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

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Chapter 7

General Discussion

Discussion

In this thesis I presented the results of my research regarding the effects of nitrogen fertilizers on soil microbial communities in agriculture. Fertilization in agricultural soils creates different soil "habitats." These habitats present different levels of soil physicochemical properties from the different fertilizer applications, which can lead to differences in the resident communities. Nitrogen fertilization appears greatly to affect the composition of the soil bacterial community, while the effect of nitrogen fertilization on the plant and soil fungal communities depends on the availability of other macronutrients, in particular P and K. Further, I identified specific microbial taxa linked to N_2O emissions under different nitrogen fertilization regimes. The choice of N fertilizer will affect the actual N_2O emissions from a soil, which is also determined by the genetic potential for N_2O emissions, e.g. the activity of resident N_2O-metabolizing microbes. Elucidating the drivers of particular microbial groups, e.g. non-N_2O producers vs N_2O producers, given the soil physicochemical levels, will enable targeted management of N_2O reduction. Here I further discuss the link between advances in sequencing technology and soil microbial ecology research as demonstrated here.

7.1 General effects of N fertilization on the soil microbial and plant communities

Under N limitation, as in natural ecosystems, plant growth is dependent upon decomposition by soil microbes of N and other nutrients bound in plant litter or soil organic matter to bioavailable forms for plant uptake (LeBauer & Treseder 2008, Aislabie & Deslippe 2013). Moreover, soil microbial communities are structured in part by plant litter and plant root deposits (Berg & Smalla 2009). This dependence between plants and soil microbes, the so-called plant-soil feedback, encompasses the ecological relationships between plants and soil microbes that can lead to species co-evolution (van der Heijden et al 2008, van der Putten et al 2013, van Nuland et al 2016, ter Horst & Zee 2016). Fertilization can upset nutrient-mediated plant-microbe symbioses by removing this nutrient limitation (Wall et al 2015). Under N fertilization, the resulting excess N availability is thought by some to decrease the plant dependence on nutrients made bioavailable by soil microbial decomposition, furthermore reducing soil microbial diversity; however, this is still under investigation by the field (Thiele-Bruhn et al 2012, Bommarco et al 2013).

In practice, plant productivity is limited by N, P and K as these macronutrients are required for growth; subsequently, these macronutrients are often added

as fertilizers for crops, which can affect existing beneficial plant-soil microbe dependencies. In Chapter 2, fertilization with NPK but not N alone affected plant community composition and diversity. The plant community in the NPK plots differed from that of the control plots in the dominance of fast-growing plant species able to cope with the high NPK inputs without growth of the medium- and slow-growing species, which led to an overall lower plant diversity. Because there was no concomitant change in the bacterial community composition in the NPK plots, we concluded that indeed, the plant and bacterial communities were disassociated due to the N input. In other words, the plant communities in the N treatment were likely limited by the lack of P and K, which in turn limited their proliferation. Moreover, the N treatment resulted in a large effect in on the soil bacterial but not the plant community, suggesting that the bulk of the added N was used by the bacterial community, resulting in their proliferation and differentiation from the bacterial communities in the control plots. In contrast to the soil bacterial community, it appeared that the soil fungal community composition co-varied with that of the plant community, with a difference in the NPK but not the N treatment (Chapter 2). This suggested that there was co-dependency between the plant species and fungal phyla in these plots, which was not affected by the long-term nutrient additions. Fungi and plant co-dependences are well-known in that fungi (e.g. arbuscular mycorrhizal fungi) form mutualistic relationships with some plant species (Vályi et al 2015). It is possible that the co-dependencies between the grasses and fungi in these plots were more robust than those of the plant and bacteria.

The microbial mining hypothesis offers a framework to interpret how the increased N to the bacterial community might lead to changes in the bacterial community composition, as I observed in Chapter 3. Organic N, or N stored in organic matter, is thought to be the main source of N for microbial growth and maintenance (Parfitt et al., 2005). Thus, microbial decomposers will usually mineralize organic N into ammonium-N to access this nitrogen. However, the N fertilization provides available N so that microbes best equipped to handle the high N levels succeed and increase their abundance in the community. We observed this in the bacterial community in the N fertilized plots in Chapter 3 as a shift to so-called copiotrophic phyla under N saturation. The copiotroph– oligotroph trade-off (Fierer et al 2007) seemed to explain our result of increased abundances of Actinobacteria and decreased abundances of Acidobacteria, Verrucomicrobia and Firmicutes in the N-saturated fields. Oligotrophs are described as those that grow slower but more efficiently and succeed during resource limitation, while copiotrophs have fast growth rates and inefficiently transform resources, hence doing better during higher resource availability (Roller & Schmidt 2015). While Acidobacteria, Firmicutes, and Verrucomicrobia are regarded as oligotrophs, Acti-

nobacteria are regarded as copiotrophs (Fierer et al 2012, Leff et al 2015). While this hypothesis is a good framework to interpret results, it is clear that this is a very simplistic approach, as within a bacterial phylum there can be copiotrophic and oligotrophic populations, perhaps even in the same genus, as evidenced by the large range of organic substrate degradation by different soil microbial species (Goldfarb et al 2011). Further, in Chapter 3 I observed that based on the metagenomic analysis, the functional potential of the soil bacterial communities did not differ based on treatment, in contrast to the taxonomic composition. This lack of difference of functional profiles, at least at a coarse-grained level, seems to be widely applicable to bacterial communities in soil (Fierer et al 2012), and suggests functional redundancy provided by different taxa. However, fine-grained analyses might reveal the specific differences in the functional potential of bacterial communities in N- and NPK- saturated plots.

7.2 Microbial populations involved in N_2O metabolism under N fertilization

Here, I add to the agricultural and microbial ecology literature cementing the idea that nitrogen addition greatly influences the soil microbial community, and further describe specific microbial taxa influenced by different N sources. In Chapter 3, the effect of the long-term inorganic fertilizations on the soil bacterial community were quite large, and the differences in the nitrogen plots clearly visible even at the phylum level. Going to Chapter 5, this chapter showcased differences at the OTU-level between the nitrogen source and control plots. The effect of nitrogen input occurs first on individual OTUs by promoting certain species, which then allows these taxa to succeed over time. Fertilization with urea appeared to select for certain groups as per the microbial mining hypothesis described previously. For example, the ammonia-oxidizing bacterial *Nitrosospira*-like population that was correlated with N_2O emissions also appeared to respond to the nitrification inhibitors co-applied with the urea. Further, also in Chapter 5, the native population appeared to be an ammonia-oxidizing archaeal *Nitrososphaera*-like population. Sugarcane agriculture practices in Brazil aim to improve nitrogen use efficiency, although this is a challenge due to the tropical climate, which provides high volumes of rainfall which contribute to nitrogen loss through NO_3^- leaching and N_2O gas production (Otto et al 2016). As seen in Chapters 4 and 5, no N application in sugarcane soils were found to result in poor N availability; the resident community of nitrifiers in the N unfertilized plots was adapted to low N conditions, being ammonia-oxidizing archaea and nitrite-oxidizing bacteria. Many studies also identified ammonia-oxidizing bacteria rather than archaea as responsible for N_2O emissions in agricultural soils (Wang et al 2016,

Meinhardt et al 2018). Recent evidence suggests that this trend of archaea producing less N_2O than bacteria is generally found, which boils down to the differences in their biochemical pathways (Stieglmeier et al 2014, Hink et al 2017, Jia & Conrad 2009). This is thought to be due to the ammonia preferences of ammonia-oxidizing archaea vs bacteria, with the former having higher affinity to ammonia and therefore preferring low ammonia availability, and the latter having lower affinity, thus preferring higher ammonia availability (Hink et al 2018).

Interestingly, as found in Chapter 6 mainly putative denitrifiers were found in the metagenome-assembled genomes of vinasse bacteria. Yang et al (2013) found that Actinobacteria were stimulated under vinasse fertilization, suggesting that copiotrophs might be stimulated by mainly the organic compounds found in vinasse. Measuring the precise nutrient conditions is vital for further research on the dynamics of N_2O emissions in soils under sugarcane. Another important point from the system of vinasse fertirrigation was our finding of potential antibiotic-resistance genes in the vinasse bacterial genomes. This is inferred to be due to the antibacterial procedure used during bioethanol distillation, but which can have serious environmental effects once used in fertirrigation (Braga et al 2017). Namely, there is a potential for the spread of antibiotic-resistance genes in the soil microbial community in sugarcane soils as occurs when antibiotic-treated livestock waste is used as fertilizer for crops (Thiele Bruhn et al 2003, Tasho et al 2016). This is an important task for future research into the environmental and health effects of vinasse fertirrigation.

Here I suggest that teasing out the drivers at a finer-grained scale can help us to further describe the ecology of all microbes involved in N_2O emissions in a system. We demonstrated in Chapter 2 that measuring micronutrients as well as macronutrients pointed to a correlation between the soil bacterial community compositions in the N fertilized plots with Fe, Al, Mg and Mn. This represents a link between abiotic and biotic ecosystem components. While the cost of metabolomics and proteomics of soil samples remains prohibitive, measuring full suites of micronutrients along with macronutrients might allow us to connect microbial populations with their drivers. Specifically, this could aid in teasing out the ecological niches of different N_2O-producing microbes and lead to designing fertilizer schemes that minimize nitrogen loss and increase nitrogen fertilizer efficiency.

7.3 Sequencing technologies paved the way for soil microbial research

Advances in next-generation sequencing technology led to decreasing costs of sequencing, widespread use and increased representation of soil microbes in sequence databases. While the cost of sequencing a bacterial genome of about 5 Mbp at 100X coverage was about EU 100 in 2012, that cost dwindled to about EU 2 in the present day (Köser et al 2012, Deurenberg et al 2017). These reductions in cost have been largely driven by clinical microbiology, but also studies of plant-associated microbes. This reduction in cost has also led to overall improvement in the field of bioinformatics, as more comprehensive studies are possible when more genomic data is deposited to the database. Further, the application of metagenomics to improving culturing conditions of microbes has led to increasing the "unculturable" fraction of represented microbes, although this reduction has been mainly in human-related microbes (Lagier et al 2012). Soil microbial communities, which reach up to 10^9 cells and between 10^4-10^6 species in one gram of soil, initially presented a challenge to fully sequence (Roesch et al 2007, Schloss & Handelsman 2006). In studies of soil microbial communities, this means that the comprehensive sequencing of the full diversity of these communities can be realized. Further, more soil genomic information represented in the databases strengthens future research of soil microbial communities by providing reference sequences with which to compare unknown sequences. In this way, researchers can move from coarse- to finer-grained analyses, and to investigate the dynamics of microbial populations.

In the present thesis, my chapters span a course of about five years, in which the advances in bioinformatics tools can be seen. The soil microbial communities in long-term fertilized grassland or sugarcane soil were evaluated at the phylum-level (coarse-grained analysis) using *16S* and *18S rDNA* amplicon (Chapter 2 & 4) and shotgun metagenomics (Chapter 3). In Chapter 3, 454 pyrosequencing was applied, while in Chapters 4 and 5 Ion Torrent was used and in Chapter 6 Illumina MiSeq sequencing was used. A fine-grained analysis (species or OTU-level) was used to investigate the ammonia-oxidizing microbial community in sugarcane soils (Chapter 5) as well as the vinasse assemblage using shotgun metagenomics and metagenome assembled genomes (Chapter 6). Bioinformatic analyses are especially useful in generating testable hypotheses that future research can address. Thus, this consideration highlights the need to study microbially-mediated events using relevant molecules at relevant time scales. For example, RNA sequencing is a good tool to examine the link between expression of N_2O-producing genes from microbes and N_2O emission peaks in the field (Theodorakopoulos et al 2017). Here I showed that the coarse-grained soil bacterial community structure was sus-

ceptible to long-term (Chapter 3) but not short-term nitrogen addition (Chapter 4) in the forms of ammonium nitrate and urea, respectively. While these studies were regarding the bacterial communities of different soils, these are considered to be comparable due to the inclusion of a control treatment in each experiment. This aspect brings up the point that experiments involving microbial community research should be carefully designed, with the inclusion of a control treatment and further, enough biological replicates to provide good statistical power, eg. a recent meta-analysis of global bacterial and fungal diversity using 189 sites and 7,560 sub-samples (Bahram et al 2018). Here, changes in OTU abundances were visible over one season in the field (Chapters 4 and 5), while differences in phylum abundances were visible after a 60+ year experiment (Chapter 3).

Because there are many pathways leading to N_2O in agricultural and natural systems it is a challenge to uncover mechanistic details regarding the microbes in control of these emissions. However, the combination of improved sequencing technologies, sequencing effort, informed experimental design and improved representation of soil microbes in public databases is closing this knowledge gap. Further, more detailed measurements of soil and environmental factors combined with gases emissions will enable us to get fuller pictures of the complex processes studied. While here I focused on nitrification and denitrification as the main sources of nitrogen fertilizer-derived N_2O emissions, it is important in the context of research into N_2O-emission reduction that all sources be considered when designing future projects. As pointed out in a recent review, microbes mediating N transformations are both metabolically and taxonomically diverse; thus, research taking microbes into account when detailing the nitrogen cycle should incorporate this diversity (Kuypers et al 2018). For investigating the role of microbes in N transformations in agricultural soils, this means zooming into the details, asking questions such as, “which functional pathway is dominant?” and further, “what are the drivers of the microbes involved in these pathways?”

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