Impact of nitrogen fertilization on the soil microbiome and nitrous oxide emissions

Cassman, N.A.

Chapter 1

General Introduction

Introduction

Nitrogen is an essential component of living systems. Nitrogen is abundant in the atmosphere as inert di-nitrogen gas and its transformation to bioavailable forms is limited by biological nitrogen fixation ($N_2 \rightarrow NH_3$; Canfield et al 2010). This latter process is carried out by a few genera of microbes and as the rate of conversion is slow, natural ecosystems are often N-limited (Kuypers et al 2018). The invention of synthetic nitrogen fixation by Fritz Haber (now called the Haber-Bosch process) in 1909 led to the mass production and widespread use of nitrogen fertilizers and corresponding high yields in agriculture in the next century (Ellis 2011). The agricultural boom of the past century has substantially attributed to a seven-fold increase in the human population, which is now over seven billion (Galloway et al 2008). About half of the world's population relies on food grown using synthetic N (Erisman et al 2008). This increased input of N fertilizers into agricultural systems has had serious impacts beyond increasing food productivity, including long-term decreases in biodiversity, in soil quality, waterway eutrophication and acidification, and greenhouse gas emissions (Foley et al 2011, Fowler et al 2015, Smith 2017).

As only about 50% of the input N to agricultural soils is used by plants, the excess nitrogen is leached out of the soil matrix and into the air and surrounding water sources, resulting in an imbalance of nitrogen in surrounding ecosystems which contributes to the degradation of surface and groundwater quality (Schlesinger et al 2009, Erisman et al 2013). Moreover, N transformations in the soil matrix include processes resulting in the greenhouse gases NO and N_2O. Before 2050, global food production is expected to double to feed the projected human population of 9 billion people (Godfray et al 2010, Tilman et al 2011). Updating nitrogen fertilizer management strategies toward long-term sustainability without decreasing crop productivity is therefore of global importance. This requires deep knowledge of the soil system, especially regarding the effects of nitrogen fertilizers on the soil microbes, which are the main players in nutrient cycling, litter decomposition and energy flows in terrestrial and agroecosystems (Baggs 2011; Hu et al 2014b). While the astronomical diversity of soil microbes has hampered detailed study of the soil microbiome, the recent advances in sequencing technology have allowed for an unprecedented glimpse into the "black box" of the microbial role in soil functioning (Torsvik et al 1990, Fierer et al 2012). Here, the overall research aim was to apply next-generation sequencing technology and associated advanced data analyses to gain detailed insight into the responses of soil microbial communities to various nitrogen fertilizer regimes,

including long term fertilization, with a focus on the potentially N_2O-producing microbial community.

1.1 Nitrous oxide emissions as a function of N fertilizer input

Reactive nitrogen generally is supplied to agricultural soils in the form of ammonium-based fertilizers, such as urea ($CO(NH_2)_2$), ammonium nitrate (NH_4NO_3), ammonium sulphate ($(NH_4)_2SO_4$) and synthetic ammonia (NH_3; Mosier 1994). About 220 Tg N yr^{-1} of nitrogen fertilizers are applied to agricultural soils globally, of which about half is lost into groundwater as soluble NO_x or as gaseous NO_x species (Gruber & Galloway 2008, Fowler et al 2015). This follows the conceptual "hole-in-the-pipe" model, also known as the nitrogen cascade, which describes soil nitrogen transformations as limited by the availability of reactive nitrogen, which then "leak" through a cascade of reactions (Galloway et al 2003). Roughly 18.8 Tg of $N\text{-}N_2O$ are emitted per year, with agricultural soils directly contributing to 16% of these emissions (Syakila & Kroeze 2011, Smith 2017). A general rule is to consider that 1% of applied fertilizer N is emitted as N_2O based on a rough estimation by IPCC (2007). However, recent studies show that this value may fluctuate from 0.2 to 4% depending on many factors, including site, soil type and management (Carmo et al 2013, Filoso et al 2015). Nitrous oxide emissions threaten the global climate because N_2O has a global warming potential 298 times that of CO_2 due to its radiative forcing and long presence (114 years/molecule) in the atmosphere (Robertson & Vitousek 2009, Snyder et al 2009). Further, once in the atmosphere it is converted to NO which reacts with tropospheric ozone; this implicates N_2O as a major ozone-depleting substance (Ravishankara et al 2009). Efforts to develop N_2O mitigation strategies focus on efficiency in N fertilizer utilization and more recently on identifying the controls and mechanisms of N_2O emissions, including the microbial role (Signor and Cerri 2013, Butterbach-Bahl et al 2013, Soares et al 2016, Pitombo et al 2016, Galloway et al 2017, Bakken & Frostegård 2017, Lourenco et al 2018, Kuypers et al 2018).

Nitrous oxide emissions from agricultural soils are mainly attributed to the cumulative effects of the biotic pathways nitrification and denitrification (Butterbach-Bahl 2013). Nitrification is the two-step oxidation of NH_4^+ to NO_2^- and NO_2^- to NO_3^-, in which N_2O is an intermediate, while denitrification is the sequential reduction of NO_3^-, NO_2^-, NO, N_2O and N_2 in which N_2O is a product of NO reduction and a reactant of N_2O reduction to N_2 (Baggs et al 2011). In ammonia oxidation, the rate-limiting step is ammonia oxidation to hydroxylamine, which is generally catalyzed by ammonia monooxygenase and encoded by the gene amoA. The other main biotic pathway leading to N_2O, denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow$

$NO \rightarrow N_2O \rightarrow N_2$), is catalyzed by a series of enzymes which are encoded by different genes (Hu et al 2015). The first step ($NO_3^- \rightarrow NO_2^-$) is carried out by the enzyme nitrate reductase, which is encoded by the *narG* or *napA* gene; the second step ($NO_2^- \rightarrow NO$) can be catalyzed by two types of nitrite reductases encoded respectively by the the nirK or nirS genes. The third step ($NO \rightarrow N_2O$) is carried out by the genes cnorB or qnorB; last, the enzyme nitric oxide reductase catalyzes the reduction of N_2O ($N_2O \rightarrow N_2$) and is encoded by the nosZ gene, which exists in two forms (nosZ I and II). The relative contributions of nitrification and denitrification to overall N_2O production are challenging to untangle due to the many interrelated reactions and microbes with overlapping function (Zhu et al 2013; Shcherbak, Millar and Robertson 2014). Other sources of N_2O emissions are denitrification by nitrifiers (nitrifier denitrification), anaerobic ammonium oxidation (anammox), complete nitrification (comammox) and dissimilatory nitrate reduction to ammonium (DNRA, or nitrate ammonification (Hu et al 2015, Kuypers et al 2018). However, due to the main contributions of nitrification and denitrification to N_2O emissions in agriculture, in the current research the focus was on nitrification and denitrification.

1.2 Nitrification and denitrification

Nitrification and denitrification are mediated by microbes (archaea, bacteria and fungi) which use these pathways to gain energy or assimilate N. Nitrifiers encompass a narrow phylogenetic range of a few bacterial and archaeal genera. Ammonia oxidation is mediated by the ammonia-oxidizing archaea (AOA), such as the Thaumarchaeota *Nitrososphaera*, and the ammonia-oxidizing bacteria (AOB), such as the Betaproteobacteria *Nitrosomonas* and *Nitrosospira*; Upon ammonium oxidation, nitrite can be formed which can be further oxidized by nitrite oxidizing bacteria (NOB), including the Nitrospirae *Nitrospira* and the Alphaproteobacteria *Nitrobacter*. Further, the process of complete nitrification by the recently discovered comammox bacteria, which have so far been found in the NOB *Nitrospira* genus, might also contribute to N_2O emissions (Liu et al 2017). Comammox bacterial genomes have revealed the full set of nitrification genes, that is for ammonia oxidation ($NH_3 \rightarrow NH_2OH$, *amoA*) and hydroxylamine oxidation (NH_2OH, *hao*), as well as the genes for nitrite oxidation ($NO_2^- \rightarrow NO_3^-$, *nxrB*; Daims et al 2015; van Kessel et al 2015; Camejo et al 2017). Both ammonia and nitrite oxidation is an obligately aerobic process, with nitrifiers being chemolitho-heterotrophic and -autotrophic.

Denitrification is a facultative anaerobic process carried out by microorganisms widely dispersed over the bacterial, archaeal and fungal domains, and deni-

trification genes can also be carried by nitrifiers in what is termed nitrifier denitrification. Some denitrifiers contain the full suite of denitrification genes and are able to reduce NO_3^- to N_2; these are known as full denitrifiers. Others contain a truncated set of denitrification pathway genes and may produce one of the intermediates, such as NO (which is rapidly converted to N_2O) or N_2O. The genetic potential of the denitrification community for full or incomplete denitrification is directly linked to the N_2O or N_2 output of the soil. A community with a higher proportion of n*osZ* to *norB* or n*irS* + n*irK* (full denitrifiers) may present a sink for N_2O (Jones, Graf et al 2013). In support, Philippot et al (2011) found increased N_2O emissions from soils when increasing dilutions of bacteria lacking *NosZ* were added to microcosms. Further, recent studies provided evidence for this as well (Domeignoz−Horta 2015 and 2018); for example, as the addition of non-denitrifier *nosZII*-containing bacteria in microcosms was linked to lower N_2O emissions (Domeingoz−Horta 2016). Thus, the overall genetic potential of a nitrifying or denitrifying community, along with environmental controls, impacts the amount of N_2O emitted.

1.3 Management factors influencing soil microbial nitrifiers and denitrifiers

The proximal, or immediate and short-term, factors influencing nitrifier and denitrifiers are carbon availability, NO_3^- concentrations, moisture levels and oxygen availability, while distal, or indirect, and long-term factors are plant growth, micronutrient availability, and pH (Hénault 2012 and Saggar, Jha et al 2013). When N fertilizers are applied, microbial decomposition can be increased or decreased, depending on recalcitrance of the organic substrate and N availability. Application of plant residues with low C:N ratios often result in high rates of N mineralization, or the conversion of organic N to plant-available NH_3 (usually by microbial death), while residues with higher C:N ratios stimulate N immobilization into microbial biomass (reviewed in Chen et al 2014). Soil organic matter directly affects N_2O production because it provides a diverse suite of substrates for heterotrophic denitrifier activity (Schmidt & Torn et al 2011). For instance, soluble sugars, or labile carbon, can easily dissolve into the water-filled spaces in soil and become available for microbial or plant uptake. In contrast, the insoluble compound lignin requires specialized microbial enzymes for degradation and otherwise remains in the soil as soil organic matter (SOM) (Swift et al 1979). Additionally, rapid decomposition can drive down oxygen levels faster than the rate of oxygen diffusion, establishing anaerobic conditions for denitrification. Parkin (1987) showed that the frequencies of N_2O emissions correlate with predictions based on the spatially heterogeneous distribution of organic compounds that are

found in soils. Nitrite levels control the nitrification and denitrification processes as it is a reaction intermediate and reactant, respectively. Therefore, N and organic matter additions -- such as in agricultural management practices of fertilization with N and plant residues -- can either promote or reduce N_2O production by their effect on the factors controlling the activity and growth of nitrifiers and denitrifiers.

1.4 Sugarcane agriculture

The N_2O emissions of sugarcane production cycles has recently drawn attention due to the use of sugarcane bioethanol as a sustainable biofuel (Crutzen et al 2008, Lisboa et al 2011, Seabra et al 2011). The largest producer of sugarcane, *Saccharum sp.*, is Brazil, which devotes almost 7.5 million hectares to sugarcane production mainly for its use as a biofuel (Christofoletti et al 2013). Sustainability of sugarcane production stems partly from the crop characteristics and partly because of efficiency in its production. After the sugarcane stalk is cut during a harvest, the regrowth yields another crop, known as the ratoon crop, during the following harvest season. The growth from the ratoon crop decreases each year, which warrants replanting of the plant crop every three to eight years, without tilling the soil in the intervening years, and this promotes SOM formation. Historically, sugarcane leaves were burned to remove the plant leaves from the sugar-containing stalks prior to harvest. Now, most Brazilian sugarcane is harvested using a 'green harvest' method in which the stalks are stripped of leaves and this so-called "straw" is left on the field (Carvalho et al 2017). The amount of dry sugarcane straw on fields in Brazil ranges between 8–30 Mg ha^{-1} dry mass of straw (Carvalho et al 2017). The green harvest method has several advantages over the burning method, namely, that application of the residues increases moisture retention and provides a long-term source of nutrients (Carvalho et al 2017) and contributes to overall lower greenhouse gas emissions (Capaz 2013). Depending on the cultivar and the conditions in which it was grown, sugarcane leaves have a C:N of roughly 125:1, which is relatively high (Carvalho et al 2017). Decomposition of plant residues with C:N of above 30 generally promotes N immobilization, or the uptake of available soil-borne N into microbial biomass. This immobilized N can turn into soil organic N following microbial death, which serves as a long-term source of N to subsequent crops (Otto et al 2013). Application of crop residues with high C:N content, such as sugarcane leaves, may lead to microbial decomposers using soil organic N for their N needs, ultimately lowering the soil N pool, unless combined with an N fertilization regime (Trivelin et al 2013, Ferreira et al 2015).

1.5 Sugarcane bioethanol and vinasse production

In the bioethanol production cycle, the sugarcane stalk is crushed, and the sugarcane juice is separated from the pulpy stalk residue. Sugarcane juice is heated, clarified with lime and cooled to crystallize sugar and molasses. The molasses is further fermented and heated to produce bioethanol and the waste product, vinasse. Up to 13 L of vinasse per liter of bioethanol may be generated (Boddey et al 2008). Essentially all of the vinasse is recycled onto the sugarcane fields as a K fertilizer source according to Brazilian agricultural practices (Moran-Salazar et al 2016). Vinasse is comprised of about 93% water and organic acids, solids and nutrients such as magnesium, calcium and potassium (Christofoletti et al 2013). It is effective as a K and P fertilizer (Moran-Salazar et al 2016) and is also used in animal feed and as a source of biogas (Christofoletti et al 2013). Benefits of using vinasse as fertilizer include improved soil quality due to the addition of moisture and micronutrients (Jiang et al 2012) and improved crop production and crop quality (Yi-Ding et al 2006, Zani et al 2018). However, when vinasse is used in conjunction with an N fertilizer, potentially detrimental effects on long-term soil fertility and greenhouse gas emissions have been observed, especially the emission of N_2O and reduction of soil C stocks due to the addition of labile C from vinasse (Fuess et al 2017, Pitombo et al 2016, do Carmo et al 2013). These negative consequences might outweigh the benefits of sugarcane bioethanol as an energy source (Lapola et al 2010, Erisman et al 2010). Further, microbial contaminants of the bioethanol process are thought to be present in vinasse (Costa et al 2015) with unknown effects on the soil microbiome upon fertilization.

1.6 Insight into microbial communities through sequencing

The soil matrix contains an astronomical number and diversity of microorganisms, which can reach up to 10^{13} cells and contain between 10^4-10^9 genotypes in one gram of soil (Torsvik and Øvreås 2002). This great diversity is a challenge to study, not least because the majority of soil microbes are unculturable. Recent advances in high-throughput sequencing technologies and computational methods, largely driven by the less diverse microbial communities of the marine and human gut environments, have enabled scientists to begin tackling the soil ecosystem (Zhou et al 2015). Briefly, a comparative metagenomics study encompasses experimental design, DNA or RNA extraction from environmental samples, sequencing, quality control of the reads, followed by taxonomic and/or functional potential identification of the reads and statistical analysis to address hypotheses.

The data subjected to the statistical analyses generally come in the form of taxonomic or functional profiles. Multivariate statistics are then applied, e.g. to identify taxa differing between groups of samples, or to find the most represented metabolic pathways in a metagenome. Several molecular methods are used to generate this data, including amplicon of phylogenetic markers or functional genes and shotgun metagenomics (Luo et al 2014, Orellana et al 2017).

The PCR of phylogenetic markers from microbial DNA in soil samples allows for the surveying of the taxonomic composition and diversity of soil microbial communities (Pace 1997, Huse et al 2008). Generally, the 16S rRNA gene is used to profile the bacterial and archaeal community while the 18S rRNA gene and/or ITS region are used for eukaryotes, including fungi. Advantages to using this method are lower cost per sample and the availability of large databases of marker genes representing sequences from millions of species. However, this strategy, so-called amplicon metagenomics, is limited by the conservation of the primers used, which can miss highly novel, divergent sequences as well as viruses; further, only taxonomic information is obtained (Logares et al 2014). Regarding the latter, several bioinformatic analysis methods have tackled gaining functional information by matching 16S taxonomy information to the functional potential of similar genomes, for example Picrust and Tax4Fun (Langille et al 2013 and Aßhauer et al 2015). These tools depend on prior knowledge of full genomes in the reference databases, which might limit the accounting of the true functional diversity of the sample. Further, the precision of reference-based methods depend on which lineages are represented in the databases.

Similar to the information derived from amplicon metagenomics, PCR of functional markers can reveal taxonomic and diversity information about a functional subgroup of the soil microbial community, e.g. the amplification and sequencing of the *amoA* gene gives insight into the ammonia-oxidizing bacterial community (Ouyang et al 2016). As functional amplicon metagenome techniques are limited to revealing relative abundances of taxa in the sample, these surveys can be supplemented by alternatives to measuring microbial biomass, such as real-time PCR, which is a quantitative method for measuring the number of copies of a gene, as a proxy for the number of cells, in a sample. The FUNGENE database is one such tool that provides a platform for functional amplicon metagenomic analysis and includes databases and Hidden Markov Models (HMMs) of a range of functional genes, including the main genes involved in nitrification (*amoA*) and denitrification (*nirS*, *nirK*, *nosZ*; Fish et al 2013). Further, the database dbCAN provides a stand-alone database for the analysis of genes encoding for enzymes involved in carbohydrate metabolism (Zhang et al 2018).

Functional potential as well as taxonomic information can be derived from shotgun metagenomes, which are genomic sequences derived from all the cells in a sample (Thomas et al 2012). Function is inferred by translating the sequences through a gene predictor followed by homology searching against a protein sequence or protein family database. Common databases for functional potential analysis include the Kyoto Encyclopedia of Genes and Genomes (KEGG), in which the genes are cross-referenced into metabolic pathways, and the protein family database (Pfam), in which protein domains are represented as HMMs (Kanehisa et al 2014, Finn et al 2016). The model organism E. coli, humans and the human gut microbiome only have 90%, 82% and 75% functionally annotated genes, respectively. In a complex, less-studied environment such as soil, the percentage of functionally annotated genes may further drop to 55% (Prakash & Taylor 2012). There are several widely used platforms for metagenomic analysis, including the MG-RAST and EBI platforms which allow users to upload and store data and to run their samples through automated pipelines. In addition to the application of amplicon and shotgun metagenomics to DNA, these analyses have also been applied to RNA transcripts (metatranscriptomics) and protein sequences (proteomics), which allow for gene expression and protein sequence levels to quantify soil microbial activity (Urich et al 2008, Hirsch et al 2010). This is useful in studies linking the activity of microbes with a potential function, e.g. the abundance of *amoA* gene transcripts, to responses, e.g. N_2O emissions (Theodorakopoulos et al 2017). Further, the sheer volume of sequencing coupled with high-throughput analytical techniques have enabled the binning of draft genomes, or metagenome-assembled genomes, from environments with low and medium diversity, with soil on the horizon (Sharon & Banfield 2013, Orellana et al 2018). Further goals are the linking of metabolomes, or all the proteins in a sample, with the metatranscriptome, metagenome and genomic information.

1.7 Research aims and thesis outline

The purpose of this dissertation was to investigate the connected system of the soil microbial community, nitrogen and organic fertilizers, and N_2O emissions. This will help to devise strategies targeting the microbes specifically affected by nitrogen fertilization. To do this, I analyzed long- and short-term studies of the effects of different N fertilizer treatments on the microbial soil communities in Dutch pasture soils and in Brazilian sugarcane fields. This was to identify the microbial taxa that responded to the treatments, with a focus on the microbial taxa that were directly involved in N_2O emissions.

In **Chapter 2** I describe potential direct and indirect effects of long-term fertilization with N, P and K on the plant and soil bacterial and fungal communities. To this end I applied co-variation analysis to the taxonomic compositions of each community across the treatments and to a suite of soil physicochemical measurements. In **Chapter 3** I focus on the effects of long-term inorganic fertilization on soil physicochemical characteristics and the soil microbial taxa in Dutch pasture soils. This was done by combining shotgun metagenomic analysis with soil physicochemical measurements using multivariate statistics.

In **Chapter 4** I investigated the effect of different urea fertilization treatments with or without nitrification inhibitors on nitrous oxide fluxes, soil physicochemical characteristics and the soil microbial community in a field experiment. Using 16S rDNA amplicon metagenomes, I evaluated the effect of these treatments on the overall bacterial community composition and diversity, and on functional subgroups using qPCR of nitrification and denitrification genes. In **Chapter 5** I describe further the effect of these urea and nitrification inhibitor treatments on the abundance of ammonia-oxidizing bacteria and other nitrifying species by the analysis of an *amoA* amplicon sequences combined with data mining of the previously published 16S rDNA dataset. Further, I identify the likely species directly responsible for the N_2O emissions in a tropical soil.

In **Chapter 6** I focused on vinasse, which contains a previously uncharacterized microbial assemblage. I obtained metagenome assembled genomes from vinasse samples taken over 1.5 years from a bioethanol factory in Brazil. Based on the functional potential described in these genomes, I describe potential effects of these vinasse bacteria on N_2O emissions in the field when used in fertirrigation.

Last, in **Chapter 7** I provide a general discussion of the research chapters, and present conclusions as well as some thoughts on future directions. This thesis showcases several advanced statistical and bioinformatic methods applied to metagenomic data. Further, the results of this thesis will contribute to the literature serving as a reference for farmers and policy-makers to steer the soil microbiome in agriculture toward long-term sustainability.

1.8 References

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