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**MICROBIAL NITROGEN CYCLE IN TROPICAL
CLOUD FOREST SOILS AND CANOPY SOILS OF
RÉUNION ISLAND**

**TROOPILISTE PILVEMETSADE MULLADE JA
VÕRAMULDADE MIKROOBSED PROTSESSID
LÄMMASTIKURINGES RÉUNIONI SAAREL**

Master`s Thesis

Environmental Governance and Adaptation to Climate Change

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Abstract of Master's Thesis			
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<p>Tropical cloud forests, known for their rich biodiversity and critical role in climate regulation, are key to understanding biogeochemical cycles. This study's main objective is to explore the intricate relationship between microbial communities and nitrogen cycling processes in the tropical cloud forest soils and canopy soils of Réunion Island with a focus on understanding the N₂O emissions. Our analysis included microbial marker abundances and gene copy numbers associated with nitrification and denitrification. Additionally, we examined soil temperature, water content, ammonium (NH₄⁺), nitrate (NO₃⁻) concentrations, and N₂O fluxes. Results showed significant variations in SWC and temperature across the erica and fern-dominated ecosystems, greatly influencing nutrient availability and microbial activity. The erica-dominated ecosystems exhibited higher NH₄⁺-N, and NO₃⁻-N concentrations, indicative of increased nitrification activity mediated by the increased abundances of AOB, AOA and COMMAMAX <i>amoA</i>. Denitrification processes characterized by <i>nirS</i> and <i>nirK</i> gene abundances, were also prominent in both ecosystems, with a notable role of fungal <i>nirK</i> in the fern ecosystem. The higher <i>nosZ</i> gene abundance in erica-dominated soils suggests a greater capacity for N₂O reduction, mitigating greenhouse gas emissions. Despite lower average N₂O fluxes, erica-dominated ecosystems showed greater variability in N₂O emissions compared to fern-dominated ecosystems. This study underscores the need for further research to elucidate the specific mechanisms driving N₂O fluxes and microbial dynamics, which is critical for developing strategies to mitigate greenhouse gas emissions in tropical cloud forests.</p>			
Keywords: Tropical cloud forest, Réunion island, microbial communities, nitrogen cycling, ecosystem, soils and canopy soils			

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<p>Troopilised pilvemetsad, mis on tuntud oma rikkaliku bioloogilise mitmekesisuse ja kriitilise rolli poolest kliima reguleerimisel, on biogeokeemiliste tsüklite mõistmisel võtmetähtsusega. Selle uuringu põhieesmärk on uurida keerulisi seoseid mikroobikoosluste ja lämmastiku ringlusprotsesside vahel Réunioni saare troopiliste pilvemetsade muldades ja võrades, keskendudes N₂O heitkoguste mõistmisele. Uurisime mulla temperatuuri, veesisaldust, ammoniumi (NH₄⁺), nitraadi (NO₃⁻) kontsentratsiooni ning N₂O voogusid. Analüüs hõlmas nitrifikatsiooni ja denitrifikatsiooniga seotud geenikoopiate arvukust. Tulemused näitasid olulisi erinevusi SWC-s ja temperatuuris Erica ja sõnajalgade domineeritud ökosüsteemides, mõjutades oluliselt toitainete kättesaadavust ja mikroobide aktiivsust. Eerika domineeritud ökosüsteemides ilmnesid kõrgemad NH₄⁺-N ja NO₃⁻-N kontsentratsioonid, mis viitab suurenenud nitrifikatsiooniaktiivsusele, mida vahendas AOB, AOA ja COMMAMAX <i>amoA</i> suurenenud arvukus.</p> <p>Denitrifikatsiooniprotsesside geenid, mida iseloomustasid <i>nirS</i> ja <i>nirK</i> geenide arvukused, olid samuti mõlemas ökosüsteemis arvukad, kusjuures seente <i>nirK</i> roll sõnajala ökosüsteemis oli märkimisväärne. Suurem <i>nosZ</i> geeni arvukus Erica domineeritud muldades viitab suuremale N₂O vähendamise võimele, mis vähendab kasvuhoonegaaside heitkoguseid. Vaatamata madalamale keskmisele N₂O voogudele näitasid Erica domineeritud ökosüsteemid N₂O heitkoguseid suuremat varieeruvust võrreldes sõnajalgade domineeritud ökosüsteemidega. See uuring rõhutab vajadust täiendavate uuringute järele, et selgitada välja spetsiifilised mehhanismid, mis juhivad N₂O vooge ja mikroobide dünaamikat, mis on kriitilise tähtsusega troopiliste pilvemetsade kasvuhoonegaaside heitkoguste leevendamise strateegiate väljatöötamiseks.</p>			
Märksõnad: Märksõnad: troopiline pilvemets, Réunioni saar, mikroobikooslused, lämmastiku ringlus, ökosüsteem, mullad ja võramullad			

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Abbreviations

ANAMMOX	Anaerobic ammonium oxidation
COMAMMOX	Complete ammonia oxidation
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
Ca	Calcium
P	Phosphorus
DNA	Deoxyribonucleic Acid
DNRA	Dissimilatory Nitrate Reduction to Ammonium
N	Nitrogen
NO	Nitric oxide
N ₂ O	Nitrous oxide
NH ₄ ⁺	Ammonium
NO ₂ ⁻	Nitrite
NO ₃ ⁻	Nitrate
NH ₃	Ammonia
P	Phosphorus
<i>nirS</i>	Denitrification indicator gene (NO ₂ ⁻ → NO stage)
<i>nirK</i>	Denitrification indicator gene (NO ₂ ⁻ → NO stage)
<i>nosZI</i>	Denitrification indicator gene (N ₂ O → N ₂ stage)
<i>nosZII</i>	Denitrification indicator gene (N ₂ O → N ₂ stage)
<i>amoA</i>	Nitrification indicator gene (NH ₄ ⁺ → NO ₂ ⁻ → NO ₃ ⁻)
SWC	Soil water content

1. Introduction

Globally, tropical cloud forests have always been one of the foremost terrestrial ecosystems (Bubb, 2004; Hedin et al., 2009; Karger et al., 2021; Pillay et al., 2022). They are not only rich with species and endemism but also play an essential role in the regulation of the climate and biochemical cycles through their immense interactions of carbon, water, energy, and nutrients with the global atmosphere (Hedin et al., 2009; Karger et al., 2021; Pillay et al., 2022). Tropical cloud forests symbolize some of the utmost biodiverse and ecologically significant ecosystems on earth, sheltering distinctive microbial communities that are substantial for nutrient cycling and the functioning of the ecosystem (Pillay et al., 2022). In places such as Hainan Island, soil nutrients in the tropical cloud forest determine above-ground biomass and leaf traits in the ecosystem (Batool et al., 2024). While soil and canopy environments make separate ecological functions and contribute exclusively to nitrogen (N) dynamics, it is imperative to note that N cycling plays a vital role in the overall productivity of the ecosystem. Canopy soil or what is also known as arboreal soil, is a type of soil that is developed from the accumulated decomposed epiphytes in bifurcation and on branches of trees in temperate and tropical forests (Haristoy et al., 2014; Van Langenhove et al., 2021). It is the layer of organic matter or soil that accumulates on the branches and leaves of trees and other vegetation in the forest ecosystem (Saeki et al., 2024)

There are, however, fundamental gaps in the knowledge and understanding of N cycling in most forests worldwide, despite the significance of biological diversity and the importance of this phenomenon in the overall global well-being of the environment. N cycle can simply be said to be the natural or a biogeochemical process where N is converted into various other forms, and the supply of N to plants and microbes helps to regulate the productivity of the entire ecosystem (Pajares & Bohannan, 2016; Z. Wang et al., 2020; J. Zhang et al., 2024). Among other challenges peculiar to practical N cycling, the most common challenge might be the imbalance of nutrients such as low phosphorus (P) or calcium (Ca) and plant species that enhance N fixation. Among other limitations, factors such as soil erosion and leaching due to excessive rainfall might cause unwanted export of nutrients from the ecosystem (Hedin et al., 2009)

It is, therefore, imperative to apply a holistic approach that considers both human impact and natural processes on N cycling in tropical cloud forest soils and canopy soils. Considering the tropical cloud forest at Réunion Island as a case study, this study will help uncover targeted conservation strategies based on the unique environmental conditions of the forest components.

The general aim of this thesis is to study the relationships between soil microbial communities and N₂O fluxes in the tropical cloud forest and canopy soils of Réunion island. The specific research questions were: what relationships exist between soil characteristics (pH, soil temperature, soil water content) and nitrogen cycling gene abundances in the tropical cloud forest and canopy soils of Réunion island?; What are the key N cycling processes in the tropical cloud forest soils and canopy soils of Réunion island.

2. Theoretical overview

2.1. Tropical and cloud forests

Tropical forests typically possess high levels of biodiversity, with abundant rainfall and warm temperatures, and as such can include various types of vegetation, such as dry forests, montane forests, mangroves, rain forests, and moist forests (FAO and UNEP, 2020; Lawrence et al., 2005; Prance, 2002; Sullivan et al., 2019). According to the United Nations (2020), approximately 30.8 percent of the world's land area is being occupied by forests. Although forests are not equally distributed globally, they cover about 4.06 billion hectares of land. Furthermore, one of the most remarkable features of tropical forests, including cloud forests, is the diversity of life they support. They harbour a vast variety of animal species and various types of plants

Tropical forests play a critical role in regulating water cycling, which is a vital aspect for rainfall and soil moisture (Betts, 2004). They facilitate water retention and the gradual evaporation of water into the atmosphere, thereby enhancing the regulation of rainfall (Betts, 2004; Chagnon et al., 2004). Tropical forests remain the most important places that must be preserved for the continuous existence of biodiversity. The presence of tropical forests has a significant impact on climate patterns (Betts, 2004). Tropical forests play a vital role in reducing the concentration of greenhouse gases, such as carbon dioxide, through the absorption and storage of carbon in both soil and biomass (Betts, 2004; Delire et al., 2004)

Cloud forests, a subset of tropical forests, are less common, and they are characterized by the persistent presence of mists and clouds, which allows them to receive additional humidity aside from rainfall, which in turn, ensures the presence of water droplets that condense on the vegetation (Bubb, 2004; Mulligan, 2011; Stadtmüller, 1987). This feature of cloud forests influences various climatic, edaphic, and ecological parameters, including the radiation and hydrological balance (Stadtmüller, 1987). Similar to other tropical forests, cloud forests provide direct and indirect benefits to humanity by helping to regulate the climate and produce essential resources for various purposes.

Despite the various benefits that cloud forests offer and their importance for ecosystem sustainability, these valuable resources are constantly disappearing. Cloud forests, like other forests, face numerous threats such as deforestation and climate change; nevertheless, their distinctive ecology and their location, usually on mountain slopes, make them particularly

susceptible to environmental fragmentation, especially due to climate change (Bubb, 2004). Over the years, increasing demand for land and natural resources to meet human needs has led to the exploration of natural resources such as trees, plants, and other forest species. This persistent pattern has led to the fragmentation of interconnected habitation and the deterioration of the quality of habitation for plants and species in cloud forests and other forest environments (Teucher et al., 2020). Cloud forests occupy about 2.5 percent of the world's tropical forests (Figure 1a), indicating their fragmentation; however, they harbour an impressively large number of the world's species, making the threat to their existence a serious concern (Bubb, 2004).

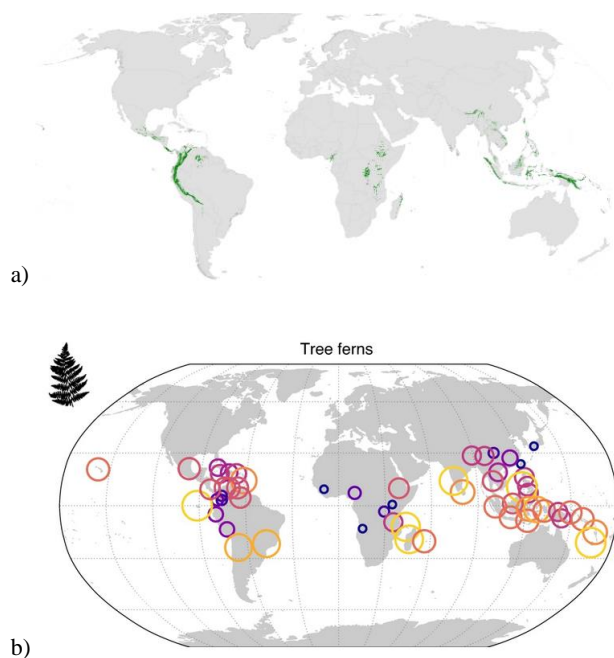


Figure 1. Distribution of tropical cloud forests in the world (a). Uniqueness in species composition of tropical cloud forest-associated species between tropical cloud forest regions (b) (Karger et al., 2021).

Cloud forests harbour exceptional biodiversity and abundant resources, despite their rarity compared to other types of forests globally (Bubb, 2004; Ramírez-Barahona et al., 2021). The quantity and diversity of orchids, mosses, ferns, and other plants growing on tree trunks, rocks, and branch surfaces in cloud forests distinguishes them from other forests (Figure 1b). The

biodiversity richness of tropical cloud forests makes them essential for ecological resilience and balance because they provide a high level of genetic diversity and endemism (Ramírez-Barahona et al., 2021). Cloud forests are valuable to downstream communities due to their distinctive features and ability to capture water from the cloud (Bubb, 2004). The people within the communities of tropical cloud forests usually rely on forest biodiversity for their livelihood (Gorczyński et al., 2023) and the products derived from the forest are often used for food, shelter, medicine, materials, and energy.

Although cloud forests and other tropical forests are essential for a variety of ecosystem services that are important for the survival of humanity, providing immeasurable support that helps them maintain a balanced ecosystem, climate stability, and sustenance of the earth's biodiversity, anthropogenic influences are still having a severe effect on the forests (FAO, 2020; Pillay et al., 2022; Ramírez-Barahona et al., 2021). Anthropogenic impact on forests refers to the several ways by which human activities affect biodiversity and ecologically essential ecosystems (Kung'u et al., 2023). Climate change and anthropogenic effects affecting the forests worldwide threaten ecosystem services and biodiversity (Anderson-Teixeira et al., 2015; Kung'u et al., 2023).

Deforestation is one of the most severe threats to tropical cloud forests (Ramírez-Barahona et al., 2021). It involves human activities, including clearing trees and other vegetation, which usually results in the conversion of forested land areas into non-forest usage of land (FAO, 2020). Aside from the disruption and loss of biodiversity, deforestation has grave consequences on the earth, such as soil erosion, disruption of the ecosystem, and climate change (Pillay et al., 2022; Seymour & Harris, 2019). Cloud forests suffer the most effects of deforestation because they are vast in the occurrence rate of endemic species, increasing the likelihood of extinction (Kung'u et al., 2023)

Moreover, deforestation is not the only threat to tropical cloud forests; over-exploitation also contributes equally to the danger to the forest habitat. The persistent exploitation of a particular species or group of species can result in a situation where such species or group of species could either be driven from that forest or get extinct outright. The distinction between overexploitation and other forms of anthropogenic activities is that a particular species is being focused on, which could result in the extinction of such species (R. J. Morris, 2010). Most species found in the cloud forests are often challenging to find in other types of forests,

therefore increasing the chance that over-exploitation could aid extinction or, in rare cases, drive such species out of their natural habitat.

Furthermore, other anthropogenic influences on forests such as invasive species, has severe consequences on cloud forests. Invasive species disrupt the ecological processes, alter the structure and function of the ecosystem, and may also result in the decline or extinction of the native species in the cloud forests (Davis et al., 2010; R. J. Morris, 2010). The climate can facilitate the movement of species causing invasion, likewise, invasive species aid climate change by the disruption of the ecosystem. These are also severe anthropogenic influences on cloud forests and their environs.

2.1.1 Soil.

Soil can simply be described as a material that enables plants to grow and which provides the plants with physical support, water, and nutrients. Historically, soil is a substance that covers the dry surface of the earth and it is formed as a result of the weathering of rocks and the breakdown of organic materials over elongated periods. It is a complex mixture of organic materials that could be living and/or dead materials. According to (van Es, 2017) soil is defined as *“The layer(s) of generally loose mineral and/or organic material that are affected by physical, chemical, and/or biological processes at or near the planetary surface and usually hold liquids, gases, and biota and support plants”*. Soil plays a vital role in different ecological processes, such as water filtration, nutrient cycling, and support for the growth of plants. The dead organic matter in soil usually includes freshly dead plant leaves, roots, and other plant litter (van Es, 2017). Also, it contains dead fauna and organic materials in different stages of decomposition from slightly reformed fairly fresh materials to complex decomposed material referred to as humus (Davis et al., 2010; Jahn et al., 2006). Soil comes in different varieties and forms and serves various purposes for different people at different points. It reflects in part, variable combinations of organic matter and minerals with varying responses both in complex combination and in isolation based on different environmental conditions as the case may be.

Soil is a pathway by which nutrients are passed onto plants, including trees of the forests. It therefore is an important component of the biogeochemical nutrient cycle in that the return of nutrients on branch shedding, leaf fall, or through tree mortality enriches the soil (L. A. Morris, 2004). The soil in the tropical forest ecosystem plays a vital role in forming the communities of the plants, structural diversity, and their species. Their conservation has fundamental relevance for biodiversity conservation and suitability land use (Gautam & Mandal, 2013). Also, the

change in diversity may be related to the early nutrient state of the soil. The nutrient cycle in soil is tightly combined through the demand for nutrients from microbial biomass during decomposition (Parton et al., 2015).

2.1.2 Canopy soil

Canopy soil or what is also known as arboreal soil, is a type of soil that is developed from the accumulated decomposed epiphytes in bifurcation and on branches of trees in temperate and tropical forests (Haristoy et al., 2014; Van Langenhove et al., 2021). It is the layer of organic matter or soil that accumulates on the branches and leaves of trees and other vegetation in the forest ecosystem (Saeki et al., 2024)

Canopy soils provide shelter for various animals and plants and may serve as habitation for invertebrate communities different from those living on the regular soil (Saeki et al., 2024). These soils retain nutrients and water on the surface and in their airspace exchange sites. Usually, nutrients can be transferred to the forest floor and become available to terrestrial vegetation as they decompose after they die when epiphytic materials ride down or fall from branches with fallen trees or broken branches (Dangerfield et al., 2017).

Canopy soil plays a crucial role in forest ecosystems by helping in the provision of nutrients for the growth of plants, moisture retention, and providing support to various microorganisms and invertebrates. Moreover, it serves a habitation for different species of mammals, birds, and insects (Ishii et al., 2018; Nadkarni et al., 2002; Saeki et al., 2024; Tong et al., 2024)

2.2. Biogeochemical cycles in tropical forest

Tropical forests play a very significant role in biogeochemical cycles, which are critical processes that help regulate the flow of elements and nutrients between living organisms and their environment. The biogeochemical cycle refers to the transfer of aspects out or into the ecosystem or can also involve an external transfer of elements among different components of the forest system (L. A. Morris, 2004). These cycles are sophisticated processes aided by the astonishing metabolic diversity of microorganisms. As a result, they drive complex nutrient and energy flows that are important to the complexity and expansion of ecosystems, which eventually enhance biodiversity. This is important because the successful operations of these cycles are reliant on the sophisticated interplay of the chemical, geological, and biological processes connecting the biosphere with the atmosphere, hydrosphere, and geosphere (Huang et al., 2024)

Forests are an important figure when it comes to water cycling because it carry out multiple essential functions that are vital for purposes such as sedimentation and reduction of erosion; influencing streamflow regulation, contributing to atmospheric water recycling and fostering groundwater recharge; and also cloud generation and precipitation through evaporation. The hydrological process is one of the most vital phenomena in interacting with forest vegetation and the ecological environment. The vegetation of the forest also has a considerable role in the spatial and temporal circulation of evapotranspiration, precipitation, runoff extent, and water balancing through the forest roots, litter, canopy, and physiological characteristics of the forest ecological system (Gianni et al., 2019).

Tropical forest soil is typically acidic ($\text{pH} < 5$), and its fertility often a reliance on the cycling of a tiny layer of organic matter associated with large volumes of plant litter materials because, among other soils, they are by and large on older geology substrates of rocks-derived elements such as cations and phosphorus (Bauters et al., 2019; Pajares & Bohannan, 2016).

Carbon plays a vital role in trapping the long-wave radiation from the sun and balancing energy. It is a biogeochemical cycle where different carbon compounds are interchanged among the different layers of the earth, such as the geosphere, biosphere, atmosphere, and hydrosphere (Thorley et al., 2015). The carbon cycle acts like cover over the earth such that if there is an interruption in it, it could amount to severe repercussions like global warming and change in the climate. Carbon is stored in trees and other plants in the forest since all green plants use sunlight and carbon dioxide for photosynthesis. Since the forest encompasses of huge number of trees and other plants, they absorb large amounts of carbon (CO_2) in the biomass, which is stored in them over many years. The recycling of carbon, therefore, takes place when the dead leaves or plant decompose carbon is stored in the soil (Huang et al., 2024)

2.2.1. Nitrogen cycle in tropical forests

Nitrogen (N) is a significant component of nucleic acid and proteins, making it an essential nutrient for every organism. The supply of N to organisms helps to regulate the primary productivity in various natural ecosystems (Z. Wang et al., 2020; J. Zhang et al., 2024). Therefore, it exists in numerous oxidation situations and chemical forms, and microorganisms characteristically enhance transformations between these forms (Aczel, 2019; J. Zhang et al., 2024).

N cycle can simply be said to be the natural or a biogeochemical process where N is converted into various other forms and moves consecutively from the atmosphere to the soil, then to the organisms, and finally back to the atmosphere (Pajares & Bohannan, 2016). The processes of the N cycle include N fixation, nitrification, denitrification, decay, and putrefaction (Bauters et al., 2019; Elrys et al., 2023). N exists in both inorganic and organic forms, such as in the atmosphere and living organisms, respectively (Lie et al., 2021). Their existence in organic forms is made accessible to living organisms through the food chain by consuming other living organisms. On the other hand, their existence in organic forms is made accessible to plants by symbiotic microbes such that they can be converted to inert N in a helpful form (Pajares & Bohannan, 2016)

For more clarity, the complexity of the biological N cycle in the soil of tropical forests can be shown by the simplified model depicted in Figure 2. The diagram shows the main processes of decomposition of dead organisms and plant litter to soil organic matter, which can be further degraded to dissolved organic N and ammonium. It also shows the assimilative processes of dissolved organic N nitrate and ammonium by microorganisms and plants for replication and growth. Lastly, it shows the process of dissimilation, including N fixation, nitrification, denitrification, and dissimilatory nitrate reduction to ammonium, as well as some newly discovered pathways like denitrification and anammox. There are lots of N available for plants and small soil organisms in many tropical forest soils compared to soils in cooler areas. This is possible because these small organisms and legumes are good at accumulating N (Pajares & Bohannan, 2016; Silver et al., 2005) Consequently, N gets into the soil more quickly because of the high rate of microbes and plants in the tropical forests. It is important to note that when there is excess N available in the ecosystem such that it becomes enormous for the capacity of the system, it can lead to N loss through processes such as leaching and release of gases like N_2O and nitric oxide, just as the shortage can deter the survival of microbes and plants (Aczel, 2019; Pajares & Bohannan, 2016).

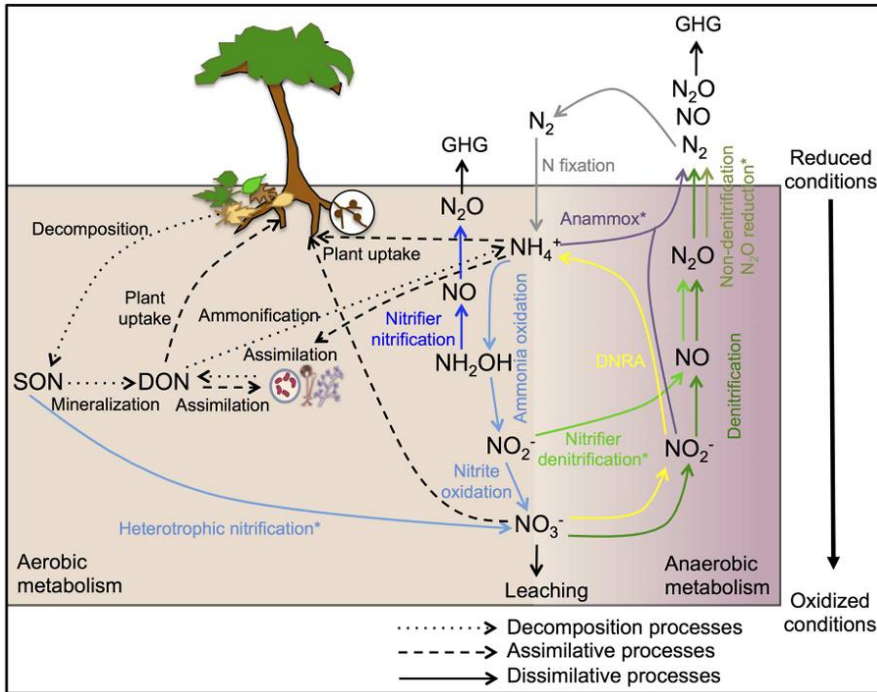


Figure 2. Diagrammatic presentation of nitrogen movement through the biological cycle in tropical forest soils (Pajares & Bohannan, 2016). The above diagram shows the arrows of dissimilative processes in various colors, while the arrows of decomposition, assimilative, and biotic processes are shown in black. Grey represented the N-fixation, light blue represented the nitrification, dark blue represented nitrifier, light green represented the nitrifier denitrification, dark green represented denitrification, olive green represented non-denitrification N₂O reduction, yellow represented DNRA, and purple represented anammox.

2.2.1. Denitrification

Denitrification is a microbial anaerobic respiration pathway by which nitrate (NO₃⁻) or nitrite (NO₂⁻) is sequentially reduced to molecular nitrogen (N₂) through the intermediate NO and N₂O (Aczel, 2019; Norton & Ouyang, 2023; Pajares & Bohannan, 2016). It is a process where N compounds return to the atmosphere to end the nitrogen cycle by converting nitrate (NO₃⁻) into the gaseous form of N (Philippot et al., 2007; Wei et al., 2015). Aside from the process of anaerobic ammonium oxidation (Anammox), denitrification is the only pathway by which reactive forms of N in aquatic and terrestrial ecosystems are transformed into inert N₂ gas (Xu

et al., 2013). The reduction of NO_2^- to NO is catalyzed by structurally separate but functionally equivalent copper-containing reductase (*NirK*) and cytochrome cd1-containing reductase (*NirS*), which is an important step in the process of denitrification because it allows dissolved N to be converted into gaseous N (Wei et al., 2015)

Denitrification comprises four reactions catalyzed by the metallo-enzymes: nitrate reductase (*Nar*), nitric oxide reductase (*Nor*), nitrite reductase (*Nir*), and nitrous reductase (*NosZ*). Among the four reactions, nitrite reductase (*Nir*) is the main enzyme of denitrification because it catalyzes the decline of nitrogen dioxide (NO_2^-) into gas products, which is the reaction that minimizes the extent of denitrification process (Pajares & Bohannan, 2016). Denitrifier organisms can possess distinct combinations of genes involved in the denitrification process, so also in the process where some microorganisms harbor all denitrification enzymes and can possibly perform the complete process; others lack the nitrous oxide reductase *nosZ* gene and produce just N_2O as the end product of denitrification.

The *nirK* and *nirS* are utilised as marker genes to investigate the ecological behaviour of denitrifying microorganisms in the ecosystem because contrasting nature of nitrite reductase (*nir*) and 16S ribosomal RNA (rRNA) genes (Pajares & Bohannan, 2016). More recently, studies have identified non-denitrifying N_2O reducers with atypical *nosZ*, which are likely contributors to the reduction of N_2O in soils. This newly discovered clade of N_2O reducers is diverse and widespread in terrestrial environments. However, the ecology of this group in tropical forest soil is still very much yet to be identified (Pajares & Bohannan, 2016). Denitrification is also a means of environmental burden because it is a means of nitrous oxide (N_2O) emission (Heinen, 2006)

2.2.2. Nitrification

Nitrification is the stepwise aerobic oxidation of ammonia (NH_3) or ammonium NH_4^+ to nitrites (NO_2^-) and nitrates (NO_3^-) (Norton & Ouyang, 2023; Pajares & Bohannan, 2016; van Kessel et al., 2015; Xu et al., 2013). It is a natural process in the habitat by specialized autotrophic bacteria and archaea where conversions are carried out (Philippot et al., 2007; Xu et al., 2013). Ammonia is considered to be oxidized first of all to nitrite by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA), and afterward oxidized to nitrate oxidizing bacteria (NOB) (van Kessel et al., 2015).

The nitrification in soil is divided into heterotrophic and autotrophic nitrification (Xu et al., 2013). Heterotrophic nitrification is performed by some heterotrophic fungi and bacteria with

the ability to oxidize both inorganic and organic N compounds, while autotrophic nitrification is performed by chemoautotrophic ammonium-oxidizing bacteria and archaea and nitrite-oxidizing bacteria (Pajares & Bohannan, 2016; Xu et al., 2013; L.-M. Zhang et al., 2010). Ammonia monooxygenase (AMO) is a vital enzyme for starting the oxidation process in chemoautotrophic ammonia oxidation. However, there are variances in the structure of AMO from AOA and AOB. Furthermore, AMO of AOB entails three subunits encoded by *amoA*, *amoB*, and *amoC* genes, respectively, while AMO of AOA has four subunits encoded by *amoA*, *amoB*, *amoC*, and *amoX* genes, respectively (Martikainen, 2022; van Kessel et al., 2015)

In recent studies, ammonia-oxidizing archaea (AOA) have been demonstrated to be ubiquitous constituents of terrestrial environments, and their discovery has transformed the paradigm of aerobic nitrification (Pajares & Bohannan, 2016). The investigation into the ecology of AOA remains ongoing because there is no simple relationship between the relative abundance of Ammonia-oxidizing bacteria (AOB) and AOA and NH₃ oxidation rates (Taylor et al., 2012).

Drivers of niche differentiation of AOB versus AOA communities have focussed extensively on N organic forms, NH₃ concentration, and pH regarding related matters to tropical forest soils (Pajares & Bohannan, 2016). The high richness of the archaeal *amoA* gene has been reported in numerous acidic soils, and multiple investigations have suggested that some groups of AOA may prefer a pH below 5.5 (Taylor et al., 2012). In strong acidic soils, the abundance of AOA increases or remains the same with the decrease of pH, while AOB abundance decreases (Pajares & Bohannan, 2016; L.-M. Zhang et al., 2010).

2.2.3. Nitrogen fixation

Nitrogen N fixation is the most essential natural pathway for introducing previously inert N-N₂ gas into the ill-managed ecosystem (Koutika et al., 2021; Van Langenhove et al., 2021). It generates the largest natural amount of new N to the terrestrial ecosystem, including the tropical forests (Cusack et al., 2009; Pajares & Bohannan, 2016; Stanton et al., 2019). Nitrogen is fixed by diazotrophs; a microorganism that can minimize N (N₂) into ammonia (Cusack et al., 2009). In this process, the atmospheric dinitrogen gas (N₂) is reactive and biologically available when it is reduced by symbiotic and free-living diazotrophic microorganisms (Van Langenhove et al., 2021).

N fixation rates in the tropical forests are usually estimated to be the same or higher between 15kg to 36 kg N ha⁻¹ yr⁻¹ than the estimate for their temperate counterparts which usually stood

between 7kg to 27kg N ha⁻¹ yr⁻¹; making them susceptible to limited nitrogen N (Pajares & Bohannan, 2016)The diazotrophs are likely to be favored in tropical forests since they acquire sufficient N to retain higher extracellular phosphatase activity, which is needed to overcome the limitation of P in the ecosystems, and because the temperature in tropical environments near the optimum for biological N fixation (Koutika et al., 2021).

Diazotrophs are divided into two groups, namely, the free-living group and the symbiotic group. The free-living N fixers are archaea or autotrophic bacteria inhabiting water, rocks, soils, or leaf litter (Koutika et al., 2021). On the other hand, the symbiotic group (fixers) are generally located in root nodules and live in mutualistic relationships with higher plants that give out C to the N fixers in exchange for N (Van Langenhove et al., 2021). Although the tropical forest ecosystem retains substantial biological Nitrogen (N) fixation to balance the huge probable N losses, this process and the microbial community involved have constantly been poorly studied in these soils (Pajares & Bohannan, 2016; Stanton et al., 2019)

Based on recent research conducted in tropical forests, there are indications that free-living N-fixing bacteria in soil and litter might be more significant as much as free-living diazotrophs are the dominant form of N-fixing bacteria in temperate forests (Pajares & Bohannan, 2016). This is because the forest canopy communities, such as mosses, lichens, and other epiphytes related to cyanobacteria, can similarly provide substantial N inputs to tropical forests. In addition, micronutrients and P play an essential part in regulating N-fixation in tropical forests. The increased P demand of diazotrophs to give the large quantities of energy needed by this process produces the likelihood for P limitation of N fixation. P is bound up with extremely weathered organic matter and soil minerals in numerous lowland tropical forests. For microorganisms to liberate the P by emanating phosphatase enzymes and their synthesis necessitates enormous quantities of N. Hence, in tropical forests, N fixing may convene a competitive advantage to diazotrophs by enabling the uptake of P (Koutika et al., 2021; Pajares & Bohannan, 2016)

3. Materials and methods

3.1. Site description and sampling

A cloud forest, namely Plaine des Cafres on Réunion Island, was selected for the study (Figure 3). The sampling was done in two different parts of the cloud forest depending upon the differences in the ecosystem mainly characterized by the dominance of tree species. The sub-

site Palmiste 1 (−21.14540, 55.56979) was dominated by the *Alsophila glaucifolia*, and the Palmiste 2 (−21.14468, 55.57137) was dominated by the *Erica reunionensis* species. Both subsites were located between 1500-1600 m above sea level.

The study site is located between Mauritius and Madagascar in the Indian Ocean and is known as the Réunion Island (Selosse et al., 2018). Réunion Island is a French overseas territory and it is home to mountains in an advanced state of dissection by short torrential rivers (Selosse et al., 2018) two sub-sites where samples are extracted are located at Plaine des Cafres, one of the high plains located at Réunion Island (de la Fournaise Volcano, 2010).

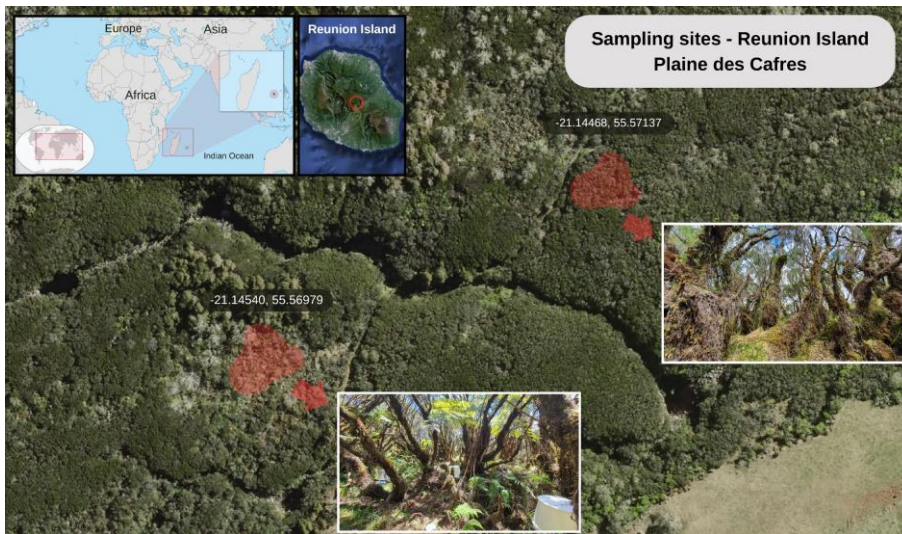


Figure 3. Study site view on map. Two sub-sites at the Plaine des Cafres on the Réunion Island, Palmiste 1 and Palmiste 2, are dominated by *Alsophila glaucifolia* and *Erica reunionensis* species, respectively.

From every sampling point, a maximum of 20 g of soil was obtained in plastic grip bags. The air in the individual bags was thoroughly squeezed out to ensure that the bags contained only the particles of soil needed for the analyses. Immediately after collection, each bag was carefully stored at a temperature of -20°C till they were required for microbial analyses. In the case of canopy soil, the soil and soil-like material present on the different branches of trees were scratched using the knife and collected in tea bags, which were later packed with active silica gel to remove the moisture from the sample. The silica gel was changed repeatedly until all moisture was removed successfully.

For chemical analyses, 100-150 g of soil was obtained from the selected sites in grip bags.

3.2. Chemical and physical parameters of soil

The chemical analyses on soil were undertaken at the Estonian Environmental Research Centre in Tartu. During the analyses, total nitrogen and carbon content and pH were determined. By using the flow-injection analysis (APHA-AWWA-WEF, 2005), ammonium ($\text{NH}_4^+\text{-N}$) and nitrate ($\text{NO}_3^-\text{-N}$) levels were determined from soil samples after extracting them with 2M KCl (1:10 ratio).

3.3. DNA extraction and concentration measurements

DNA extraction of 0.25 g of each of the twelve soil samples and 0.12 g of the canopy soil samples was done using the DNeasy® PowerSoil® Pro Kit with careful adherence to the instructions by the manufacturers. The samples at a rate of 5000 rpm were homogenized by utilizing Precellys 24 for 20 seconds. The DNA extracted was therefore analyzed for concentration and quality utilizing the TECAN Infinite 200 pro and NanoQuant Plate. The extracted DNA was stored at a temperature of $-20\text{ }^\circ\text{C}$ pending further analyses.

3.4. Quantitative PCR

After DNA extraction from soil and canopy soil samples, qPCR was utilized as an evaluation mechanism for both bacterial and archaeal abundances by quantifying the abundance of bacterial and archaeal 16S rRNA genes. Bacterial *amoA* (ammonia monooxygenase gene), archaeal *amoA*, and COMAMMOX (complete ammonia oxidation) *amoA* genes were quantified by qPCR to check for nitrifier abundance. Similarly, denitrification genes *nirS* (cytochrome cd1-type nitrite reductase gene), *nirK* (copper-containing nitrite reductase gene), *nosZI* (clade I nitrous oxide reductase gene), and *nosZII* (clade II nitrous oxide reductase gene) were also quantified with qPCR.

The polymerase chain reaction (qPCR) was performed using RotorGene (Qiagen) equipment. The reaction mix was prepared within the laminar flow. The component mixture consisted of SYBR Green, both forward and reverse primers, nuclease-free water, and DNA samples with varying concentrations of primers depending on the target genes. The total volume in each tube was maintained at 10 μl , with adjustments made only to the volume of water to accommodate changes in primer concentration while keeping the SYBR Green constant. Details of primers and their qPCR profile, such as the primer concentration gene names, are shown in Table 1.

The results were assessed using Rotor-Gene Q software v.2.0.2 (Qiagen). The number of gene copies per gram was calculated using a standard curve obtained from the successively diluted mixture of target sequences. Detailed description of qPCR method (Espenberg et al., 2018).

Table 1. Primers used in qPCR, their quantities and qPCR programs

Marker gene	Primer ID	Primer Concentration (μM)	qPCR program	References
Bacterial 16S rRNA	Bact517F	0.6	95°C 10 min; 35 cycles: 95°C 30 s; 60°C 45 s; 72°C 45 s	(Liu et al., 2007)
	Bact1028R			(Dethlefsen et al., 2008)
Archaeal 16S rRNA	Arc519F	0.6	95°C 10 min; 45 cycles: 95°C 15 s; 56°C 30 s; 72°C 30 s	(Espenberg et al., 2016)
<i>nifH</i>	Ueda19F	0.8	95°C 10 min; 45 cycles: 95°C 30 s; 53°C 45 s; 72°C 45 s	(Ueda et al., 1995)
	Ueda407R			
Bacteria <i>amoA</i>	amoA-1F	0.8	95°C 10 min; 45 cycles: 95°C 30 s; 57°C 45 s; 72°C 45 s	(Rotthauwe et al., 1997)
	amoA-2R			
Archaeal <i>amoA</i>	CrenamoA 23F	0.8	95°C 10 min; 45 cycles: 95°C 30 s; 54°C 45 s; 72°C 45 s	(Tourna et al., 2008)
	CrenamoA 616R			
COMMAMOX <i>amoA</i>	comamoA AF	0.8	95°C 10 min; 40 cycles: 95°C 15 s; 55°C 30 s; 72°C 30 s	(M. Wang et al., 2018)
	comamoA SR			
<i>nirS</i>	<i>nirSCd3af</i>	0.8	95°C 10 min; 45 cycles: 95°C 15 s; 55°C 30 s; 72°C 30 s; 80°C 30	(Kandeler et al., 2006)
	<i>nirSC3cd</i>			
<i>nirK</i>	<i>nirK876</i>	0.8	95°C 10 min; 45 cycles: 95°C 15 s; 58°C 30 s; 72°C 30 s; 80°C 30	(Hallin & Lindgren, 1999)
	<i>nirK1040</i>			
Fungal <i>nirK</i>	FnrK-F3	0.8	95°C 10 min; 45	(Chen et al., 2016)

	FnirK-R2		cycles: 95°C 15 s; 56°C 30 s; 72°C 30s	
<i>nosZ1</i>	nosZ2F	1.2	95°C 10 min; 45 cycles: 95°C 15 s; 60°C 30 s; 72°C 30 s; 80°C 30	(Henry et al., 2006)
	nosZ2R			
<i>nosZ11</i>	nosZ11F	1.2	95°C 10 min; 45 cycles: 95°C 30 s; 54°C 45 s; 72°C 45 s; 80°C 45	(Jones et al., 2013)
	nosZ11R			

3.5. N₂O gas measurements

Using 65 L polyvinyl chloride chambers on pre-installed plastic collars, the gas samples were obtained in pre-vacuumed glass vials (50 ml). Gas sampling was repeated twice daily in each subsite and in each sampling point, four times periodically in the span of one hour to get the standard curve. Each gas sample underwent testing for N₂O concentration using two Shimadzu-2014 gas chromatographs featuring an electron capture detector (GC-ECD), a thermal conductivity detector (GC-TCD), and a Lofthfield-type autosampler. Fluxes were determined by calculating the slope of the least-squares linear regression of the N₂O concentration change in the chamber headspace throughout the measurement period. Detailed description of gas measurements method (Espenberg et al., 2024)

3.6 Statistical analysis and author's contribution

Statistical software programs R (version 4.0.4) were used for statistical analysis and to create the figures. Spearman's rank correlation coefficient measured the association between N₂O emissions and gene abundances and environmental factors. Gene abundances were log-transformed to normalize the datasets, minimize skewness and produce more statistically significant results.

The author of the master thesis participated in the extraction of DNA from the canopy soil samples, performed qPCR measurements and calculated the number of gene copies. In addition, the author performed the data analysis and writing the thesis.

4. Results

This research aspect focuses on the forest floor soil and canopy environments. By doing the analysis of soil samples gathered from these different habitats, we intend to unravel the

compound interplay between nitrogen cycling processes, environmental conditions, and the abundance of microbial communities.

4.1. Soil physicochemical parameters

The temperature in both ecosystems ranged between 13.73 °C and 15.7 °C. SWC in Erica's dominant ecosystem ranged from 0.34 m³ m⁻³ to 0.55m³ m⁻³, while in the Fern-dominated ecosystem, the mean value was 0.45 m³ m⁻³ with a range from 0.25 m³ m⁻³ to 0.61m³ m⁻³. Figure 4 shows the relationship between soil temperature and soil water content by ecosystem. As expected, the figure depicts a negative relationship between SWC and soil temperature. This indicates that soil in more temperate sites has lower SWC.

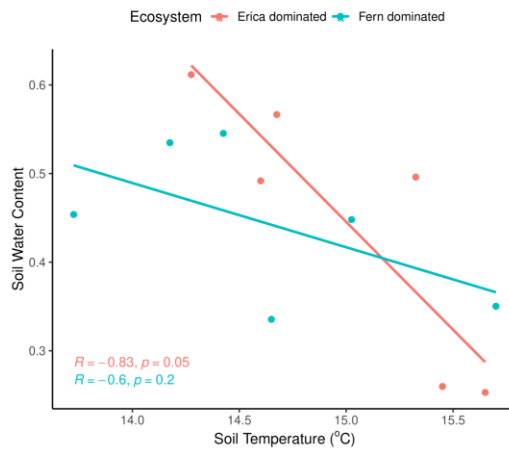


Figure 4. Relationship between soil temperature and soil water content by ecosystem. Different shapes and colours represent the observations in different dominated areas. The slopes and lines represent the best fit for the relationship between SWC and soil temperature in different ecosystems.

4.2. Soil N₂O fluxes

The boxplot analysis (Figure 5) revealed that erica-dominated ecosystems had a lower median N₂O flux of 0.9 µg N m⁻² h⁻¹ compared to fern-dominated ecosystems, with a median flux of 1.2 µg N m⁻² h⁻¹. Additionally, the boxplot for erica-dominated ecosystems showed a larger range of N₂O flux values, with some samples reaching a flux of up to 3.1 µg N m⁻² h⁻¹, compared to fern-dominated ecosystems where the highest observed flux was 1.4 µg N m⁻² h⁻¹.

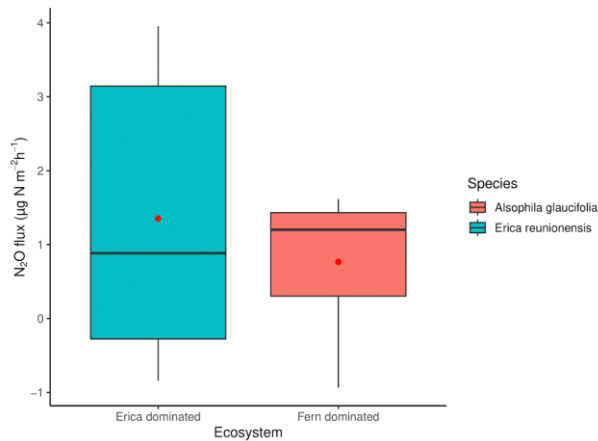


Figure 5. Soil N₂O emissions in studied ecosystems. The box in the box plot indicates the data points between the 25th and 75th percentile, while whiskers show the range of all data points, excluding the outliers. The intersected line in the box represents the median, while the red dots represent the mean.

Erica-dominated ecosystems had a slightly higher median NH₄⁺ concentration of 14 mg N kg⁻¹ than fern-dominated ecosystems, with a median concentration of 12 mg N kg⁻¹. Additionally, the boxplot for erica-dominated ecosystems exhibited a wider range of NH₄⁺ concentrations, with the highest observed concentration reaching 39 mg N kg⁻¹, compared to fern-dominated ecosystems, where the highest concentration was 13 mg N kg⁻¹.

There was also a significant difference in soil nitrate concentration between erica-dominated and fern-dominated ecosystems (Figure 6). Erica-dominated ecosystems had a higher median soil nitrate concentration of 51 mg N kg⁻¹ than fern-dominated ecosystems, with a median concentration of 26 mg. Furthermore, the boxplot for erica-dominated ecosystems exhibited a wider range of soil nitrate concentrations, with the highest observed concentration reaching 124 mg N kg⁻¹, compared to fern-dominated ecosystems, where the highest concentration was 41 mg N kg⁻¹. Figure 4.2 shows the box plot of the soil nitrate and ammonium in the ecosystem.

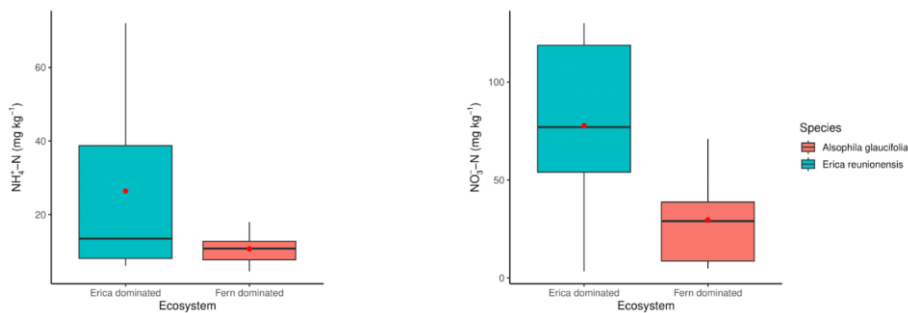
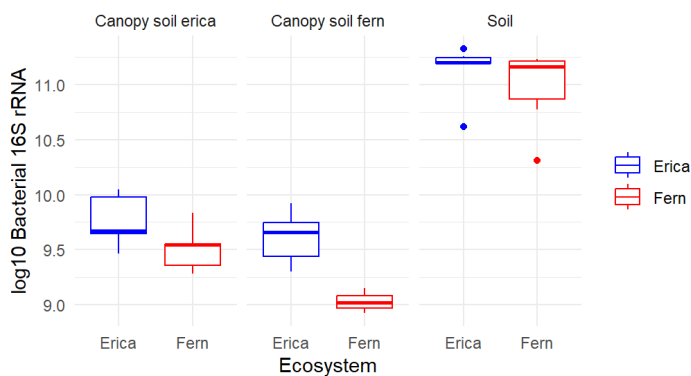


Figure 6. Variations in soil nitrate and soil ammonium by ecosystem. The box in the box plot indicates the data points between the 25th and 75th percentile, while whiskers show the range of all data points, excluding the outliers. The intersected line in the box represents the median, while the red dots represent the mean.

Erica-dominated ecosystems generally exhibited higher abundance levels across all ecosystem types regarding bacterial and archaeal communities (Figure 7). Bacterial 16S rRNA was consistently more abundant across all ecosystem types than archaeal 16S rRNA. Soil ecosystems, irrespective of vegetation dominance, displayed the highest bacterial 16S rRNA abundance. Additionally, soil ecosystems demonstrate considerable archaeal 16S rRNA abundance, with fern-dominated soil showing slightly higher values than erica-dominated soil.



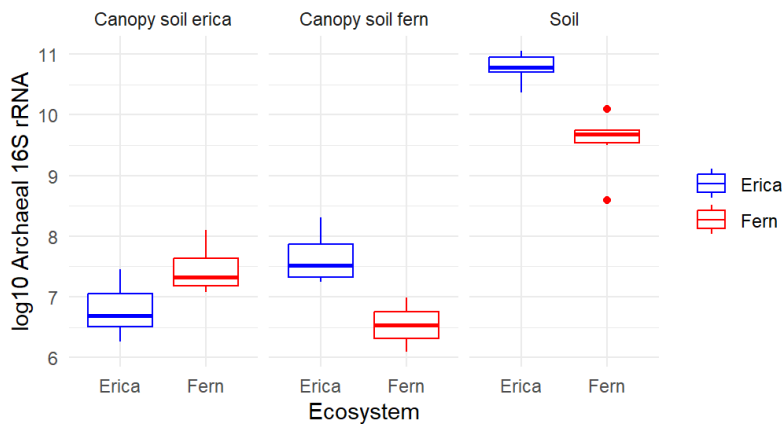


Figure 7. Abundances of bacterial and archaeal 16S rRNA genes. The box in the box plot indicates the data points between the 25th and 75th percentile, while whiskers show the range of all data points, excluding the outliers. The intersected line in the box represents the media

4.3. Abundances and proportions of functional genes in soil and canopy soil

The boxplot revealed significant differences in microbial marker abundances between the Erica and fern species of both canopy and soil ecosystems. Figure 8 compare the abundance of archaeal *amoA*, bacterial *amoA*, and COMAMMOX *amoA* across canopy soil in Erica, canopy soil in fern, and soil ecosystems. Bacterial *amoA* abundance exhibited higher values in Erica's canopy soil than in fern's. Soil ecosystems, particularly erica-dominated soil, displayed the highest bacterial *amoA* abundance. This suggests potentially higher levels of bacterial-mediated ammonia oxidation and nitrification in erica-dominated soil ecosystems. Additionally, variations in archaeal *amoA* abundance were noted between erica's canopy soil and fern's canopy soil, with slightly higher values observed in fern's canopy soil. Soil ecosystems, especially erica-dominated soil, exhibited the highest archaeal *amoA* abundance. Furthermore, for COMAMMOX *amoA*, both canopy soil ecosystems displayed similar values, while soil ecosystems, particularly erica-dominated soil, exhibited the highest values.

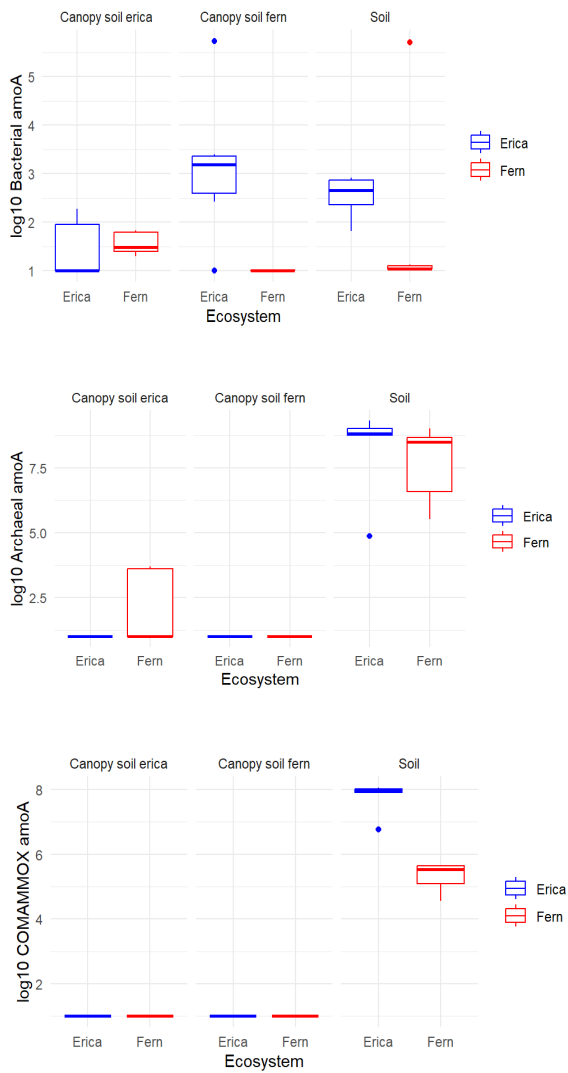


Figure 8. Abundances of bacterial, archaeal and COMAMMOX *amoA* genes. The box in the box plot indicates the data points between the 25th and 75th percentile, while whiskers show the range of all data points, excluding the outliers. The intersected line in the box represents the median.

In erica-dominated canopy soil, the proportion of *nirS* (%) (Figure 9) was lower compared to fern-dominated canopy soil. In fern-dominated canopy soils, erica and fern-dominated ecosystems exhibited low or negligible proportions. The soil of these ecosystems displayed a higher abundance of *nirS*. The abundance of *nirK* in the erica-dominated canopy soil was

significantly higher in the erica-dominated ecosystem compared to the fern ecosystem, while for soil ecosystems, *nirK* gene abundance was fairly comparable between the two vegetation types. In fern-dominated canopy soil, the fern ecosystem exhibited a higher significant abundance of fungal *nirK*; negligible proportions were displayed in the soil ecosystem. The highest abundance of *nosZ* was seen in the erica-dominated soil ecosystem while the lowest abundance was observed in the erica ecosystem of both erica-dominated canopy soil and fern-dominated canopy soils.

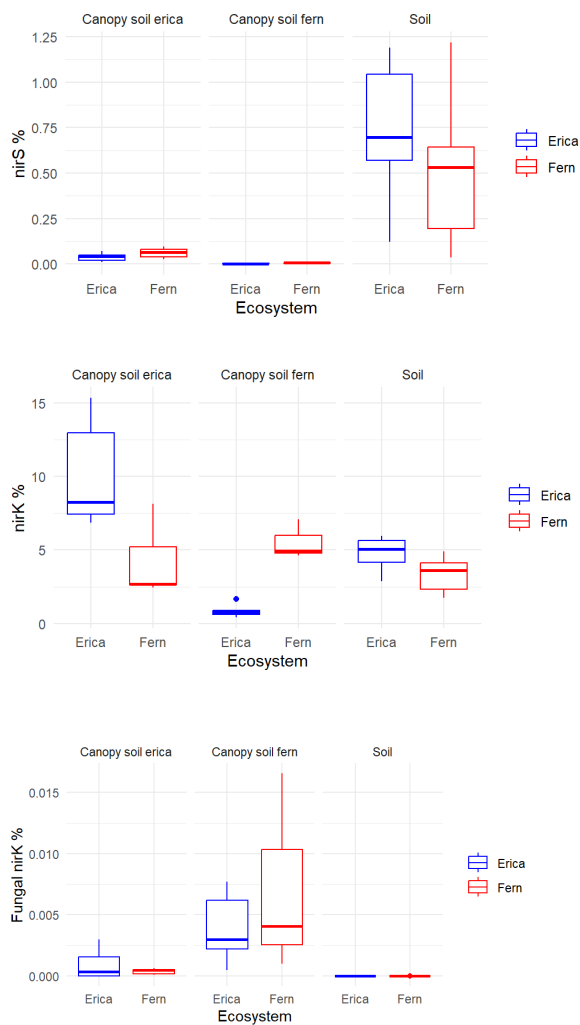
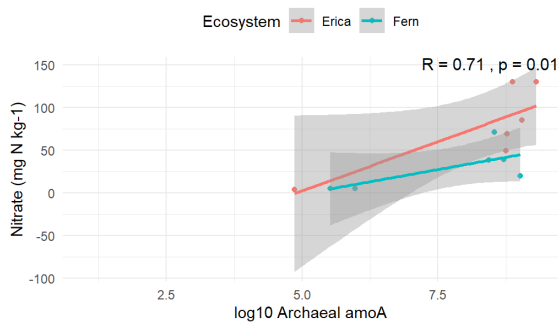
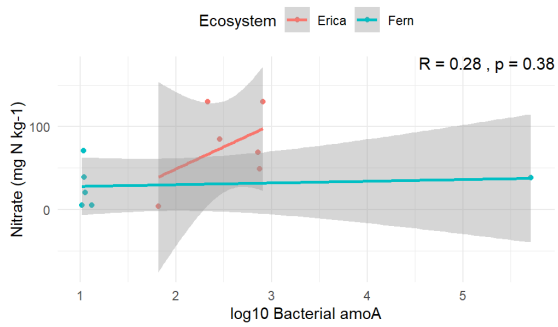


Figure 9. Abundances of *nirS*, *nirK*, fungal *nirK* and *nosZ*. The box in the box plot indicates the data points between the 25th and 75th percentile, while whiskers show the range of all data points, excluding the outliers. The intersected line in the box represents the median.

The result of the correlation analysis between soil nitrate concentration and the abundance of functional genes is shown in Figure 10. A weak positive correlation is observed between soil nitrate and bacterial *amoA*. In contrast, a strong positive correlation was found between soil nitrate and archaeal *amoA* and COMAMMOX *amoA*. The regression lines depicting the relationship between soil nitrate and bacterial *amoA* exhibit a steeper slope in erica-dominated soils compared to fern-dominated soils.



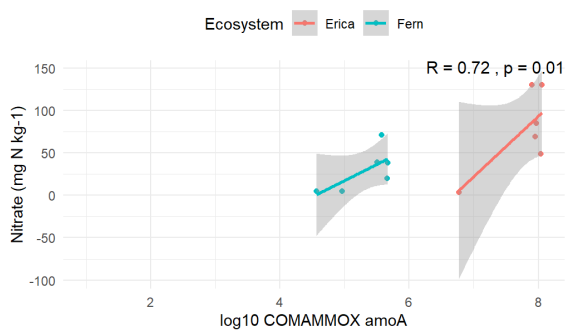


Figure 10. Correlation between soil nitrate and nitrification genes.

4.4. Relationship between gene abundances and N₂O emissions

Multivariate regression analysis between N₂O emissions and functional gene's abundances shows that only the coefficients of *nirS* and *nirK* were statistically significant ($p < 0.05$) (Figure 11), which indicates that these genes have a significant impact on N₂O flux, with *nirS* exhibiting a negative relationship and *nirK* showing a positive relationship, facilitating the reduction of NO₂⁻ to NO at the initial stages of denitrification. However, the coefficients for bacterial *amoA* and archaeal *amoA* are not statistically significant, suggesting that their abundance does not directly affect N₂O emissions in this analysis.

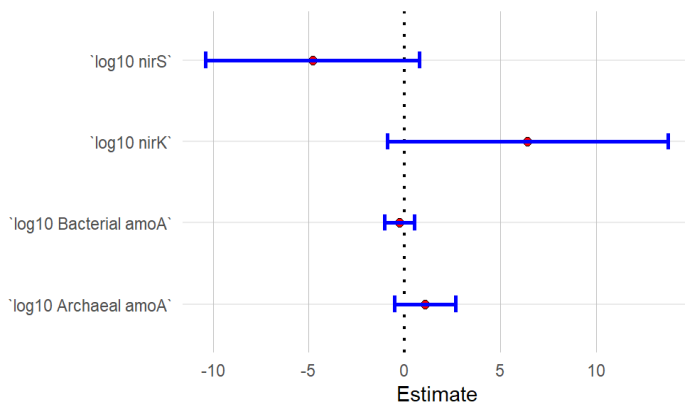


Figure 11. Coefficient plot from the multivariate regression analysis of N₂O emission on different functional genes.

5. Discussion

This study aimed to shed light on the relationships between microbial communities of soil and canopy soil and the nitrogen cycling processes in a sub-tropical cloud forest, primarily focusing on Réunion Island. Several key insights have emerged through comprehensive analyses of the soil's physicochemical parameters, functional gene distribution, and their correlation with soil nitrate concentrations and N₂O fluxes. Understanding this phenomenon in the N-rich soil of tropical cloud forests will help forecast and simulate future trends of N₂O emissions in climate change discussions.

Our results show variations in soil physicochemical parameters such as temperature and soil water content (SWC) across the studied ecosystems. Despite similar temperature ranges between the erica-dominated and fern-dominated ecosystems, the fern-dominated ecosystem tends to have a slightly higher mean of SWC than the erica-dominated ecosystem. This variability affects the availability of oxygen and other nutrients in the soil of both ecosystems, thereby influencing the dominance of the underlying microbial process (Kazmi et al., 2023). The pH from the soil ecosystem ranged from 3.8 to 4.6, which is acidic and impacts the activity of nitrifying and denitrifying microbes (Gieseke et al., 2006; Šimek & Cooper, 2002). At low pH, the *nirS* activity is reduced (Saleh-Lakha et al., 2009). Although nitrification tends to occur optimally at a pH between 7 and 8 (Tarre & Green, 2004), previous studies have demonstrated nitrification occurring at lower pH (Yaying et al., 2018). During this study, we observed notable differences in nutrient concentrations between erica-dominated and fern-dominated ecosystems, particularly with respect to NH₄⁺-N and NO₃⁻-N. The broader range of NH₄⁺-N in erica-dominated ecosystems suggests a more dynamic turnover by AOB and AOA, which uses the NH₄⁺-N to provide more substrate for their metabolic activity. This enhances ammonia oxidation rates, the first step in the nitrification process. Similarly, the higher median concentration of NO₃⁻-N in the erica-dominated ecosystem (Figure 6), suggests active nitrification as NO₃⁻-N is the end product of the nitrification process. The observed temperature range in erica-dominated and fern-dominated ecosystems is moderate enough to favor the activity of AOB and AOA. The range of the SWC indicates that the soil remains adequately moist, providing the necessary aeration for nitrifying microbes. Nutrient availability and a suitable environment for microbial activity increase the soil's N₂O emission potential (Highton et al., 2023). The wider range for N₂O flux values in erica-dominated ecosystems indicates more variability in N₂O emissions. Additionally, increased SWC in erica-dominated ecosystems compared to fern-dominated ecosystems leads to minor production of N₂O, creating conditions for nitrification and incomplete denitrification. Previous studies have noted a comparable rise in N₂O emissions as SWC increases (Lin & Hernandez-Ramirez, 2022).

Distinct patterns in microbial abundances and functional gene distributions between erica- and fern-dominated canopy soils, as well as in their respective soil ecosystems, were observed in our study. Erica-dominated canopy soils and the soil ecosystems exhibited a higher abundance of bacterial *amoA* genes, indicating a higher potential for ammonia oxidation and nitrification processes. Previous studies have highlighted the numerical abundance and transcriptional activity of certain microbial genes with nitrification potential (Leininger et al., 2006). However,

despite their prevalence, it remains to be conclusively demonstrated whether these microbes also dominate in terms of their nitrification activities. Similar to bacterial *amoA*, archaeal *amoA* gene abundance was also higher in the erica-dominated canopy and soil ecosystems than in fern-dominated canopy and soil ecosystems. The lower abundance in fern-dominated ecosystems reflects a lesser role of AOA in ammonia oxidation in these ecosystems compared to erica-dominated soils. Hence, more bacterial-mediated functional genes are active in the ammonia oxidation step of nitrification processes. The enhanced nitrification capacity of our studied ecosystem genes was further supported by the presence of COMMAMOX *amoA*, which was more prevalent in the erica-dominated canopy and soil ecosystems.

A significant abundance of denitrifiers (*nirS* and *nirK*) was also observed. In canopy soils, the proportion of *nirS* is quite lower in the erica-dominated canopy soil compared to fern-dominated canopy soil. This indicates that the denitrifier communities responsible for reducing NO_2^- to NO through the *nirS* pathway are less prevalent in the erica-dominated canopy soils. Contrastingly, fern-dominated canopy soils revealed a higher proportion of *nirS*. Despite the differences in canopy soils, both erica- and fern-dominated ecosystems exhibited high abundances of *nirS* genes in their soils, which suggests that regardless of the canopy type, the soil layer itself favors significantly microbial activities for the reduction of NO_2^- to NO, a very important stage of denitrification. The *nirK* gene abundance was also significantly higher in the fern-dominated ecosystem, which points to the dominant role of *nirK*-type denitrifiers in the fern ecosystem. Previous studies have also highlighted this dominance of *nirK* gene abundance (Clark et al., 2012). Furthermore, within the fern-dominated ecosystem, a higher abundance of fungal *nirK* was noted, highlighting the importance of fungal communities in denitrification. The higher abundance of *nirK* in erica-dominated canopy soils correlates with higher maximum N_2O . The variation in the abundance of *nosZ* also reveals the potential that exists in the reduction of N_2O to N_2 which is a great factor in mitigating greenhouse gas emissions. The higher *nosZ* abundance in our study, as observed in the canopy soils of both erica fern-dominated ecosystems, suggests why these ecosystems displayed an increased capacity for N_2O , resulting in lower N_2O emissions from these sites. The moderate SWC supported this observation as no flooded condition, which creates an anaerobic condition that allows N_2O reducers to thrive, was seen across both sites of erica and fern-dominated ecosystems.

The weak positive correlation between soil NO_3^- and bacterial *amoA* indicates a modest contribution of bacterial ammonia oxidation to NO_3^- production, suggesting that although these bacteria are present, their role is not dominant. In contrast, we observed a strong positive correlation between soil NO_3^- and archaeal and COMMAMOX *amoA*, indicating that these functional microbes are the key drivers for NO_3^- accumulation in the soil. The strong positive correlation between soil NO_3^- concentrations and the abundance or activity of archaeal and COMMAMOX *amoA* genes have been corroborated by previous studies (Leininger et al., 2006). Higher NO_3^- particularly predominant in the erica-dominated soil ecosystem, stimulates the microbial communities involved in denitrification. The significant correlation between soil NO_3^- and *nosZI* suggests an enhanced capacity for complete denitrification.

The interactions between microbial abundance, nitrate, soil ammonium, and N₂O fluxes suggest that nitrification and denitrification were active in our study. The nitrification process is more active in the erica-dominated canopy and soil ecosystems due to the higher abundance of bacterial and archaeal *amoA* and the presence of COMMAMOX *amoA*. Although there is a significant abundance of fungal *nirK* in fern-dominated canopy soils, the overall denitrification activity is lower compared to erica-dominated canopy soils.

Conclusion

This study found significant relationships and complex interplay between soil temperature, SWC, and nutrient concentrations in shaping the nitrogen cycling processes within the tropical cloud forest soils and canopy soils of Réunion island. Variations in SWC and temperature across the erica and fern-dominated ecosystem greatly influence nutrient availability and microbial activities. Notably, the erica-dominated canopy and soil ecosystem displayed higher concentrations and broader ranges of NH₄⁺-N and NO₃-N, indicating a robust turnover and higher nitrification activity, which are mediated by increased abundance of AOA, AOB and COMMAMOX *amoA*. The denitrification process was also more prominent in both ecosystems with higher abundances of *nirS* and *nirK*. *nirK* played the dominant role in the fern ecosystem. The abundance of *nosZ* highest in the erica-dominated ecosystem showed a greater capacity for N₂O mitigation. In addition, the higher abundances of fungal *nirK* predominant in the fern-dominated ecosystems also emphasize the role of fungi in the denitrification process within these ecosystems.

Although the N₂O emissions in our study were less based on the values of nitrate and ammonium observed, N₂O fluxes were compared between erica-dominated and fern-dominated ecosystems. The higher observed N₂O fluxes in fern-dominated ecosystems showed that, on average, fern-dominated ecosystems release more N₂O into the atmosphere than erica-dominated ecosystems. On the other hand, a wider range of N₂O in erica-dominated ecosystems suggests greater variability in N₂O emission within these ecosystems. This implies that while erica-dominated ecosystems may not consistently emit as much N₂O as fern-dominated ecosystems on average, they can experience occasional spikes in N₂O emissions, leading to the observed wider range of N₂O flux values. Overall, the soils under study were found to be weak sources of N₂O.

Future research should investigate the mechanisms of N₂O fluxes in erica and fern-dominated ecosystems and the microbial dynamics driving the observed differences in the nitrification and denitrification pathways. Further research would deepen our understanding of the complex relationships between microbial communities, nitrogen cycling processes, and environmental factors influencing N₂O emissions in the sub-tropical cloud forests and shape our approach to mitigating greenhouse gas emissions and combating climate change.

Summary

Cloud forests, often situated at high altitudes in tropical and subtropical regions, play essential roles in global biodiversity and climate regulation through their immense interaction with water, carbon, energy, and nutrients within the global atmosphere. These ecosystems are characterized by persistent cloud cover that maintains high moisture levels and nutrient supply that fosters the growth of plants and diverse microbial communities. The nitrogen cycle within these forests is particularly significant as it involves complex interactions between soil, plants, and microorganisms, which are essential for nutrient availability and overall ecosystem productivity. Microbial processes, including nitrification and denitrification, play crucial roles in the soil's transformation and movement of nitrogen compounds. These processes affect plant growth and soil health and contribute immensely to the emissions of potent greenhouse gases like nitrous oxide.

In this master's thesis, we conducted an in-depth analysis of microbial communities and nitrogen cycling processes within sub-tropical cloud forest ecosystems on Réunion Island, a French overseas territory in the Indian Ocean. Known for its unique geographical features and diverse ecosystems, Réunion Island provides an ideal setting for exploring the intricate interactions between microbial communities, environmental factors, and nutrient cycling processes, particularly nitrogen cycling processes that lead to N₂O emissions.

Our study area, the Plaine des cafres on Réunion Island, was carefully selected due to its distinct ecological features characterized by the dominance of tree species; *Alsophila glaucifolia* and *Erica reunionensis*. These two sub-sites, Palmiste 1 and Palmiste 2, both situated at an elevation of 1500 - 1600m above sea level, provided contrasting vegetation types and environmental conditions.

Our research questions centered on understanding the relationships between microbial communities, nitrogen cycling processes, and environmental factors in sub-tropical forests. We aimed to answer these questions by investigating microbial abundances, functional gene distributions, soil physicochemical parameters, and N₂O emissions across vegetation types and environmental conditions.

Previous studies have examined microbial-mediated nitrogen cycling processes in several terrestrial ecosystems like sub-tropical peatlands, wetlands, and significant ecological sites. These past studies have helped in providing valuable context and theoretical frameworks for this study, guiding our methodologies and results interpretations.

Methodologically, we employed a robust approach to sample collection, DNA extraction, quantitative PCR, gas measurements, and statistical analyses. Soil and canopy soils were carefully collected, processed, and stored properly at an appropriate condition for further analysis of their physicochemical parameters, microbial abundances, and functional gene distributions. Gas sampling using polyvinyl chloride chambers was used to quantify N₂O fluxes.

Our results showed significant variations in SWC and temperature across the erica and fern-dominated ecosystems, greatly influencing nutrient availability and microbial activity. We observed that erica-dominated ecosystems exhibited higher $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ concentrations, indicative of increased nitrification activity mediated by the increased abundances of AOB, AOA, and COMMAMAX *amoA*. Denitrification processes characterized by *nirS* and *nirK* gene abundances were also prominent in both ecosystems, with a notable role of fungal *nirK* in the fern ecosystem. The higher *nosZ* gene abundance in erica-dominated soils suggests a greater capacity for N_2O reduction, mitigating greenhouse gas emissions. Despite lower average N_2O fluxes, erica-dominated ecosystems showed greater variability in N_2O emissions compared to fern-dominated ecosystems. This study underscores the need for further research to elucidate the mechanisms driving N_2O fluxes and microbial dynamics. This is critical for developing strategies to mitigate greenhouse gas emissions in tropical cloud forests.

Kokkuvõte

Pilvemetsad, mis asuvad sageli troopilistes ja subtroopilistes piirkondades kõrgel, mängivad ülemaailmses bioloogilises mitmekesisuses ja kliimaregulatsioonis olulist rolli, kuna neil on tohutu koostoime vee, süsiniku, energia ja toitainetega globaalses atmosfääris. Neid ökosüsteeme iseloomustab püsiv pilvkate, mis säilitab kõrge niiskustaseme ja toitainetega varustatuse, mis soodustab taimede ja mitmekesiste mikroobikoosluste kasvu. Nende metsade lämmastikuringe on eriti oluline, kuna see hõlmab keerulisi koostoimeid pinnase, taimede ja mikroorganismide vahel, mis on toitainete kättesaadavuse ja ökosüsteemi üldise tootlikkuse jaoks olulised. Mikroobsed protsessid, sealhulgas nitrifikatsioon ja denitrifikatsioon, mängivad otsustavat rolli mulla muundumisel ja lämmastikuühendite liikumisel. Need protsessid mõjutavad taimede kasvu ja mulla tervist ning aitavad tohutult kaasa tugevate kasvuhooenergiaasid, nagu dilämmastikoksiidi, emissioonile.

Selles magistritöös analüüsisime põhjalikult mikroobikooslusi ja lämmastiku ringluse protsesse subtroopiliste pilvemetsade ökosüsteemides Réunioni saarel, Prantsusmaa ülemereterritooriumil India ookeanis. Tuntud oma ainulaadsete geograafiliste tunnuste ja mitmekesiste ökosüsteemide poolest. Réunioni saar pakub ideaalset keskkonda mikroobikoosluste, keskkonnategurite ja toitainete ringlusprotsesside, eriti lämmastiku ringlusprotsesside, mis põhjustavad N_2O heitkoguseid, vahelisi keerulisi koostoimeid.

Uurimisala, Plaine des cafres Réunioni saarel, valiti hoolikalt selle eristuvate ökoloogiliste tunnuste tõttu, mida iseloomustab puuliikide domineerimine; *Alsophila glaucifolia* ja *Erica reunionensis*. Need kaks alakohta, Palmiste 1 ja Palmiste 2, mis asuvad mõlemad 1500–1600 m kõrgusel merepinnast, pakkusid vastandlikke taimestikutüüpe ja keskkonnatingimusi.

Uurimisküsimused keskendusid subtroopiliste metsade mikroobikoosluste, lämmastiku ringlusprotsesside ja keskkonnategurite vaheliste suhete mõistmisele. Nendele küsimustele püüdsime vastata, uurides mikroobide arvukust, funktsionaalseid geenide jaotusi, mulla füüsikalisi-keemilisi parameetreid ja N_2O heitkoguseid taimestikutüüpide ja keskkonnatingimuste lõikes.

Varasemates uuringutes on uuritud mikroobide vahendatud lämmastiku ringlusprotsesse mitmetes maismaaökosüsteemides, nagu subtroopilised turbaalad, märgalad ja olulised

ökoloogilised alad. Need varasemad uuringud on aidanud luua selle uuringu jaoks väärtuslikku konteksti ja teoreetilised raamistikud, mis on suunanud meie meetodikat ja tulemuste tõlgendamist.

Metodoloogiliselt kasutasime proovide kogumisel, DNA ekstraheerimisel, kvantitatiivsel PCR-il, gaasi mõõtmisel ja statistilistel analüüsidel lähenemisviisi. Pinnase ja võramullad koguti hoolikalt, töödeldi ja säilitati nõuetekohaselt sobivates tingimustes nende füüsikalise-keemiliste parameetrite, mikroobide arvukuse ja funktsionaalse geenijaotuse edasiseks analüüsimiseks. N₂O voogude kvantifitseerimiseks kasutati gaasiproovide võtmist polüvinüülkloriidkambrite abil.

Tulemused näitasid olulisi erinevusi SWC-s ja temperatuuris Erica ja sõnajalgade domineeritud ökosüsteemides, mõjutades oluliselt toitainete kättesaadavust ja mikroobide aktiivsust. Täheleandmatusena, et Erica domineeritud ökosüsteemides oli kõrgem NH₄⁺-N ja NO₃⁻-N kontsentratsioon, mis näitab suurenenud nitrifikatsiooniaktiivsust, mida vahendab AOB, AOA ja COMMAMAX *amoA* suurenenud arvukus. Denitrifikatsiooniprotsessid, mida iseloomustasid *nirS* ja *nirK* geenide arvukus, olid samuti mõlemas ökosüsteemis silmapaistvad, kusjuures seente *nirK* roll sõnajala ökosüsteemis oli märkimisväärne. Suurem *nosZ* geeni arvukus Erica domineeritud muldades viitab suuremale N₂O vähendamise võimele, mis vähendab kasvuhoonegaaside heitkoguseid. Vaatamata väiksematele keskmistele N₂O voogudele näitasid Erica domineeritud ökosüsteemides N₂O heitkogused suuremat varieeruvust võrreldes sõnajalgade domineeritud ökosüsteemidega. See uuring rõhutab vajadust täiendavate uuringute järele, et selgitada välja mehhanismid, mis juhivad N₂O vooge ja mikroobide dünaamikat. See on kriitilise tähtsusega strateegiate väljatöötamiseks kasvuhoonegaaside heitkoguste vähendamiseks troopilistes pilvemetsades.

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