sources and human activities.

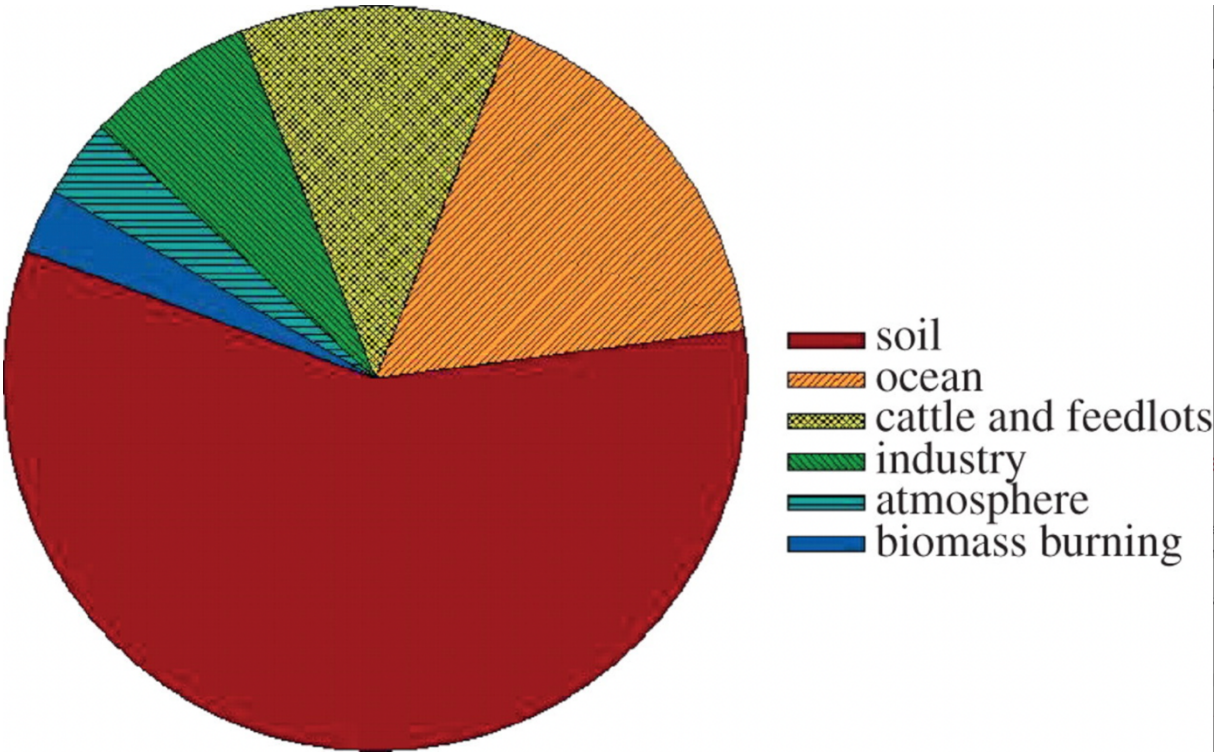


Figure 3: The distribution of global nitrous oxide emissions from different sources and human activities. This is copied from Thomson et al., (2012)

3.1 N_2O Contribution to global warming and destruction of stratospheric ozone

N_2O holds significant importance as a long-lived greenhouse gas and a prominent contributor to global climate change, as noted by Ravishankara et al. (2009) and J. Luo et al. (2018). It is also recognized as a key stratospheric ozone-depleting substance. Despite being the third most significant anthropogenic greenhouse gas, following carbon dioxide and methane, N_2O possesses a potent warming effect, with a global warming potential approximately 265-298

times stronger than carbon dioxide over a 100-year timeframe, according to IPCC (2013) (Intergovernmental Panel on Climate Change) and Martens (2005).

Its extended atmospheric lifetime of about 120 years further contributes to its impact. N_2O emissions primarily stem from agricultural soils globally, making them a substantial source of anthropogenic greenhouse gas emissions. Estimates suggest that N_2O accounts for approximately 6.2% of the total anthropogenic radiative forcing, as highlighted by IPCC (2013) and Edenhofer et al. (2014). Given its potency and long lifespan, N_2O poses a significant threat as a contributor to climate change. Human activities account for roughly 40% of N_2O emissions, with agriculture being the main source (IPCC, 2013).

Around 62% of the total global N_2O emissions are believed to originate from natural and agricultural soils. This includes roughly 6 Tg N yr−1 from natural soils and 4.2 Tg N yr−1 from agricultural soils, primarily due to bacterial denitrification and ammonia oxidation, as indicated by Thomson et al. (2012). The intensification facilitated by synthetic nitrogen fertilizers and manure also contributes to these emissions, as highlighted by Davidson et al. (2014). The remaining one-third of N_2O emissions is attributed to the ocean, primarily through nitrification and denitrification processes. Other anthropogenic sources of N_2O include the production of nitric acid, power plants fueled by fossil fuels, and vehicle emissions, as outlined by Thomson et al. (2012).

Furthermore, Thomson et al. (2012) notes that N_2O emissions are additionally influenced by the reintroduction of animal waste into the soil and the process of wastewater treatment. Over the course of the past century, these cumulative effects have contributed to an estimated 20% increase in atmospheric N_2O concentration, which continues to rise at a rate of about 0.2-0.3% per year. Bacterial and fungal respiratory processes in soils, collectively known as denitrification and nitrification, account for more than two-thirds of these emissions.

According to Davidson et al., (2014) N_2O emissions from wastewater were estimated to be 0.2 Tg N_2O-N per year in 2010, constituting approximately 3% of total gross anthropogenic emissions. These emissions encompass direct N_2O release from wastewater effluent, as well as emissions from bioreactors used in the removal of nitrogen in biological nutrient removal plants However, Barbita et al. (2022), writes that the main source of N_2O release in this context is the biological nitrogen removal process, which occurs in different reaction zones of various configurations of biological nutrient removal (BNR) plants. These BNR plants aim to achieve

effective nitrogen removal from wastewater by facilitating nitrification and denitrification processes.

Between 1990 and 2018, the total N_2O-N emissions increased from 5.175 Tg to 6.548 Tg per year. Currently, net anthropogenic N_2O emissions stand at approximately 5.3 Tg N_2O -N per year. Projections based on emission scenarios suggest that anthropogenic N_2O emissions could nearly double by 2050. It is predicted that future anthropogenic N_2O-N emissions will reach around 9.7 Tg per year in 2050, accounting for 46% of the total global N_2O-N emissions for that year (Davidson et al., 2014). Furthermore, estimations indicate that total global anthropogenic N_2O emissions will increase by 83% between 2005 and 2050 (UNEP, 2013; Barbita et al., 2022).

The IPCC emphasizes the importance of reducing N_2O emissions in order to meet the objectives outlined in the Paris Agreement. The agreement aims to limit global warming to well below 2 degrees Celsius above pre-industrial levels and to pursue efforts to limit the temperature increase to 1.5 degrees Celsius. To achieve these goals, it is crucial to address the reduction of N_2O emissions, among other greenhouse gases. By implementing measures to mitigate N_2O emissions, we can contribute to the collective effort of combating climate change and ensuring a sustainable future.

Furthermore, IPCC mentions that reducing N_2O emissions plays a critical role in global endeavors to mitigate climate change. It entails implementing various measures, including enhancing agricultural practices, minimizing fossil fuel combustion, and improving industrial process efficiency. By actively working towards decreasing N_2O emissions, we can contribute to slowing down the pace of global warming and mitigating the adverse effects of climate change. These efforts are essential in safeguarding our planet and creating a more sustainable future.

3.2 Biologically driven N_2O emissions

In the environment, nitrous oxide is mainly produced as an intermediate during denitrification and as a by-product of nitrification processes (Yoon et al., 2016). The release of N_2O into the atmosphere occurs when there is a kinetic imbalance between the rates of reactions producing and consuming N_2O during denitrification. In addition to denitrification, nitrifiers, which are responsible for ammonia oxidation, have been identified as a potential primary source of N_2O in agricultural soils. Other processes that contribute to N_2O formation include respiratory ammonification (DNRA), which involves the dissimilatory reduction of nitrate/nitrite to

ammonium, and chemo-denitrification, an abiotic reaction of nitrite with ferrous iron. While there are diverse pathways for N_2O generation, the main biological pathway for N_2O removal is reduction to nitrogen gas (N_2) (Yoon et al., 2016).

According to Thomson et al. (2012), the respiratory nitric oxide reductase enzyme, which is present in denitrifying bacteria and some ammonia-oxidizing organisms, plays a significant role in the biological production of nitrous oxide in many environments. NOR catalyzes the reduction of nitric oxide to N_2O, contributing to the overall N_2O production in these organisms.

N_2O production in soils occurs as an intermediate product in various microbially driven processes, namely nitrification and denitrification, as highlighted by Ryan et al. (2017) and Gesche et al. (2011). These two key microbial and biochemical processes play a significant role in governing N_2O production in both terrestrial and aquatic ecosystems. The regulation of these processes is influenced by a range of environmental and biological factors, including temperature, water and oxygen levels, acidity, substrate availability (which is influenced by nitrogen fertilizer use and livestock manure management), and recycling dynamics, as outlined by Tian et al. (2020). These factors collectively influence the rates of N_2O production and contribute to its emissions in natural systems.

Microbial production processes in soils, sediments, and water bodies are the primary sources of N_2O. These processes are closely associated with the activity of microorganisms. Ultimately, N_2O is released back into the atmosphere through microbial denitrification processes occurring in soils, sediments, freshwater environments, and marine waters. These microbial-driven processes play a significant role in the N_2O cycle and contribute to its emissions into the atmosphere.

3.3 Factors Affecting N_2O Emissions

There are various environmental factors that can influence the emissions of nitrous oxide. Factors such as soil moisture, temperature, pH, nitrogen inputs, land use, and water quality can all impact the production and release of N_2O into the atmosphere. For the purpose of this master thesis the focus will be amongst others on pH, C and N and temperature. Microbial activity in soils, which includes processes like denitrification and nitrification carried out by diverse microbial communities, is recognized as a significant contributor to the atmospheric loading of N_2O. The interplay between these environmental factors and microbial activity influences the magnitude of N_2O emissions from terrestrial ecosystems.

Furthermore Thomson et al. (2012) mentions that the cellular abundance and activities of the enzymes involved in N_2O production and consumption play a crucial role in N_2O emissions from bacterial populations. The abundance of these enzymes is regulated by the expression of their corresponding genes, which is controlled by regulatory systems and signal transduction pathways within the cells. These regulatory systems respond to various intra- or extracellular signals, allowing bacteria to adjust their enzyme expression in response to environmental conditions and physiological cues. The coordinated regulation of gene expression and enzyme activities is important for maintaining a balance between N_2O production and consumption, thereby influencing the overall emission of N_2O from bacterial populations.

The genes involved in denitrification can differ among species, and not all denitrifying organisms possess the complete set of genes associated with this process. Additionally, the production of N2O through denitrification can be influenced by soil pH and the concentration of organic carbon. These factors have been found to have an impact on the amount of N2O generated during denitrification (Zumft, 1997; Bahram et al., 2022).
Understanding the influence of genetic diversity and environmental factors on denitrification processes is crucial for accurately predicting and managing N2O emissions in different ecosystems.

The emission of N_2O part can be summarized as follows:

Agricultural soils are a major source of N_2O emissions, accounting for approximately 62% of global emissions. The use of fertilizers and intensification of agricultural practices have increased atmospheric N_2O levels. N_2O emissions also originate from the ocean, wastewater treatment, and other anthropogenic sources.

Understanding the behavior and underlying factors of N_2O emissions is crucial for effective mitigation. N_2O emissions are influenced by microbial processes such as nitrification and denitrification, which are affected by environmental and biological factors. These include temperature, water and oxygen levels, substrate availability, and recycling dynamics that will be discussed in the next chapter. Microbes play a significant role in N_2O production and its release into the atmosphere.

Reducing N_2O emissions is essential for meeting the goals of the Paris Agreement and mitigating climate change. It involves implementing measures such as enhancing agricultural practices, minimizing fossil fuel combustion, and improving industrial process efficiency. By

actively addressing N_2O emissions, we contribute to global efforts to combat climate change and create a sustainable future. The regulation of microbial processes and understanding the factors influencing N_2O production are key in managing N_2O emissions from natural systems.

3.3.1 The Influence of Soil pH

This chapter focuses on the role of soil pH in the context of denitrification, specifically its impact on the ratio of nitrous oxide to dinitrogen produced by denitrifiers. Numerous studies have demonstrated that low pH inhibits the reduction of N_2O to N_2, suggesting that soil liming could be an effective strategy to mitigate N_2O emissions (Liu et al., 2010). Additionally, a negative correlation between soil pH and N_2O emissions has been observed, indicating that higher pH levels are associated with lower N_2O emissions (Frostegård et al., 2022). The regulatory mechanisms and phenotypic variations in denitrifying bacteria are crucial for understanding and managing greenhouse gas emissions.

Studies have shown that pH modulation does not significantly affect the transcription of the NosZ gene in Pa. denitrificans, but it does impact N_2O reductase activity, suggesting a post-translational effect on translation and assembly (Bergaust et al., 2010; Liu et al., 2010). Research conducted by Liu et al. (2010) on agricultural soils consistently observed a decrease in the N_2O /N_2 ratio with increasing pH, highlighting the importance of pH-mediated control on this ratio, particularly in regions experiencing soil acidification due to nitrogen fertilizer use (Bergaust et al., 2010).

The relationship between pH and denitrification extends beyond N_2O emissions. Bakken et al. (2012), referenced by Thomson et al. (2012), emphasize that pH controls the product ratio of denitrification, with higher acidity leading to increased $N_2O/(N_2 + N_2O)$ ratios. They highlight the importance of N_2O -reductase activities in maintaining low N_2O emissions, which can be enhanced by liming materials (Samad et al., 2016). Other studies have also reported greater N_2O emissions in acidic soils compared to neutral pH soils (Qu et al., 2013).

The impact of pH on denitrification is not limited to N_2O emissions but also affects the activity and abundance of denitrifying bacteria, the availability of electron acceptors and substrates, and the expression of denitrification genes (Gaimster et al., 2018). The functional efficiency of N_2O -reductase increases at higher pH levels, resulting in effective denitrification and reduced N_2O emissions (Liu et al., 2014). Consequently, the application of liming materials has been found to reduce N_2O emissions by increasing soil pH (Shaaban et al., 2020).

The study by Frostegård et al. (2022) provides valuable insights into the effects of soil pH on denitrification. Their research demonstrated that low pH delays N_2O reduction and affects the maturation of NosZ apo-protein. Acidic soils showed lower NO_2^- concentrations due to biological activity, while neutral soils exhibited high Nar expression leading to NO_2^- accumulation. The study also revealed discrepancies in gene and transcript quantifications when predicting community phenotypes, highlighting the diversity of bacteria involved in denitrification-driven gas emissions.

3.3.2 Temperature Effects

This section explores the influence of temperature and moisture levels in soil and their impact on N_2O production and release in soil systems.

Soil temperature directly affects N_2O emissions by influencing microbial growth and the kinetics of reactions. It also controls the consumption of oxygen by microbial communities, leading to decreased soil oxygen levels and increased anaerobic conditions. Denitrification peaks between 40 and 60 °C, although this can vary across different climates (Wang et al., 2021).

Increasing soil temperature also results in a higher N_2/N_2O ratio. Studies by Lai and Denton found that higher temperatures lead to increased N_2O emissions and a significant conversion of N_2O to N2, resulting in lower N_2O/N_2 ratios. Additionally, soil temperature impacts nitrogen availability during freeze-thaw cycles, influencing N_2O and N_2 release. In regions with regular soil freezing, a significant portion of annual N_2O emissions occurs after thawing due to anaerobic microbial activity and the release of carbon and nitrogen compounds from microbial biomass when soil moisture is high (Wang et al., 2021).

Soil moisture plays a crucial role in N_2O emissions, especially under high water-filled pore space (WFPS) conditions. Higher soil moisture enhances N_2O emission by promoting organic matter decomposition and increasing substrate availability for microbial activities. Moist soils sustain prolonged N_2O emissions due to the abundant availability of carbon (C) substrates. No tillage practices can further increase WFPS compared to conventional tillage, contributing to elevated N_2O emissions (Hassan et al., 2022).

Understanding the complex interplay between temperature, moisture, microbial activity, and N_2O dynamics is crucial for comprehending the environmental controls on N_2O emissions from soil systems. This knowledge can contribute to the development of strategies for mitigating greenhouse gas emissions and managing soil fertility.

3.3.3 Carbon and Nitrogen

Organic carbon concentration in soil is another important factor. Higher organic carbon levels can provide a greater supply of substrates for denitrifiers, promoting denitrification and potentially increasing N_2O production. Conversely, low organic carbon concentrations may limit the availability of electron donors and substrates, leading to reduced denitrification rates and lower N_2O emissions.

The presence of carbon in the soil serves as a fuel for soil microorganisms, leading to enhanced microbial activity. Both nitrifiers and denitrifiers rely on an accessible carbon source to carry out the oxidation of ammonium (NH_4^+) and the reduction of nitrate (NO_3^-). The level of soil organic carbon (SOC), particularly the amount of water-soluble carbon (WSC), plays a crucial role in promoting soil nitrification and denitrification. As the SOC content increases, so does the capacity for these processes to occur (Wang et al., 2021).

Furthermore, Wang et al., (2021) have looked at several research studies that have suggested that SOC can enhance microbial activity and reduce the emission of N_2O. Similar findings have been reported in other studies as well. However, it should be noted that the influence of SOC on the ratio of N_2 to N_2O can vary depending on the soil nitrogen levels. This is supported by other studies that have examined N_2 and N_2O emissions from soils with different carbon-to-nitrogen (C/N) ratios. In cases where soil N is low, the low N_2O emissions can be attributed to the positive impact of SOC input on N_2O reduction.

In their investigation they (Wang et al., 2021) suggest that the effectiveness of biochar in suppressing N_2O emissions is influenced by the specific C/N ratio established by biochar and the limited availability of N in the soil following its application. The investigation of biochar's effect on N_2O emissions in maize fields revealed a consistent pattern: as the application rates of biochar increased, there was a corresponding decrease in N_2O emissions. This suggests that higher levels of biochar application have a mitigating impact on N_2O emissions from maize fields (Wang et al., 2021).

They further suggest that any form of nitrogen input into agricultural soils has the potential to contribute to the availability of N substrates for N_2O emissions. Denitrification primarily relies on the presence of nitrate molecules. The concentration of soil NO_3^- is constantly changing and is influenced by factors such as net mineralization and nitrification rates, plant N uptake,

microbial immobilization rate, as well as NO_3^- movement through the soil via leaching and lateral flow.

Weier et al. (1993) conducted experiments to examine the effect of different soil NO_3^- concentrations on the N_2/N_2O ratio. Their findings revealed that higher soil NO_3^- concentrations suppressed the activity of N_2O reductase, resulting in a reduced N_2/N_2O ratio.

To facilitate efficient denitrification, it is important to provide an adequate amount of carbon source. However, surpassing the optimal carbon concentration only leads to marginal increases in denitrification rate. Thus, maintaining an appropriate carbon-to-nitrogen (C/N) ratio is essential (Bhatia and Pathania, 2019).

3.3.4 Oxygen and Soil Texture

The presence of oxygen plays a crucial role in the global nitrogen cycle as it directly affects the processes of nitrification and denitrification, which control the production of nitrous oxide in the atmosphere (Aryal et al., 2022).

The rates of nitrous oxide emissions resulting from the reduction of nitrite and nitrate were estimated to be significantly higher, ranging from ten to one hundred times, compared to N_2O production from ammonium. In environments with sufficient oxygen, the production of N_2O through the nitrite pathway can be attributed to the process of nitrite-denitrification. In both terrestrial soil and marine water environments, the concentrations of N_2O exhibit an exponential increase as oxygen concentrations decrease (Aryal et al., 2022).

Soils with finer textures have the potential to release higher amounts of nitrous oxide compared to sandy soils. This is due to the presence of more capillary pores within aggregates, which allows for tighter water retention. Consequently, anaerobic conditions can be more readily achieved and sustained for longer periods in the aggregates of finer textured soils compared to sandy soils (Wang et al., 2021). Weier et al. (1993) observed that denitrification generally intensified as soil texture became finer and as the water-filled pore space (WFPS) increased.

3.3.5 Metabolic Preference of N2O over Nitrate in Denitrification

The N_2O -reducing potential of bacterial strains can be influenced by variations in metabolic capabilities, even when they possess comparable denitrification capacities. These metabolic differences may arise from variations in enzyme activities, gene expression levels, or other factors affecting the efficiency of N_2O reduction within the denitrification pathway (Mania et al., 2020; Gao et al., 2021).

Mania et al. (2020) observed an intriguing phenomenon where bacterial strains capable of nitrogen gas production exhibited significant inhibition of nitrate reduction in the presence of nitrous oxide. This inhibition stemmed from competition among different electron transport pathways, with a strong preference for N_2O reduction over nitrate. This suggests that bacteria with this characteristic could potentially serve as important sinks for N_2O. In agriculture, enhancing the abundance of these bacteria could be achieved through the development of inoculants for legume crops, incorporating efficient nitrogen fixation and effective N_2O reduction capabilities.

Experimental results by Mania et al. (2020) clearly demonstrated that N_2O inhibits nitrate reduction, even in the presence of functional NapAB. Inhibitor-based experiments, such as using myxothiazol to inhibit the cytochrome bc1 complex, indicated competition between the electron transport pathways to NapAB and NosZ as the cause of nitrate reduction inhibition by N_2O. This finding was further supported by experiments utilizing TMPD, facilitating surplus electron transfer to NosZ via cytochrome c and enabling direct electron transfer from the cellular quinol pool to NapC.

The primary objective of this study was to investigate the denitrification characteristics of a collection of Bradyrhizobium isolates, focusing on the competition for electrons between Nap and Nos enzymes. Refined analyses of electron flow kinetics and proteomics were employed to determine the relative abundance of Nap and Nos. The study's findings revealed a significant phenomenon in which nitrate reduction was strongly inhibited in the presence of N_2O for all Bradyrhizobium strains exhibiting complete denitrification. Importantly, proteomic analysis provided evidence that this inhibition was not due to low Nap abundance. Comparisons of denitrification kinetics between Bradyrhizobia and the model bacterium Paracoccus denitrificans, which possesses Nap and Nar in its denitrification pathway, revealed distinct denitrification phenotypes. Specifically, P. denitrificans carrying NarG exhibited simultaneous reduction of nitrate and N_2O, aligning with expectations (Gao et al., 2021).

Denitrifying bacteria facilitate the transfer of electrons from the quinol pool to various enzymes, including Nap, Nos, Nor, and Nir. Specifically, the electron transfer to Nap occurs through the membrane-bound NapC, while the transfer to Nos, Nor, and Nir involves the membrane-bound bc1 complex and periplasmic cytochromes. Notably, the electron pathway to NosZ via bc1 exhibits higher competitiveness compared to the pathway to NapAB via NapC. This heightened competitiveness can be attributed to several factors, including a stronger affinity for quinol and a higher turnover rate (kcat) of bc1 compared to NapC (Gao et al., 2021). These findings provide insights into the intricate metabolic preferences within denitrifying bacteria and contribute to

our understanding of the metabolic dynamics underlying the N_2O preference over nitrate during denitrification processes.

Liu et al. (2015) suggested that the accumulation of denitrification intermediates can be attributed to electron competition among the nitrogen oxide reductases involved in the four-step denitrification process. These key enzymes obtain electrons from a shared source in the electron transport chain, as supported by studies by Pan et al. (2015) and Richardson et al. (2009). When the supply rate of electrons falls short of the demand for electron consumption by the four reduction steps, electron competition can occur during hydrogenotrophic denitrification. Consequently, factors that reduce the rate of hydrogen oxidation, such as the supply rate of hydrogen and carbon dioxide, can result in the accumulation of intermediates like N_2O during hydrogenotrophic denitrification (Li et al., 2017).

The findings of this study contribute to the initial understanding of the accumulation of intermediates, specifically N_2O, during hydrogenotrophic denitrification. This research provides valuable insights into the factors influencing electron competition and the potential mechanisms underlying the build-up of N_2O during this process. Further investigation in this area can help unravel the complexities of hydrogenotrophic denitrification and provide a more comprehensive understanding of intermediate accumulation dynamics.

The aim of the present study was to investigate the variability of denitrification phenotypes among different taxonomic groups of bradyrhizobia, with a specific emphasis on their ability to reduce N_2O. The study also aimed to elucidate the cellular mechanisms contributing to these organisms potentially acting as sinks for N_2O. By examining the denitrification capabilities and N_2O reduction capacity of various Bradyrhizobium strains, the researchers sought insights into the factors influencing their potential as mitigators of N_2O emissions.

Numerous taxonomic groups within the genus Bradyrhizobium have been found to possess denitrification capabilities. However, it has been observed that many strains within this genus possess truncated denitrification pathways, lacking one or more of the sequential steps involved in the complete denitrification process. In other words, these strains may not possess the complete set of enzymes required for the entire denitrification pathway. This variation in the denitrification pathway among different strains of Bradyrhizobium highlights the diversity and complexity of denitrification within this taxonomic group.

In a recent study conducted by Mania et al. (2020), strains within the genus Bradyrhizobium were screened to evaluate their denitrification phenotypes. The results revealed that the majority of tested strains were capable of performing two or more denitrification steps.

The metabolism of N_2O can be summarized as follows:

Variations in metabolic capabilities among Bradyrhizobium strains can influence their ability to efficiently reduce nitrous oxide, despite having similar overall denitrification capabilities. These variations can be attributed to differences in enzyme activities, gene expression levels, and other factors affecting N_2O reduction efficiency. Bacterial strains capable of producing nitrogen gas exhibit a strong preference for reducing N_2O over nitrate, resulting in the inhibition of nitrate reduction in the presence of N_2O. The competition between electron transport pathways leading to Nap and Nos enzymes contributes to this preference. Understanding the metabolic dynamics underlying N_2O preference during denitrification processes can have implications for developing strategies to enhance N_2O reduction in agricultural systems. Additionally, electron competition among nitrogen oxide reductases during hydrogenotrophic denitrification can lead to the accumulation of N_2O intermediates. Further investigation is needed to unravel the factors influencing electron competition and the mechanisms underlying N_2O accumulation during hydrogenotrophic denitrification. The study also highlights the diversity and complexity of denitrification capabilities among Bradyrhizobium strains, emphasizing the variation in denitrification pathways and the potential for certain strains to act as important sinks for N_2O.

4 Future perspectives -> application (N_2O mitigation)

4.1 N_2O specialists, direct application

Gao et al. (2022) investigated whether the preference for nitrous oxide reduction over nitrate reduction, observed in bradyrhizobia under optimal conditions, is retained when the organisms are starved for carbon. They found that starved cultures of a Bradyrhizobium strain with significantly reduced respiration rates compared to well-fed cultures were able to completely reduce all available N_2O before utilizing provided NO_3^-. These starved organisms performed complete denitrification, possessing the periplasmic nitrate reductase NapA but lacking the membrane-bound nitrate reductase NarG.

Proteomics analysis revealed similar levels of NapA and NosZ (N_2O reductase), indicating that the lack of NO_3^- reduction was not due to low NapA abundance. Instead, the study suggests that at the metabolic level, the bc1 complex, which channels electrons to NosZ through cytochromes, outcompetes the NapC enzyme that provides electrons to NapA via NapB. This finding challenges the common understanding that NosZ activity diminishes under carbon limitation. It suggests that bradyrhizobia carrying NosZ can efficiently consume N_2O under natural conditions, making them effective sinks for this greenhouse gas. This finding has implications for the development of biofertilizers, indicating that the ability of rhizobia to reduce N_2O should be considered as a criterion in their selection and use (Gao et al. 2023).

The following section is a summary from the work by Jonassen et al., (2022): In their study, Jonassen et al. (2022) investigate the potential application of nitrous oxide respiring bacteria (NRB) for mitigating N_2O emissions in agricultural soils. They propose the use of digestates, byproducts of biogas production, as substrates and carriers for inoculating NRB, aiming to enhance their abundance and activity in order to reduce N_2O emissions. Through gas-kinetics and meta-omic analyses, the researchers identify and recover metagenome-assembled genomes (MAGs) of enriched NRB, revealing their growth by utilizing fermentation intermediates from the methanogenic consortium present in the digestates. They also isolate NRB strains, including a dominant N_2O -reductase producer identified as a Dechloromonas bacterium. These isolated strains possess the necessary denitrification genes, enabling them to both produce and sequester N_2O.

Experimental validation demonstrates that the isolated NRB strains can act as sinks for N_2O in soil. Growing the isolates aerobically in digestates and fertilizing the soil with NRB-enriched digestates results in reduced N_2O emissions, confirming the effectiveness of the NRB isolates in mitigating N_2O release from the soil.

The study highlights the potential of utilizing digestates as a practical and scalable strategy for introducing NRB into soil to reduce N_2O emissions. This approach not only offers cost savings but also provides a framework for engineering the soil microbiome for various objectives, such as promoting plant growth or facilitating bioremediation processes. The findings contribute valuable insights and lay the foundation for future applications in microbiome engineering.

The following section is a summary from the work by Jonassen et al., (2021) on manipulation of soil metabolism through a high-density inoculation of microbes. In their study, Jonassen et al. (2021) aimed to develop a strategy for reducing nitrous oxide emissions in agricultural soil by manipulating soil metabolism through microbial inoculation. They proposed a novel approach using digestate, a byproduct of anaerobic digestion, as a substrate and carrier for microbial enrichment. Through a sequential anaerobic enrichment process, they monitored gas kinetics and community composition to identify operational taxonomic units (OTUs) with nitrous oxide-respiring capabilities.

The researchers isolated several nitrous oxide-respiring bacteria associated with the dominant OTUs from the enriched cultures. These bacteria underwent genome sequencing and phenotyping to assess their genetic potential and effectiveness as N_2O sinks in soil. Notably, two specific strains, Cloacibacterium sp. and Pseudomonas sp., exhibited strong N_2O sink activity when introduced into soil. Pseudomonas sp. showed a particularly long-lasting sink effect, indicating its potential for reducing N_2O emissions over time.

The utilization of waste, such as digestate, to engineer the soil microbiota holds promise for decreasing N_2O emissions and improving soil metabolic functions. This waste-based approach offers a valuable strategy for addressing N_2O emissions in agriculture. However, further research and validation are needed to refine and implement this strategy effectively in different agricultural systems. The findings highlight the potential of microbial inoculation and waste utilization in mitigating N_2O emissions and promoting optimal microbial activity in agricultural soil.

4.2 N_2O reducing rhizobia as inoculants

Rhizobial inoculants may provide a promising method of reducing soil N_2O emissions.

In their work Hénault et al., 2022 refer to the investigation done by Bakken and Frostegård, 2020 about N_2O reducing organisms and strategies to enhance their abundance and activity in soil

In recent years, there has been increasing recognition of the importance of strongly N_2O reducing organisms and strategies to enhance their abundance and activity in soil. Two approaches have emerged to address this. One approach involves developing inoculants tailored for legume crops that possess efficient N_2O reduction capabilities. By introducing these inoculants into the soil, it is expected that the population of N_2O reducing organisms will increase, leading to reduced N_2O emissions. Another strategy involves utilizing digestates, the byproducts of biogas production, as a growth medium for N_2O reducing organisms. Digestates can provide a nutrient-rich environment that promotes the growth and activity of these organisms. However, further research is needed to validate and refine these approaches, assessing their effectiveness and feasibility in different agricultural systems.

Strategies aimed at mitigating N_2O emissions in agriculture often focus on promoting the conversion of N_2O to N_2, which reduces the greenhouse gas potential of N_2O. Rhizobial inoculants have been identified as a potential option for mitigating N_2O emissions in food production by manipulating the soil microbiota. These inoculants, introduced to enhance nitrogen fixation in legume crops, may also contribute to N_2O reduction in the soil. Specifically, it has been noted that certain rhizobia, such as Bradyrhizobia, exhibit strong N_2O sink capabilities compared to other denitrifying bacteria. This is due to the competition between their nitrate reductase and N_2O reductase, with nitrate reductase having a weaker competitive advantage. Consequently, a preference for N_2O reduction over nitrate reduction occurs. Moreover, the N_2O reduction activity of inoculated rhizobia can persist in the soil over an extended period, indicating a potential long-term impact on reducing N_2O emissions.

The following sections about the strategy survival of rhizobia is from Gao et al., (2023). Their study focuses on the survival strategies of rhizobia, which are bacteria residing in nodules of legume plants. While rhizobia have a stable carbon supply within nodules, they also need to survive as free-living bacteria in carbon-limited soil. Many rhizobia can perform denitrification, an anaerobic respiration process using nitrogen oxides as electron acceptors. However, the

regulatory mechanisms and metabolic shifts in carbon-limited rhizobia during denitrification are not well understood.

In this research, a starved culture of Bradyrhizobium strain was investigated. The starved cultures showed a preference for reducing N_2O over NO_3^-. These cultures possessed the nitrate reductase NapA but lacked the membrane-bound nitrate reductase NarG. Proteomics analysis revealed comparable levels of NapA and NosZ (N_2O reductase), suggesting that the reduced NO3- reduction was not due to low NapA abundance. The electron competition between the bc1 complex and NapC enzyme was found to contribute to the preference for N_2O reduction in starved Bradyrhizobium cultures.

These findings challenge the conventional understanding that N_2O reduction decreases under carbon-limited conditions. They suggest that bradyrhizobia with NosZ can effectively reduce N_2O even with limited carbon availability. This has implications for biofertilizer development, highlighting the importance of considering rhizobia's impact on N_2O emissions in agriculture. Incorporating the N_2O reduction ability of rhizobia into biofertilizer strategies can help mitigate greenhouse gas emissions in agricultural systems.

5 Conclusions

In conclusion, nitrous oxide plays a significant role as a greenhouse gas and ozone-depleting substance, contributing to global climate change. The majority of global N_2O emissions, approximately 62%, originate from natural and agricultural soils, primarily through bacterial denitrification and ammonia oxidation processes. Denitrification, a microbial dissimilatory process, converts nitrate and nitrite into gaseous compounds. Various environmental factors, including soil moisture, temperature, pH, nitrogen inputs, land use, and water quality, influence N_2O emissions, with microbial activity in soils being a major contributor to atmospheric N_2O levels (Thomson et al., 2012).

A promising approach to reduce N_2O emissions in soil is the utilization of rhizobial inoculants. Recent advancements have led to the identification of an increasing number of highly efficient N_2O -reducing organisms, and strategies to enhance their abundance and activity in soil are being developed. Developing inoculants specifically for legume crops with strong N_2O reduction capabilities is one potential avenue. Mitigating N_2O emissions from agricultural soil can be achieved by promoting the conversion of N_2O into N_2 through various strategies. Implementing such strategies holds promise for effectively addressing N_2O emissions and contributing to sustainable agricultural practices.

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