Impact of changes in environmental parameters (pH and elevated CO_2) on soil microbial communities involved in N-cycling

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Wer ohne die Welt auszukommen glaubt, irrt sich.
Wer aber glaubt, dass die Welt nicht ohne ihn auskommen könne, irrt sich noch mehr.
François de La Rochefoucauld

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Summary

Microorganisms involved in the nitrogen (N)-cycle in soils are the major drivers of N-transformation changes and the main source of the potent greenhouse gas nitrous oxide (N_2O) from soil, which has a global warming potential of 298 times that of carbon dioxide (CO_2). Accordingly, it is of great interest to explore shifts in the rates, balances and reactions of the N-cycle impacted by climate changes, in order to offer more accurate predictions. Particularly, since increases in CO_2 concentrations or changes in the pH of agricultural fields due to anthropogenic influences often lead to changes in the N-transformation rates, along with an increase of N_2O emissions. However, the N-cycle and its corresponding pathways are very complex and the response to different environmental changes is difficult to predict. Many of the interactions between microorganisms and their contribution to N-transformation rates as well as N_2O emission are not well understood, controversially discussed and plenty of important interactions remain puzzling. Therefore, the main objective of this thesis was to shed light on the interaction of the overall and active microbial communities involved in the N-cycle in response to pH shifts or elevated atmospheric CO_2 concentrations in soils, two variables known to influence N_2O fluxes from soils.

In the first part we studied the influence of an acidic pH on a denitrifier community from an initial neutral pH. We followed the abundance and composition of an overall and active denitrifier community extracted from soil (pH = 7.1) when exposed to pH 5.4 and drifting back to pH 6.6. When exposed to pH 5.4, the denitrifier community was able to actively grow, but only reduced N_2O to N_2 after a near neutral pH was reestablished by the alkalizing metabolic activity of an acid-tolerant part of the community. The genotypes proliferating under these conditions differed from those dominant at neutral pH. Denitrifiers of the *nirS*-type appeared to be severely suppressed by low pH whereas *nirK*-type and *nosZ*-containing denitrifiers showed strongly reduced transcriptional activity and growth, even after

restoration of neutral pH. Our study suggests that low pH episodes alter transcriptionally active populations which shape denitrifier communities and determine their gas kinetics.

The second part of this thesis analyses the effect of elevated CO_2 (eCO_2) on the N-cycle to reveal the underlying microbial mechanisms and process inside the N-cycle causing the enhanced emission of N_2O. To gain a better understanding of the impact of eCO_2 on soil microbial communities, we applied a molecular approach targeting several microbial groups involved in soil N-cycling (N-fixers, denitrifiers, archaeal and bacterial ammonia oxidizers, and dissimilatory nitrate reducers to ammonia) at the Gießen Free Air Carbon dioxide Enrichment (GiFACE) site. Remarkably, soil parameters, overall microbial community abundance and composition in the top soil under eCO_2 differed only slightly from soil under ambient CO_2. We concluded that +20% eCO_2 had little to no effect on the overall microbial community involved in N-cycling. Based on these findings, in a third part we conducted a comprehensive study monitoring N-transformation rates, nutrient fluxes, and gaseous emission, while analyzing the dynamics in microbial communities involved in N-cycling under eCO_2 accompanied with simultaneous addition of N-fertilizer. We could show that long-term fumigation with eCO_2 influences the response of the soil microbial communities to N inputs via fertilization. Compared to aCO_2 distinct parts of the community were transcriptionally activated. Here, *nirS*-type denitrifiers showed the greatest positive feedback to eCO_2, which correlated with increasing N_2O emissions. This stimulation may be an effect of higher labile C input in the rhizosphere by increased photosynthesis. However, the input of N by fertilization rather seems to exert short term effects on the expression of functional marker genes with consequences for N-transformations which do not translate into the development of distinct communities under eCO_2 in the long-term. In conclusion this thesis provides evidence that already small changes in abundance and composition of the microbial community involved in N-cycling are sufficient to strongly influence emission of N_2O from soil under changing environmental parameters such as pH and elevated CO_2.

Zusammenfassung

Die hauptsächliche Quelle des Treibhausgases Distickstoffmonoxid (N_2O) sind in Böden vorkommende Mikroorganismen, die an der Umsetzung von Stickstoffverbindungen und damit am Stickstoffkreislauf beteiligt sind. N_2O hat im Vergleich zu CO_2 ein 298-fach erhöhtes Treibhauspotential. Aus diesem Grund ist die Erforschung der durch die Klimaerwärmung veränderten Reaktionsraten und –gleichgewichte des Stickstoffkreislaufs essentiell um akkuratere Vorhersagen bestimmen zu können. Insbesondere der anthropologisch begründete Anstieg des CO_2-Gehalts in der Atmosphäre, sowie pH Veränderungen durch landwirtschaftlich genutzte Flächen, beeinflussen die Stickstoffumsetzung in Böden und resultieren in erhöhten N_2O Emissionen. Die Komplexität des Stickstoffkreislaufs erlaubt jedoch nur ungenaue Prognosen darüber, wie sich einzelne Umwelteinflüsse auf ihn niederschlagen. So sind beispielsweise die Interaktionen und Beiträge einzelner Mikroorganismen zu Stickstoffumsatz und N_2O Emission kaum bekannt oder werden kontrovers diskutiert. Aus diesen Gründen ist das hauptsächliche Ziel dieser Arbeit die Reaktion der gesamten und transkriptionell aktiven Mikroorganismengemeinschaft, die am Stickstoffkreislauf beteiligt ist, auf pH Veränderungen und höhere CO_2 Partialdrücke zu untersuchen.

Im ersten Teil dieser Arbeit wurde der Einfluss einer Ansäurung auf eine denitrifizierende Gemeinschaft untersucht. Dabei wurde sowohl die Abundanz als auch die Zusammensetzung der gesamten und aktiven denitrifizierenden Gemeinschaft eines neutralen Bodens (pH = 7,1) während einer Veränderung des pH zu 5,4, gefolgt von einer graduellen Verschiebung zu pH 6,6, analysiert. Auch bei pH 5,4 war ein Wachstum der denitrifizierenden Gemeinschaft zu verzeichnen, allerdings wurde N_2O erst vollständig zu N_2 reduziert, nachdem ein nahezu neutraler pH, erreicht wurde. Diese pH Verschiebung lässt sich vermutlich auf alkalisierende metabolische Prozesse einer säuretoleranten Population

zurückführen. Die unter diesen Bedingungen identifizierten wachsenden Genotypen unterschieden sich von denen in neutralen pH Bereichen gefundenen. Dabei waren Denitrifizierer des *nirS*-Typs stärker von niedrigen pH Werten beeinträchtigt, als die des *nirK*- und *nosZ*-Typs, die zumindest niedrige Wachstums- und Transkriptionsraten zeigten, auch nachdem der pH wieder einen fast neutralen Wert eingenommen hatte. Die vorliegende Studie impliziert, dass niedrige pH Werte die transkriptionell aktive Population nachhaltig verändert, wodurch sich die gesamte Gemeinschaftsstruktur und deren Gaskinetiken ändert.

Der zweite Teil dieser Thesis beschäftigt sich mit dem Einfluss eines erhöhten CO_2 Partialdrucks (eCO_2) auf den Stickstoffkreislauf und die übergeordneten mikrobiologischen Mechanismen und Prozesse, die in einer erhöhten N_2O Emission resultieren. Um diesen Einfluss besser zu verstehen, wurde verschiedene mikrooganismische Gruppen des Stickstoffkreislaufs (Stickstofffixierer, Denitrifizierer, archeale und bakterielle Ammoniumoxidierer und dissimilatorische Nitratreduzierer) der Gießen Free Air Carbon dioxide Enrichment (GiFACE) Anlage gezielt untersucht. Erstaunlicherweise unterschieden sich die Bodenparameter, sowie die Abundanz und Zusammensetzung der gesamten Mikroorganismengemeinschaft der mit CO_2 begasten Böden kaum von denen ohne spezielle Begasung. Daraus ist zu schließen, dass +20% eCO_2 keinen oder nur einen geringen Effekt auf die am Stickstoffkreislauf beteiligten Mikroorganismen hat. Basierend auf diesen Ergebnissen wurde im dritten Teil dieser Arbeit eine umfassende Studie der Stickstoffumsätze, Nährstoffkreisläufe sowie Gasemissionen kombiniert mit der Analyse der Dynamik innerhalb der Mikroorganismengemeinschaft unter eCO_2 Bedingungen und während der Zugabe von Stickstoffdüngern durchgeführt. Wir konnten zeigen, dass die langfristige Begasung mit eCO_2, die Reaktion der mikrobiellen Gemeinschaften während des Eintrags von N durch Düngung beeinflusst. Im Vergleich zu aCO_2 wurden verschiedene Teile der Gemeinschaft transkriptionell angeregt. Dabei zeigten *nirS*-Typ Denitrifizierer die größte positive Resonanz zu eCO_2, die mit der zunehmenden N_2O-Emission korreliert. Diese

Beeinflussung könnte auf einen erhöhten Eintrag von Kohlenstoffverbindungen durch die Rhizosphäre, ermöglicht durch eine erhöhte Photosyntheseleistung der Pflanzenbiomasse bei eCO_2, beruhen. Allerdings scheint der Eintrag von N durch Düngung nur kurzfristige Auswirkungen auf die Expression von funktionellen Marker-Genen auszuüben. Dies führt zu Veränderung in der N-Transformation, welche sich langfristig allerdings nicht in der Entwicklung von verschiedenen Gemeinschaften unter eCO_2 wiederspiegeln. Zusammenfassend zeigt diese Arbeit, dass bereits kleine Änderungen in der Abundanz und Zusammensetzung der mikrobiellen Gemeinschaft aus dem Stickstoffkreislauf ausreichen, um einen starken Einfluss auf die Emission von N_2O aus Böden unter wechselnden Umgebungsparameter wie pH-Wert und erhöhtem CO_2 auszuüben.

Chapter I

1. Introduction

1.1. Nitrogen cycle and N transformation in soil

Nitrogen (N) is the most abundant element in our atmosphere with 78%. N is of great importance as a component of e.g. DNA and amino acids for animals, humans, plants and microbes. The total amount of N on earth is ~ 10^{15} t, the main amount of N (~ 94%) is bound in the lithosphere followed by the N in the atmosphere (~ 6%) (Sweeney *et al*., 1978). N-transformations constitute one of the most complex cycles on earth reflected in the highly intricate biogeochemical cycle, where N occurs in valence states from -3 (ammonia (NH_4^+)) to +5 (nitrate (NO_3^-)) and where many of the transformations are carried out by a few organisms under standard conditions (STP). Gaseous losses are also associated with the N cycle such as nitrous oxide (N_2O) which is of environmental concern, being a long-lived trace gas in the atmosphere, with a global warming potential of 298 times that of carbon dioxide (CO_2) on a 100 year basis and a half-life time of 120 years (IPCC, 2013). Atmospheric N_2O concentrations increased since the industrial revolution by about 20% (Fig. 1.1). Emissions from natural and agricultural soils emission contribute approximately 56-70 % to the global N_2O budget due to the conversion of fertilizer and manure N (Syakila and Kroeze, 2011).

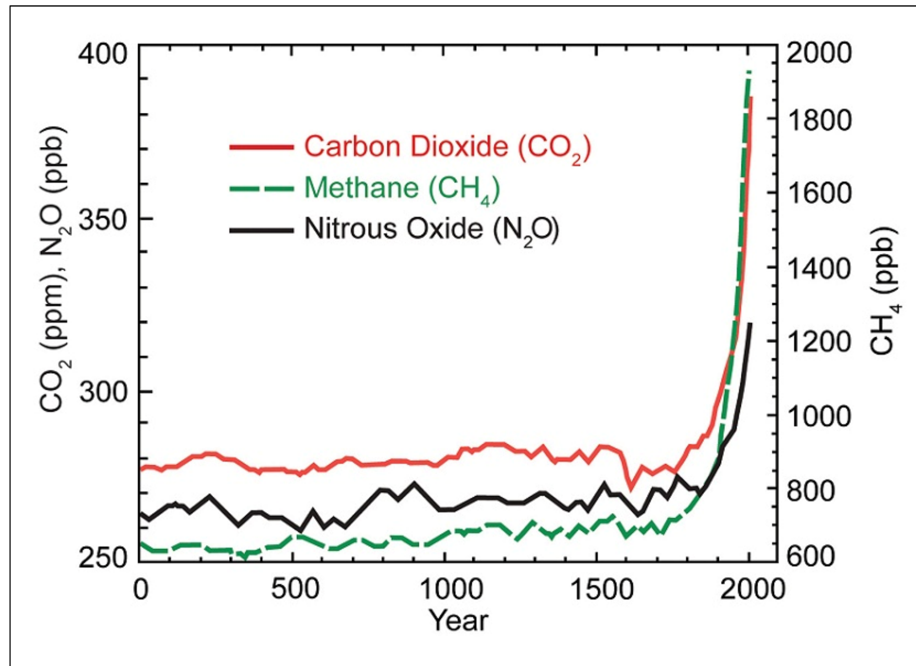

Figure 1.1. Increase in greenhouse gas (GHG) concentrations in the atmosphere over the last 2,000 years. Concentration units are parts per million (ppm) or parts per billion (ppb), indicating the number of molecules of the greenhouse gas per million or billion molecules of air. (USGCRP, 2009).

N transformations in soil (Fig. 1.2) are complex and carried out by diverse organisms. For instance fixation of nitrogen gas (N_2) to ammonium (NH_4^+) is carried out by specialist N-fixing microorganisms (Burns and Hardy, 1975). Ammonium is produced by the mineralisation of organic substrates and by dissimilatory nitrate reduction (DNRA) (Tiedje, 1988). Ammonium together with nitrite (NO_2^-) can produce molecular nitrogen via anaerobic ammonium oxidation (ANAMMOX) (Strous *et al*., 1997) which mainly occurs in aquatic systems or can be oxidized to NO_3^- in a two-step process via specialized nitrifiers via a two step process: $NH_4^+ \rightarrow NO_2^-$ and $NO_2^- \rightarrow NO_3^-$ (Hart *et al*., 1994). Nitrate can be reduced anaerobically via dissimilatory nitrate reduction (denitrification) to N_2 (Knowles, 1982). A range of microorganisms (bacteria, archaea or fungi) are responsible for the N transformations (Fig. 1.2). Additionally, some microorganisms can catalyze different processes in the N cycle. For instance, functional marker genes for denitrification were found in ammonia oxidizers and

vice versa (Bartossek *et al.*, 2010; Cantera and Stein, 2007; Casciotti and Ward, 2005; Garbeva *et al.*, 2007; Shaw *et al.*, 2006). Functional marker genes are frequently used to analyze the composition and abundance of the microorganisms involved in the N cycle, since 16S rRNA genes as an universal marker gene is not inevitably related to the physiology of target organisms (Calvo and Garcia-Gil 2004; Kowalchuk and Stephen, 2001). Therefore, functional markers like the genes encoding key enzymes that are involved in a specific metabolic pathway have been used in microbial ecology studies (Rotthauwe *et al.*, 1997).

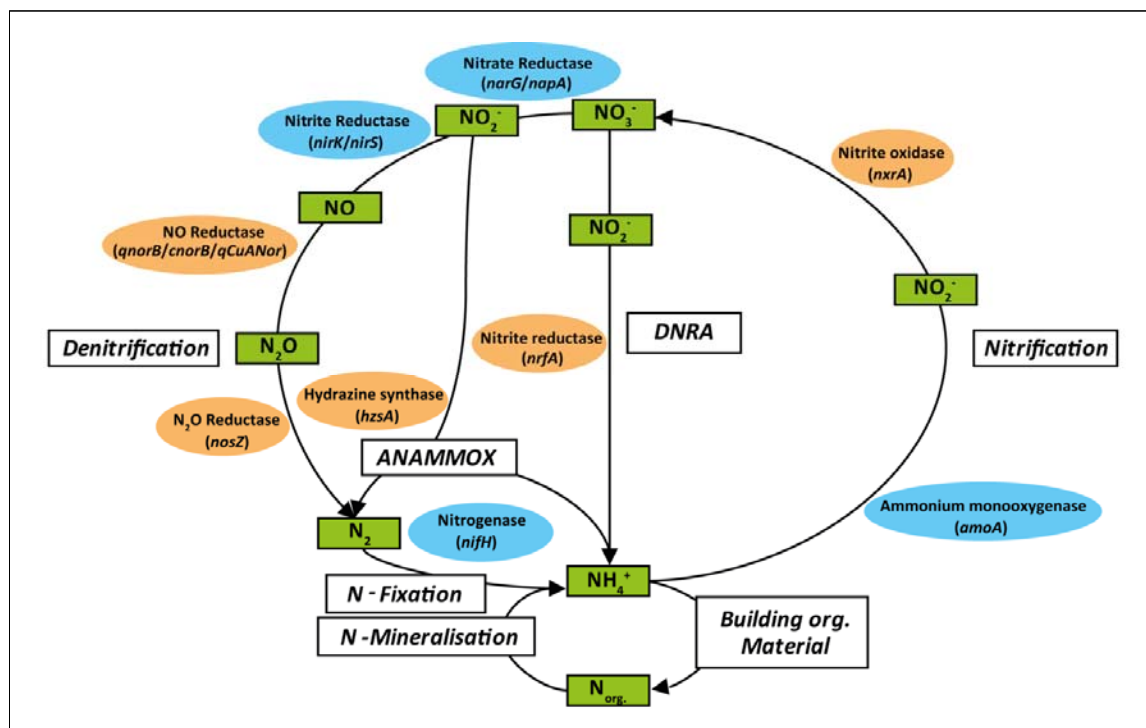

Figure 1.2. Pathways in the biological nitrogen cycle. In circle are listed the enzymes which catalyze each pathway along with the specific functional marker gene, respectively. Orange = exclusively performed by bacteria; Blue = performed by bacteria and archaea; DNRA = dissimilatory nitrate reduction; ANAMMOX = anaerobic ammonium oxidation.

1.2. Pathways in the nitrogen cycle and their functional marker genes

Denitrification

Denitrification (Fig. 1.3) is the stepwise reduction of nitrate (NO_3^-) via nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O) to molecular nitrogen (N_2). Together with ANAMMOX, denitrification is the main pathway for the reduction of N compounds to N_2.

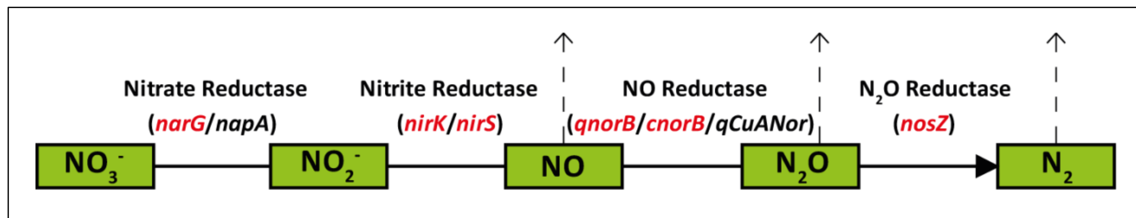

Figure 1.3. Reduction steps from nitrate to nitrogen during denitrification. Between the steps, catalyzing enzymes together with their functional marker genes are indicated. Dashed lines indicate an emission of nitrogen gas into the atmosphere. Red = most frequently used marker genes.

From an ecological and economical point of view denitrification has positive and negative consequences. A major issue is the production of N_2O as an intermediate product. As stated before, N_2O is a powerful greenhouse gas, which is also involved in the destruction of the ozone layer. On the other hand, as a greenhouse gas, it reflects the infrared light back to the earth surface, contributing to global warming (Crutzen, 1970; Dickinson and Cicerone, 1986; Ravishankara *et al*., 2009). Denitrification, together with nitrification among a range of other processes, is the major sources of N_2O from soils (Conrad, 1996; Butterbach-Bahl *et al*., 2013). Denitrification converts reactive N to gaseous products including N_2O and N_2 and therefore reduces the availability of NO_3^- for plant N uptake. (Ambus and Zechmeister-Boltenstern, 2006; De Klein and Logtestijn, 1994; Mogge *et al.*, 1999). This stimulates increasing application of N-fertilizers on farm fields, to avoid a loss of yield. Apart from fertilizer also dairy cattle and the cultivation of legumes increases the amount of mineral N and can therefore have an effect on gaseous emissions (Tilman *et al*., 2002). Thus, the use of

fertilizer over the last 150 years is a major reason for the increase of the N_2O concentration (Fig. 1.1) in the atmosphere from 275 ppbv (pre-industrialization) to 319 ppbv (after-industrialization) (IPCC, 2013). It is estimated that the N_2O concentration will continue to increase by about 0.3% per year (Fig. 1.4; WMO, 2014). However, on the other hand, denitrification together with nitrification plays a significant role in the elimination of N compounds from waste water treatment, to counteract the eutrophication of waters that serve as receiving water bodies and also reduces leaching of NO_3^- from soil (Grady *et al*., 2011).

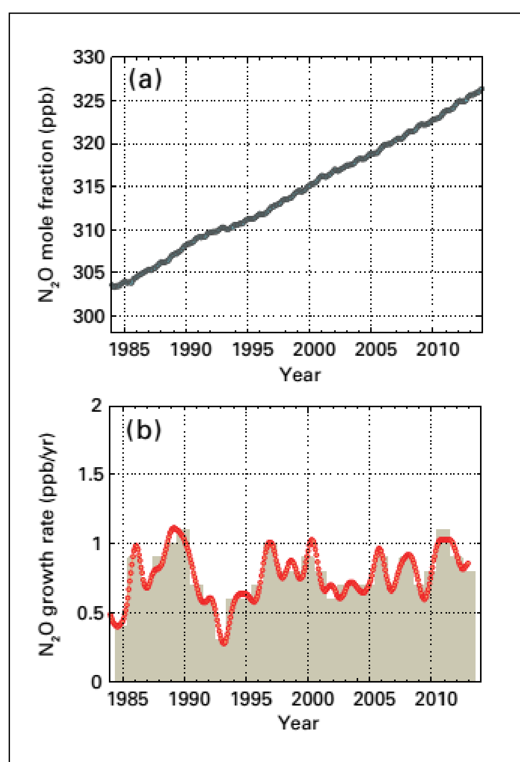

Figure 1.4. Globally averaged N_2O mole fraction (a) and its growth rate (b) from 1984 to 2013. Differences in successive annual means are shown as shaded columns in (b). (WMO, 2014)

Denitrification itself is a microbial respiratory key process, in which electron transport phosphorylation is coupled to a stepwise reduction of nitrogen oxides (Tiedje, 1994). It is a facultative anaerobic using mainly organic compounds as electron acceptors (Zumft, 1997). The first step of denitrification is the reduction of NO_3^- to NO_2^-, which can be catalyzed by

one of two types of molybdenum-containing enzymes, a membrane-bound (*narGH*) (Bonnefoy-Orth *et al*., 1981) and a periplasmatic nitrate reductase (*napAB*) (Siddiqui *et al*., 1993).

The enzymes which catalyze the next reduction step from NO_2^- to NO, are the key enzymes of denitrification, because the bound N is transformed into a volatile form and cannot be assimilated by microorganisms anymore (Henry *et al*., 2004). Two periplasmatic nitrite reductases with different prosthetic groups are known, a cytochrome cd_1-reductase encoded by *nirS* and a cooper containing reductase encoded by *nirK*. Both enzymes catalyze the same reaction, but have a different evolutional origin (Heylen *et al*., 2006). Recently, in contrast to previous studies (Tiedje, 1994), it was shown that approximately 80% of nitrite reductases, possess a *nirK* gene (Graf *et al*., 2014). Additionally, 10 bacterial strains were found with a copy of both *nirK* and *nirS* (Graf *et al*., 2014), which contradicts the previous assumption that the two nitrite-reductases are mutually exclusive (Zumft, 1997). Nevertheless, the functionality of both gene products in these strains could not be demonstrated so far. Most strains possess one copy of either *nirK* or *nirS*, but genome analyses revealed strains with more than one copy of *nirK* or *nirS* (Etchebehere and Tiedje, 2005; Graf *et al*., 2014) and being expressed under different conditions (Etchebehere and Tiedje, 2005).

In the third step of denitrification, the reduction of NO to N_2O is catalyzed by NO-reductase. A high affinity of the reductases for NO ensures a most efficient conversion to N_2O. Three types of NO-reductases with different electron donors are known yet. Cytochrome *c* or pseudoazurin is the electron donor for cNor, a quinol reservoir for qNor and menaquinones for qCu_ANor. Two different types of *norB* encode cNor and qNor. While cNor additionally requires *norC*, which encodes the second subunit of the NOR protein, NorC is lacking in the qNor enzyme (Cramm *et al*., 1999; Hendriks *et al*., 2000). The third NO-

reductase qCuANor was to date only isolated from *Bacillus azotoformans* (Suharti *et al*., 2001) but the gene is still unknown.

N_2O-reductase catalyzes the last step of denitrification from N_2O to N_2. This step is the only known biological process which can reduce N_2O to N_2. The gene *nosZ* encodes this soluble, copper-containing periplasmic protein (Zumft *et al*., 1990; Henry *et al*., 2006). By now *nosZ* is known as the only enzyme to catalyze the reduction. It was postulate for a long time that there exist only one family of N_2O reducers, but recently a new clade of nosZ containing denitrifiers were observed (Jones *et al*., 2013).

Denitrifiers are facultative anaerobic microorganisms capable of either stoichiometrically reducing NO_3^- or NO_2^- to N_2O or N_2 in the absence of oxygen (O_2) (Tiedje, 1994). Some microorganisms are catalyzing the whole reduction, while others are able to perform only single steps of denitrification. Especially NO_3^-- and N_2O-reduction are the most independent ones and can be run as autonomous processes by microorganisms (Zumft, 1997), e.g. nitrate reduction by *Thioalkalivibrio nitratireducens* (Sorokin *et al.*, 2003b) and N_2O-reduction by *Wolinella succinogenes* (Simon *et al.*, 2004) or *Halomonas chromatireducens* sp. (Shapovalova *et al.*, 2009). Other microorganisms lack the first (NO_3^--reduction) or the last step of denitrification (N_2O-reduction), e.g. *Thioalkalivibrio denitrificans* (Sorokin *et al.*, 2003a) and *Agrobacterium tumefaciens* (Baek and Shapleigh, 2005). Two special cases are *Rhizobium sullae* HCNT1 and strains of *Mesorhizobium* spp., which possess only a nitrate reductase and additionally a functional NirK. However, due to the production of cytotoxic NOs these organisms are not able to grow under denitrifying growing conditions (Falk *et al.*, 2010; Monza *et al.*, 2006; Toffanin *et al.*, 1996).

Denitrifiers are an important group of microbes in soil, with an amount of up to 10% of the total microbial community in terrestrial ecosystem (Henry *et al.*, 2004; Henry *et al.*, 2006; Tiedje, 1988; Brenzinger *et al.*, in preparation). Denitrifiers can be found in nearly all

phylogenetically main groups except the *Enterobacteriaceae* and obligate anaerobic species. They were detected in over 50 genera and more than 130 species (Philippot *et al*., 2007). The denitrifiers belong mainly to the phylum of Proteobacteria, but can be also found in *Firmicutes*, *Actinomycetes*, *Bacteroidetes*, *Aquificaceae* and also in Archaea (Völkl, 1993). For decades, it has been assumed that only prokaryotes were capable of denitrification, but reductases for the reduction of NO_3^-, NO_2^- and NO were also detected in the mitochondria of fungi (Takaya, 2002). More recent studies also showed the existence of Eukarya (Foraminifera and *Gromiida)* with the ability to denitrify (Piña-Ochoa *et al.*, 2010; Risgaard-Petersen *et al.*, 2006). The widespread ability for denitrification is probably due to horizontal gene transfer, convergent evolution of various structural types or lineage sorting of gene duplication (Heylen *et al.*, 2006; Heylen *et al.*, 2007; Jones *et al.*, 2008). Denitrifiers can be found in many different habitats such as soil, activated sludge and marine-/freshwater-sediments. Recently, the ability of denitrification was even found in tank reservoirs of bromeliads (Suleiman *et al*., in preparation) and in leaf axils of oil palm trees (Suleiman *et al*., in preparation).

Nitrogen fixation

Nitrogen fixation (Fig. 1.5) is the process in which atmospheric nitrogen (N_2) is converted into ammonium (NH_4^+) (Burns and Hardy, 1975). The process of N-fixation is very important, since nitrogen (N_2) is relatively inert and cannot be taken up by plants. To make N available again three different ways of fixation are known so far, through geochemical processes, i.e. lightning (Gruber and Galloway, 2008), industrially through the Haber-Bosch process and biologically by N-fixing bacteria via the enzyme nitrogenase (Lineweaver *et al*., 1934; Burk *et al*., 1934). The contribution of microbes to N-fixation is ~ 200-300 Mtons of fixed N per year, including marine and terrestrial systems (Galloway *et al*., 1995).

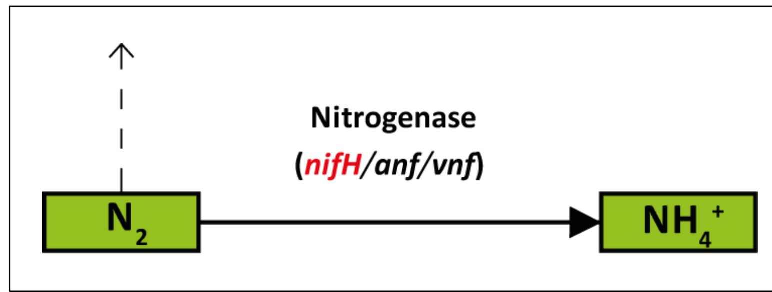

Figure 1.5. N-fixation with the enzyme and the functional marker genes. Dashed lines indicate an emission of this component into the atmosphere. Red = most frequently used marker gene.

Three different types of nitrogenases were observed so far, a molybdenum-dependent nitrogenase (encoded by a *nifHDK*), a vanadium dependent nitrogenase (encoded by *vnf*) and an iron-only nitrogenase (encoded by *anf*). There are only few microorganisms harboring the last two types of nitrogenases and they were only detected in a strain together with the *nif* operon. Under shortage of molybdenum, the alternative forms are used (Pau, 1989; Pau *et al*., 1991). The common nitrogenase which is encoded by *nifHDK* consists of two components, a MoFe protein (commonly: dinitrogenase or component I) and the electron transfer Fe protein (commonly: dinitrogenase reductase or component II) (Winter and Burris, 1976; Hageman and Burris, 1978; Dean *et al*., 1993 *nifH* encodes component II and is commonly used as a functional marker gene to detect N-fixing bacteria in the environment (Kirshtein *et al*., 1991; Widmer *et al*., 2000; Poly *et al*., 2001a; 2001b).

The whole process of N-fixation is highly endothermic (Bayliss, 1956) and usually it is an anaerobic process, because the nitrogenase is very oxygen sensitive (Goldberg *et al*., 1987). Cells developed several different mechanisms to protect the nitrogenase from oxygen, e.g. cells are surrounded with a thick mucilaginous layer that inhibited oxygen diffusion or a high respiration rate lower the free oxygen (Poole and Hill, 1997; Ureta and Nordlung, 2002). Cyanobacteria and *Burkholderia* are the only bacteria that can tolerate oxygen while they fix N_2 (Stal and Krumbein, 1985; Estrada-De Los Santos *et al*., 2001).

N-fixers are also known as diazotrophs, which are widespread along several bacterial taxonomic groups and can also be found in Archaea (Murray and Zinder 1984; Belay *et al*., 1984; Leigh, 2000). N-fixers can occur free living (e.g. *Azotobacter*, *Bacillus*, *Clostridium*, *Rhodopseudomonas*, *Klebsiella* and *Methanosarcina)* or as symbionts (e.g. *Anabaena*, *Frankia*, *Rhizobium* and *Bradyrhizobium*), which requires a close relationship with a host to carry out N-fixation (Postgate, 1998).

Dissimilatory nitrate reduction to ammonium (DNRA)

DNRA is the direct reduction from NO_3^- or NO_2^- to NH_4^+, in contrast to the required combined reduction by denitrification and N-fixation (Fig. 1.6). DNRA is in direct competition to denitrification as it also requires NO_3^- as an electron acceptor. It was shown that under conditions with high availability of labile carbon and/ or low NO_3^--concentration DNRA has an advantage over denitrification, because NO_3^- is used much more effectively, consuming eight moles of electrons per one mole of NO_3^- compared to five moles of electrons during denitrification (Bonin, 1996; Fazzolari *et al*., 1998; Nijburg *et al*., 1997; Tiedje, 1982; Yin *et al*., 2002). Recent studies postulate that a C/NO_3^- ratio of > 12 favors DNRA (e.g. Rütting *et al*., 2011). Even though the calculated free energy in the process of denitrification is higher than from DNRA (-2669 kJ mol^{-1} glucose for denitrification over -1796 kJ mol^{-1} glucose for DNRA; Gottschalk, 1986), studies with pure cultures showed that the real free energy yield from DNRA is actually higher than from denitrification (Strohm *et al*., 2007). DNRA resulted in a two-fold higher cell mass production per mole NO_3^- compared to denitrification (Strohm *et al*., 2007). To date, the importance of denitrification relative to DNRA and vice versa is not well understood, especially under field conditions. It is presumed that some of the NO_3^- reduction, which was attributed to denitrification, actually results from DNRA. DNRA has been shown to occur predominantly in anaerobic sludge and sediments

(Ambus *et al*., 1992; Bonin, 1996; Nijburg *et al*., 1997; Tiedje *et al*., 1982). Nowadays, studies showed that DNRA also plays a more important role in terrestrial ecosystems (Silver *et al*., 2001; Müller *et al*., 2004; 2007; Rütting *et al*., 2011).

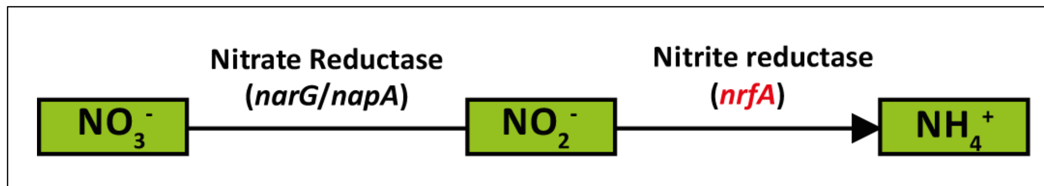

Figure 1.6. DNRA with catalyzing enzymes and the associated functional marker genes. Red = most frequently used marker gene.

In addition to the conversion from NO_3^-/NO_2^- to NH_4^+, N_2O is produced as a byproduct, mainly to avoid intoxication by NO_2^-. A $^{15}NO_3^-$ labeling experiment proved evidence that several microorganisms were capable of simultaneously producing N_2O and NH_4^+ via dissimilatory pathways, but NH_4^+ accounted typically for a majority of total product with > 90 % (Bleakley and Tiedje, 1982; Smith and Zimmerman, 1981). Nevertheless, the production of N_2O by DNRA ranges around 1% of NO_3^-/NO_2^- (Cole, 1988). However, $^{15}NO_3^-$ labeling studies alone cannot resolve the real contribution from DNRA to the overall N_2O emission from the environment, since DNRA as well as denitrification use the same initial electron acceptor (NO_3^-). For this purpose, it is necessary to use additional molecular techniques together with analytical approaches to investigate the activity of the microorganisms that are involved in N_2O emission from soil (Rütting *et al*., 2011).

A pentaheme cytochrome *c* nitrite reductase (NrfA) is the key enzyme catalyzing the reduction of NO_3^- or NO_2^- to NH_4^+ (Einsle *et al*., 1999). The functional marker gene *nrfA* can be found in diverse groups of bacteria including *Proteobacteria*, *Planctomycetes*, *Bacteroides*, and *Firmicutes* (Mohan *et al*., 2004). *nrfA* is commonly used as functional marker gene to detect microbes with the capability to perform DNRA (Smith *et al*., 2007; Song *et al*., 2014; Welsh *et al*., 2014). However, some bacteria are even capable of DNRA without possessing

nrfA, they process a putative reverse hydroxylamine:ubiquinone reductase module pathway (Hanson *et al*., 2013), for which so far no functional marker gene was found.

Anaerobic ammonium oxidation (ANAMMOX)

ANAMMOX is the anaerobic microbial process to convert NH_4^+ together with NO_2^- to N_2 (Fig. 1.7). Since it requires both oxidized and reduced inorganic N-compounds and anoxic conditions, it occurs at oxic/anoxic interfaces (Kuypers *et al*., 2003). ANAMMOX was first described in a laboratory-scale denitrification reactor (Mulder *et al*., 1995). Afterwards, ANAMMOX was mainly discovered and analyzed in aquatic environments (Kuypers *et al*., 2003; Stevens and Ulloa, 2008). In marine sediments, ANAMMOX can account for up to 79% of the total N_2 production (Engström *et al*., 2005). It is estimated to be insignificant in soils, since other processes which use the same substrates contribute to N_2 production as well (Long *et al*., 2013). Even though there are several recent studies that focus on ANAMMOX in different soil related environments (paddy soil: Zhu *et al*., 2011; Wang *et al*., 2012; peat soil: Hu *et al*., 2010; reductisol, agricultural soils: Humbert *et al*., 2010), the importance of ANAMMOX in soil N-cycling is not fully understood so far.

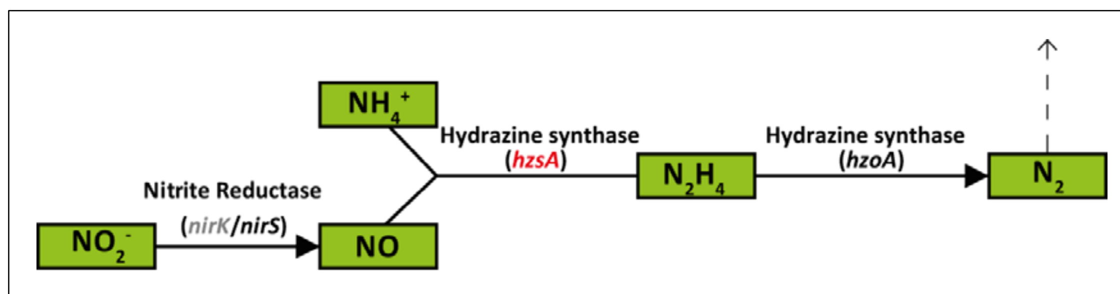

Figure 1.7. The anaerobic ammonium oxidation (ANAMMOX) pathway and the associated enzymes together with their functional marker genes. Dashed lines indicate an emission of this component into the atmosphere. Grey = gene were only found in one organism so far. Red = most frequently used marker gene

The reaction of ANAMMOX takes place inside the anammoxosome: an intracytoplasmic compartment formed by a single ladderane lipid-containing membrane (Van Niftrik *et al*., 2004). Three enzymes are important for the conversion from NH_4^+ + NO_2^- to N_2 in the anammoxosome: nitrite reductase (*nirS* [Strous *et al*., 2006] or *nirK* [Hira *et al*., 2012]), hydrazine synthase (*hzs*) and hydrazine oxidoreductase (*hzo*). NO_2^- is reduced by nitrite reductase to NO and with NH_4^+ further metabolized by *hzs* to hydrazine (N_2H_4). Afterward N_2H_4 is oxidized by *hzo* to N_2. The gene *hzsA* encoding a part of the hydrazine synthase is used as a functional marker gene for ANAMMOX, because the whole cluster is unique to ANAMMOX bacteria (Harhangi *et al*., 2012; Russ *et al*., 2013). The ANAMMOX reaction is only performed by autotrophic bacteria of the phylum *Planctomycetes* (Fuerst and Sagulenko, 2011) within the order *Brocadiales* (Jetten *et al*., 2010).

Nitrification

Nitrification is the oxidation from NH_4^+ to NO_3^- with the intermediate product NO_2^- (Fig. 1.8). Nitrification includes two steps, ammonium oxidation and nitrite oxidation. Hydroxylamine (NH_2OH), NO and N_2O are produced as byproducts. As mentioned before, nitrification together with denitrification, contributes up to 70% of global N_2O emission from soils (Conrad, 1996, Butterbach-Bahl *et al*., 2013).

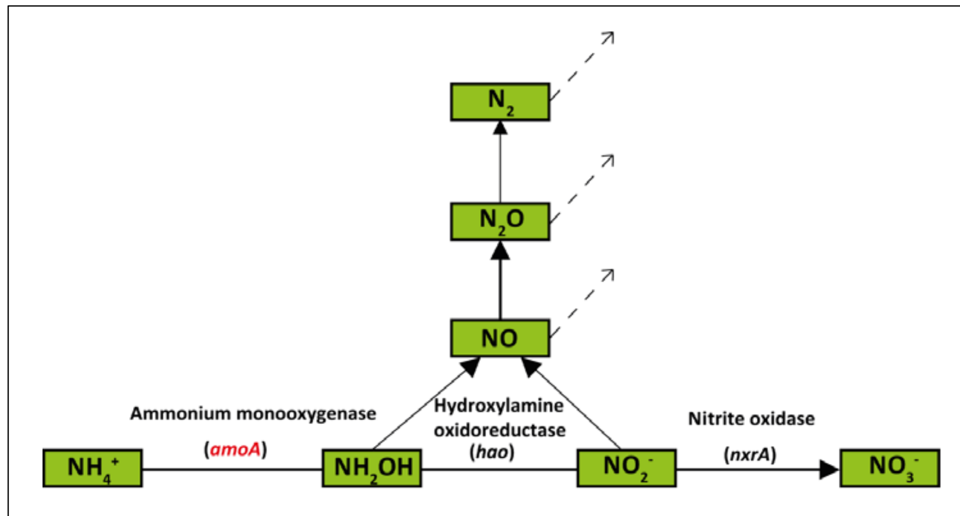

Figure 1.8. The nitrification pathway with intermediates and side products. The enzymes of the main process with their functional marker genes are stated between each step. Dashed lines indicate an emission of this component into the atmosphere. Red = most frequently used marker gene.

Ammonium oxidation is catalyzed by two different enzymes; NH_4^+ is oxidized to NH_2OH by the membrane-bound ammonium monooxygenase (AMO encoded by the *amoABC* operon (Sayavedra-Soto *et al*., 1998; Hommes *et al*., 1998). The *amo* operon occurs in multiple, nearly identical copies in all ammonia oxidizer strains that have been examined to date (Norton *et al*. 1996; 2002). The second step, the oxidation from NH_2OH to NO_2^- is catalyzed by the periplasm-associated enzyme hydroxylamine oxidoreductase (HAO). Bacteria as well as Archaea were found to be capable of ammonia oxidation, they are termed AOA (ammonia oxidizing archaea) and AOB (ammonia oxidizing bacteria). Both possess an AMO, but Archaea are lacking the HAO (Stahl and Torre, 2012). It is still unclear how AOA convert NH_2OH to NO_2^-. A possible scenario is that nitroxyl (HNO) is the intermediate product from AOA instead of hydroxylamine (Walker *et al*., 2010). Additionally, in genome analyses of two AOAs two plastocyanin-like proteins were found which are shared between all AOAs. These redox-active copper proteins may participate in electron transfer from the unknown product of ammonia oxidation (e.g., hydroxylamine or nitroxyl) to a membrane-bound electron transfer chain (Stahl and Torre, 2012). By chemical decomposition, NH_2OH

can also be reduced to NO and N_2O (Frame and Casciotti, 2010; Hooper and Terry, 1979; Wrage *et al*., 2005). However, formation of NO_2^- is always the main pathway, while concentrations of NO and N_2O produced are several orders of magnitude lower (10^3–10^6) than those of NO_2^- (Arp and Stein, 2003). Several studies have observed a difference in the behavior of AOA and AOB to environmental factors, such as pH (Nicol *et al*., 2008), salinity (Mosier and Francis, 2008) and heavy metal concentrations (Li *et al*., 2009; Mertens *et al*., 2009), suggesting that these two groups might occupy distinct ecological niches (Kelly *et al*., 2011).

The second step of nitrification from NO_2^- to NO_3^-, nitrite oxidation is catalyzed by the membrane-bound nitrite oxidoreductase (NXR). NXR contains multiple subunits (NxrABC), iron-sulfur centers and a molybdenum cofactor (Kirstein and Bock, 1993; Meincke *et al*., 1992; Sundermeyer-Klinger *et al*., 1984). Till now only nitrite oxidizing Bacteria (NOB) were found to possess the *nxr* cluster.

NO_2^- can also be reduced by autotrophic nitrifier denitrification (ND) to N_2O via NO. Thereby, the N_2O is mainly produced by AOB, because some AOA are capable to produce NO, but not N_2O. For N_2O production, homologues of enzymes as in denitrification are used (NirK and NorB) (Kowalchuk and Stephen, 2001; Cantera and Stein, 2007). Even though it is possible that N_2O is further reduced to N_2 (Poth, 1996), the main product is NO_3^-.

The most common functional marker gene to detect ammonia oxidizers in soils is *amoA* (Rotthauwe *et al*., 1997; Junier *et al*., 2010; Hernandez *et al*., 2014; Li *et al*., 2015). It is mainly used, because AOB and AOA possess both an exploit homologous *amoA* and can be compared to each other, even though different sets of primers are used. Furthermore, ammonia oxidation is the first and rate-limiting step of nitrification in soils.

1.3. Parameters that can influence nitrogen transformations in soils and the microorganisms involved

Many physical and chemical parameters (e.g. pH, oxygen availability, N-compounds accumulation, temperature, etc.) in the environment can influence the transformation of N, by interaction with the microbial communities involved in the N cycle and their functioning (Fig. 1.9). Conditions that favor one pathway are often counterproductive to other processes in the N-cycle.

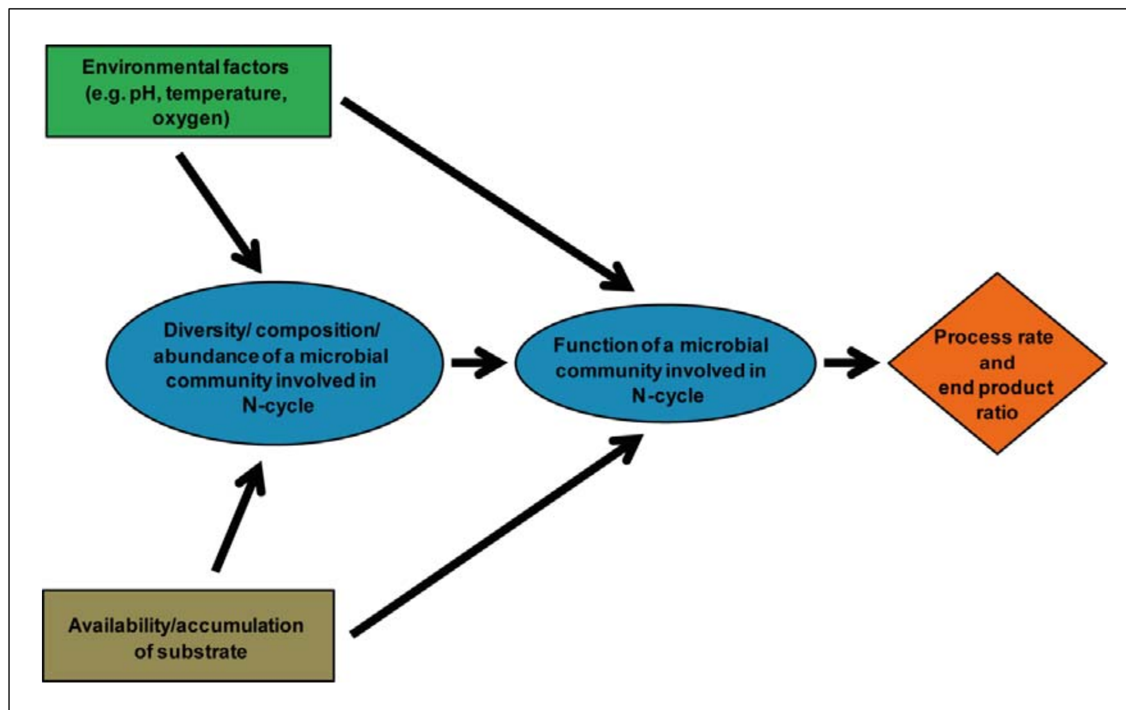

Figure 1.9. Hypothetical connection between the environment, the microbial community and their influence on the nitrogen processes (modified from Balser *et al*., 2006 and Braker, 2012).

Oxygen availability and N-compounds in the soil

The major factor which controls the different processes in the N-cycle is the availability of oxygen. While nitrification requires oxygen, denitrification, DNRA and ANAMMOX need an anoxic habitat. Only N-fixation can occur under both oxic and anoxic

conditions (Goldberg *et al*., 1987; Stal and Krumbein, 1985). In soils one of the major regulators of oxygen partial pressure is the water content. Hence, nitrification is the main source of N_2O fluxes from well-aerated soils (water-filled pore space, WFPS < 60%), while N_2O production in wet soils (WFPS 60–90%) is predominantly derived from anaerobic denitrification (Bateman and Baggs, 2005; Mathieu *et al*., 2006; Skiba *et al*., 1997). Nevertheless, also well aerated soils can have a tight linkage between denitrification and nitrification, in the form of 'hot spots' which provide anoxic zones in soil aggregates for denitrification (Parkin, 1987; Kremen *et al*., 2005). The presence of NO_2^- and low oxygen partial pressure are the predominant exogenous signals that induce the activation of the denitrification system (van Spanning *et al*., 2007). However, considerable variability exists among microbial strains in their response to these signals and thus in N_2O production (Bergaust *et al*., 2008; Ferguson, 1994; Ka *et al*., 1997; Miyahara *et al*., 2010; Saleh-Lakha *et al*., 2008; Zumft, 1997). Oxygen partial pressure is also a significant factor in differencing between archaeal and bacterial ammonia oxidation, because AOA often have a higher affinity for oxygen than AOB (Chen *et al*., 2008; Jung *et al*., 2011; Pitcher *et al*., 2011). However, different ecotypes appear even within the AOA which are better adapted to suboxic conditions (Gleeson *et al*., 2010; Molina *et al*., 2010).

Soil physical parameters such as texture and clay content can affect N turnover in soils in several ways. Sandy soils have a lower water holding capacity than fine-textured soils and tend to have higher soil organic carbon concentrations (Sutton *et al*., 2011). The most important soil chemical parameters which influence the rates of N-cycling are soil organic carbon (SOC), carbon:nitrogen (C:N) ratio and total NO_3^-/NH_4^+-concentrations (Sutton *et al*., 2011). Especially, increasing SOC leads to higher N_2O emission rates from soils (Li *et al*., 2005; Keeney and Sahrawat, 1986). A reason for N leaching and gaseous N losses on the ecosystem scale was identified in soil C:N ratios (Gundersen *et al*., 1998; Klemedtsson *et al*., 2005). Additionally, as mentioned before a high C:N ratio, with a low NO_3^--concentration

favors DNRA over denitrification (Tiedje, 1982; 1988). The C:N ratios has also an impact on the AOA community, which are promoted by lower C:N ratios (Bates *et al*., 2010). In contrast, a high relative availability of NO_3^- is likely to stimulate denitrification, while a high amount of NH_4^+ favors nitrification. The increase in NH_4^+-concentration in a soil through fertilization does not only lead to an increase of nitrification activity, but also of denitrification activity (Avrahami *et al*., 2002). The overall AOB community was relatively unaffected by increasing NH_4^+-concentration, only the transcriptionally active community was influenced (Avrahami *et al*., 2003). Only if a high amount of NH_4^+ (200 µg NH_4^+-N g^{-1} soil) is added AOB abundance seem to increase, in contrast abundance AOA rise already after the addition of a 10-fold lower NH_4^+ concentration (Prosser and Nicol, 2012).

In general, fertilization stimulates denitrification and nitrification and leads to an increase of N_2O-emission from soils. Here, denitrification benefits more from organic fertilizer (e.g. compost, manure) than from mineral forms (e.g. extracted from minerals or produced industrially) (Dambreville *et al.*, 2006; Ellis *et al.*, 1998; Enwall *et al.*, 2005; Wolsing and Priemé, 2004). Fertilizer also influenced denitrifier and nitrifier community structure and abundance (Hallin *et al.*, 2009; Avrahami *et al*., 2003). In the root-rhizosphere complex (part of the soil which is influenced by plants) the addition of a high amount of NH_4^+/NO_3^- fertilizer lead to an increase of AOB abundance compared to AOA (Kastl *et al*., 2015). Furthermore, addition of tons of fertilizer and the long-term agricultural land use resulted in significant shifts of AOB community. The application of nitrification inhibitors in agricultural soils is one of the most promising approaches for increasing N-utilization efficiency and reducing N_2O emission to environment (Yi *et al*., 2014). The diversity and abundance of N_2-fixing bacteria tended to increase with periods of organic agricultural management. For instance, in a comparative study on different field types, the highest abundance of *nifH* was observed in the bulk soil and rhizosphere after five years of organic

management. Additionally, C:N ratio was the most important factor influencing the community composition and abundance of N_2-fixing bacteria (Shu *et al.*, 2012).

Even though, a change in soil parameters has an impact on the microbial community involved in N-cycling, in most cases these effects occur mainly through accessory effects, such as pH changes following fertilization (Enwall *et al.*, 2005).

pH

pH is often mentioned as one of the most important factor in the N cycle, especially with regard to denitrification. Acidic pH leads to an accumulation of N_2O by denitrification processes (Liu *et al.*, 2010; 2014; Simek and Cooper, 2002). This is believed to occur mainly through a post-translational inhibition of N_2O reductase (Bergaust *et al.*, 2010). Additionally, also the energy gains increased by -20 [kJ/mol N] in denitrification under a decrease of pH from 7 to 4 (Wrage *et al.*, 2001). Acidic pH also has negative effects on the expression of the denitrifier genes. A less diverse denitrification gene pool was observed in acidic soil compared to neutral soils (Čuhel *et al.*, 2010; Fierer and Jackson, 2006; Braker *et al.*, 2012). It was also shown, that transcriptional activation under acidic pH was reduced in an incubation of a denitrifier community extracted from a soil with an initially neutral pH (Brenzinger *et al.*, 2015). Especially *nirS*-type denitrifiers seemed to be impaired by acidic pH (Čuhel *et al.*, 2010). Nitrification was also directly influenced by acidic pH, AOA were favored over the growth of AOB (Nicol *et al.*, 2008; Robinson *et al.*, 2014; Yao *et al.*, 2011). However, the opposite occurred in soils with a high N-amount, such as in grazed grassland soils under urine patches, where AOB being primarily responsible for NH_4^+ oxidation (Di *et al.*, 2009; 2010). For AOA, a detailed phylogenetic analysis showed the coherence between composition of AOA in soil and the respective pH value (Gubry-Rangin *et al.*, 2011; Oton *et al.*, 2015).

Thereby, several lineages of AOA seem to be adapted to specific pH ranges (Gubry-Rangin *et al*., 2011). Also, N_2O production from autotrophic nitrification can be increased by acidic pH (Martikainen and de Boer 1993). Several studies to explore the effect of pH on DNRA yielded partly contradictory results. Higher DNRA was associated with alkaline conditions (Nommik, 1956; Stevens *et al*., 1998; Fazzolari-Correa and Germon, 1991; Gamble *et al*., 1977). In contrast other studies showed that DNRA increased at lower pH (< 4) in poorly drained soils, which was related to soluble C content (Waring and Gilliam, 1983). Therefore, contrasting findings of pH effects on DNRA may partly be related to soil C availability and, hence, be of indirect nature (Rütting *et al*., 2011). A reason for acidification of soil could be N_2-fixation by legumes. Short term proton excretion into the rhizosphere can lower soil pH, with significant variation in the acidification potential of different legume species (McLay *et al*., 1997).

Temperature and soil moisture

Anthropogenic induced climate changes have also an impact on N_2O emissions. It was shown that biochemical processes which result in N_2O emissions are strongly influenced by water content and temperature rise. For example, as soils get warmed, microbial decomposition increase (Bond-Lamberty and Thomson, 2010), which further leads to higher N_2O emission rates. Therefore, temperature and moisture are major influences on temporal and spatial scales, but temperature stimulating effect on the microbial N cycling is greater if soil moisture concentration is not a limiting factor (Sutton *et al*., 2011). It was previously reported that temperature together with soil moisture concentration is another important factor influencing nitrification (Allen *et al*., 2005; Avrahami *et al*., 2003; Liu *et al*., 2015; Tourna *et al*., 2008; Yuan *et al*., 2005). It was also shown that nitrification can occur at very low temperatures (Jones and Morita 1985; Jones *et al*., 1988) as well as high temperatures (Lebedeva *et al*., 2005). Two thermophilic AOA were cultivated recently (Hatzenpichler *et*

al., 2008; De la Torre *et al*., 2008). Furthermore, temperature was the most important factor in controlling growth and diversity of AOA and AOB in aquarium biofilters. However, the role of AOA in this system is still unclear (Urakawa *et al*., 2008; Wu *et al*., 2013). The source of NO_3^- depends also on the temperature, at low temperature (15°C) NO_3^- results from heterotrophic nitrification, while autotrophic nitrification is the source at higher temperatures (25/30°C) (Liu *et al*., 2015). However, the optimal temperature range for nitrification is narrow between 15 to 25°C (Dalias *et al*., 2002; Grundmann *et al*., 1995). Nitrification as well as the N_r mineralization increase with rising temperatures up to ~ 30°C (Shaw and Harte, 2001).

The optimum temperature for denitrification lies between 25°C and 35°C (Kesik *et al.*, 2006; Saad and Conrad, 1993a; Saad and Conrad, 1993b). Temperature plays also an important role with regard to denitrification rates, the ratio between the end products N_2O/N_2 and denitrification activity, especially at moderate temperature locations (Malhi *et al.*, 1990; Paul and Clark, 1989; Saad and Conrad, 1993a; Maag and Vinther, 1996). With increasing temperature denitrification activity also increases (Nömmik, 1956; Gödde and Conrad, 1999; Braker *et al*., 2010). Increasing temperatures led to higher NO-production from denitrification as well as from nitrification from clay and silt loam soil (Gödde and Conrad, 1999), higher nitrate reductase activity and N_2O-production in a forest soil (Szukics *et al.*, 2010) and a generally higher activity of a denitrifier community in an agricultural soil (Braker *et al*., 2010). Additionally, Braker *et al*. (2010) showed that the composition of *nirK-/nirS*-type denitrifier communities changed and that the abundance of nitrate reducers increase with higher temperatures. For both, nitrification and denitrification increased temperatures resulted in higher N_2O emission, due to the increase of absolute nitrification rate, denitrification rate and their N_2O/NO_3^- ratios (Benoit *et al*., 2015). Whereas below 20°C, N_2O was essentially produced by denitrification rather than by nitrification, the ratio of N_2O emitted per unit of nitrate reduced or produced steadily increases with temperature (Benoit *et al*., 2015). The

downside of higher temperature is the increase of N losses by higher N_2O emissions; this effect can be buffered by the stimulation of DNRA at higher temperatures, which led to a fresh increase of NH_4^+ into the system (Rubol *et al*., 2013). For instance, in a North Sea estuary DNRA and denitrification occurred at all temperatures, but DNRA was favored at the extremes of the temperatures applied (< 14 and > 17°C) while temperatures in between (14 to 17°C) favored denitrification (Kelly-Gerreyn *et al*., 2001). The influence of temperature on DNRA was also observed for coastal sediments, where a large seasonal variation of DNRA was attributed to a temperature increase in summer, which increases sediment oxygen consumption, thus creating more reduced conditions in the sediment (Ferrón *et al*., 2009; Gardner and McCarthy, 2009; Smyth *et al*., 2013). Previous results showed that the relative importance of DNRA rates is higher in temperate climates (Rütting *et al*., 2011). Under tropical temperatures DNRA rates increased more than 10 fold relative to denitrification, due to the higher affinity for NO_3^- (Dong *et al*., 2011).

Temperature is also for N_2 fixation one of the most important control factors (Hartwig, 1998). Under both, low (e.g., in arctic and alpine regions) and high temperatures N_2-fixation and nodulation are increasingly handicapped (e.g., due to nodulation failure) and N_2-fixation can be more affected than plant growth (Hartwig, 1998).

Vegetation and roots exudates

N is a very important nutrient for plants. Thus, there is a competition for N between plants and microbes involved in soil N cycling. Especially denitrifiers and nitrifiers compete with plants for the main N-compounds NH_4^+ and NO_3. Amino acids or other monomers play only a role in extremely N-poor and cold ecosystems where N-mineralization from soil organic matter is limited (Schimel and Chapin, 1996). As a consequence, microorganisms

have a higher affinity for NO_3^- and NH_4^+ at low concentrations of mineral N compared to plants (Kuzyakov and Xu, 2013). Studies showed that after addition of ^{15}N, the N uptake by microorganisms was higher than uptake by plants, not just because of higher substrate affinities, but also due to their larger surface area to volume ratios as well as faster growth rates compared to plants (Hodge, 2004; Schimel and Bennett, 2004). In longer trial periods, the plant uptake of ^{15}N supplements increased, based on the gradual release of microbial ^{15}N into the soil (Harrison *et al*., 2007). Nevertheless, addition of ammonium nitrate fertilizer of up to 100 kg ha^{-1} resulted in a depletion of NH_4^+ and NO_3^- in the root-rhizosphere layer, only the addition of higher amounts of fertilizer increased NH_4^+ and NO_3^- concentration in the RRC (Kastl *et al*., 2015). Plant N-uptake relies on a transport system in the plasma membrane of root cells and mechanisms that regulate the activity of N transport systems and root growth according to plant growth requirements. External factors, such as soil NH_4^+/NO_3^-, organic N compounds, soil pH, light, temperature as well as internal factors such as C and N metabolites have an influence on the plants and regulate their N uptake (Jackson *et al*., 2008).

However, plants have also a positive feedback on the microbial communities associated with the roots or rhizosphere. With the excretion of low and high molecular compounds (primarily by exudation of C-compounds) as root exudates and rhizodeposition (Brzostek *et al*., 2013; Whipps and Lynch, 1990), the activity of microbial communities and the activity of the soil N-cycle can be stimulated (Bird *et al*., 2011; Cheng, 2009). Additional studies showed that it is probably the labile C input into the soils that increases the N cycling and the activation of microbial biomass (Holz *et al*., 2014). Further, Holz *et al*. (2014) observed that NH_4^+ over NO_3^- is the preferred N source for roots and microorganisms. Plants positively influenced DNRA rates and impaired autotrophic nitrification by the release of nitrification inhibitors and by influencing ammonium availability. Through the release of oxygen and labile organic carbon from the rhizosphere, also nitrate reducers were stimulated in their diversity and abundance (Kofoed *et al*., 2012).

It is still not clear if the plant species or the presence of a plant has a greater impact on the N-cycle and the microbial community involved in N-cycling. In shortgrass steppe Vinton and Burke (1995) stated that the presence of a plant had a greater impact on the N-cycle than the plant species differences. This result was supported by other studies which showed presence or absence of a plant is more important than plant species for nutrient, especially N availability (Charley and West, 1975; Clarholm, 1985; Groos et al., 1995; Jackson and Caldwell, 1993a; 1993b; Robertson *et al*., 1988). Nevertheless, also plant species can have an impact on the N cycle, as it has been demonstrated by a 16-week laboratory incubation of soils with different plant species. There, it was shown that different plant species can significantly influence soil C and N cycling rates, but even after 15 yr the magnitude of the effect was still very small (Chen and Stark, 2000).

Higher C input from plants into the soil led to an increase of abundance, activity and growth of microbes in the rhizosphere (Blagodatskaya *et al*., 2009; 2011; Kapoor and Mukerji, 2006; Oger *et al*., 2004; Saharan and Nehra, 2011), which consequently consume the remaining available nutrients through microbial uptake and immobilization (Zak *et al*., 2000). In addition, not solely the amount but also the composition of the C substrate can have an effect on microbial community composition (Nielsen *et al*., 2011). Not only the C input originating from root exudates, but also degraded plant litter influence in the N cycle. A high C:N ratio in plant litter increases microbial N-immobilization, which then increases NH_4^+ and NO_3^- availability for plants (Booth *et al*., 2005). Additionally, the oxygen flux through the taro sterm and root system into flooded sediment can be an important driver for nitrification and coupled denitrification (Penton *et al*., 2013). Higher *nosZ*/*amoA* abundance and a domination of *nirS*-type nitrite reducers in sediments were observed in treatments with vegetation compared to ones without (Penton *et al*., 2013). Not only the plant itself plays a major role, but also mycorrhizas that are associated with plants have a great impact on the microbial community. Arbuscular mycorrhizal fungi (AMF) form a symbiotic association

with the majority of plants. Their presence leads to a negative correlation with *nirK* and a positive correlation with *nosZ*, which leads to a decrease in N_2O emissions (Bender *et al.*, 2014). The same study pointed out that disruption of the AMF symbiosis through intensification of agricultural practices may contribute to increased N_2O emissions. Other studies observed changes in the denitrification activity in the presence of plants (Bremer *et al.*, 2007; 2009; Cavigelli and Robertson, 2000; Dandie *et al.*, 2007). Bremer *et al.* (2009) reported that the combination of sampling time and plants as well as presence of plants had an effect on the composition of the *nirK*-type denitrifier community and denitrification enzyme activity. For example, the presence of specific plant species had an influence on the structure of a nitrate reducing community (Patra *et al.*, 2006). The higher carbon availability in the rhizosphere is another important factor stimulating denitrification and emissions of the greenhouse gas N_2O (Henry *et al.*, 2008). For example, plant roots were reported to increase denitrification rates in the rhizosphere up to 22-fold in comparison to unplanted soil (Philippot *et al.*, 2009). Legumes or the decomposed parts seem to have a particularly stimulating effect on the denitrification activity (Kilian and Werner, 1996; Scaglia *et al.*, 1985; Aulakh *et al.*, 1991), presumably due to the symbiosis with rhizobia, in which many are capable of denitrification. The impacts of plants on the two dissimilatory NO_3^- reducing pathways (DNRA and denitrification) are not well characterized. Both pathways were strongly dependent on the presence of plants in wetland soils (Matheson *et al.*, 2002). In unplanted wetland soil, DNRA was the primary mechanism of NO_3^- removal, accounting for almost half of the added ^{15}N-NO_3^-, whereas in planted wetland soils denitrification was the principal mechanism of NO_3^- removal and DNRA were insignificant (Matheson *et al.*, 2002). Contrary to these results, Nijburg *et al.* (1997) reported that DNRA was dominant in planted pots compared to unplanted ones.

Stimulation of the activity and abundance of AOB in the rhizosphere of O_2 releasing plants suggests that nitrification is common in places where N is not limited (Bodelier *et al.*,

1996; Briones *et al*., 2002; Engelaar *et al*., 1995). However, nitrification can either be stimulated or inhibited depending on the composition of the root exudates (Hawkes *et al*., 2006; Subbarao *et al*., 2009). Exotic grasses can increase the nitrification rates in soil, which seem to be an important ability for invasive plants (Lee *et al*., 2012). The higher rates are thereby related to an increase in abundance and changes in the composition of AOB. On the other hand the invasive species *Andropogon gayanus*, which prefers NH_4^+ over NO_3^- as a N source, inhibits nitrification but stimulates ammonification (Rossiter-Rachor *et al*., 2009). Some studies reported that invasive plants could also modify denitrification and N_2-fixation (Wardle *et al*., 1994; Dassonville *et al*., 2011). Together, these studies show that plants can cause altered microbial N-transformations, but additionally can also benefit from them, which is of importance for ecosystem functioning and plant community structure. Also nitrification, like denitrification, was influenced by planted or unplanted treatments. Breidenbach *et al*. (2015) observed a higher abundance of some taxa involved in nitrification in unplanted soil compared with soil planted with rice after fertilization with NH_4^+. A possible explanation is the lack of competitors on NH_4^+ in the unplanted pot. Rhizodepostion and root exudates can also have a negative feedback on microbial communities, as observed for archaeal/ bacterial *amoA* and *nosZ*. These groups were significantly less abundant in rhizosphere soil compared with bulk soil, because under N limitation, the growth of rhizosphere nitrifiers and denitrifiers depended on their competition with rice roots for N (San-An *et al*., 2014).

Bacterial and archaeal communities involved in the N-cycle

The microorganisms that are involved in the N-cycle constitute a diverse community. In addition to the previously mentioned parameters microorganisms are crucial for the rates and activity in the N-cycle, because all products in the N-cycle are directly produced by microorganisms. But all of these influences are also affecting each other. Thus, changes in

activity in the N-cycle can in some cases be attributed to a concatenation of variables that influence each other. In the end, to decide which boundary determines the function is difficult, if not impossible. Presumably, each parameter has an equally decisive role. It is also assumed that the relationship between denitrifiers and their functioning may be ecosystem specific (Rich and Myrold, 2004). Wallenstein *et al.* (2006) stated that the activity of the denitrification enzymes may depend either on environmental factors and/ or denitrifier community composition. Significant correlations between potential denitrification rates and microbial community patterns in wetlands also underlined role of denitrifier composition for ecosystem functioning (Peralta *et al*., 2010; Song *et al*., 2011; Rich *et al*., 2003). However, a general correlation between denitrifier community structure and denitrification rates in soils does not exist (Boyle *et al.*, 2006; Enwall *et al.*, 2005; Hallin *et al.*, 2009; Rich and Myrold, 2004, Song *et al*., 2012). Previous studies have shown that pH-dependent responses in denitrification product ratios in soils were related to the size and composition of the underlying denitrifier communities (Dörsch *et al.*, 2012; Čuhel *et al.*, 2010). In contrast, other studies found no significant relationship between microbial communities and microbial processes including denitrification (Boyle *et al*., 2006; Ma *et al*., 2008). However, there is a substantial agreement that as denitrification potential and rates changes with time and site, the dynamics of denitrifying communities must have an impact on these denitrification processes (McGill *et al*., 2010; Philippot and Hallin, 2005). In some cases only parts of the denitrifier community showed a direct effect for the rates of denitrification, e.g. an influence of *nirS*-type but not *nirK*-type denitrifiers (Enwall *et al*., 2010) or the opposite (Bremer *et al*., 2009; Braker *et al*., 2012; Dörsch *et al*., 2012). Cavigelli and Robertson (2000) suggested that different physiological characteristics between denitrifier communities, including enzyme kinetics and enzyme sensitivity to environmental parameters, could lead to different denitrification rates or N_2O production rates. This assumption is based on a study of two geomorphologically similar soils, which had different denitrification rates and end product

ratios, even though the parameters that regulate the denitrification activity were optimal. In a following study, Cavigelli and Robertson (2001) analyzed the communities of these two soils and found differences in the composition of the denitrifier communities. Additionally, isolates from these two soils also showed physiological differences in their denitrification rates. However, more isolates are needed to get a better understanding about the relationship between denitrification rates/ end product ratios and denitrifier diversity, because even strongly related species often showed different denitrification activities (Falk *et al.*, 2010; Fesefeldt *et al.*, 1998; Hashimoto *et al.*, 2009). It is even more important to identify single isolates from the environment, under the assumption that already one specialized species can change the function of the surrounding (Salles, *et al*., 2009). With these isolates, the role of single species in the N-cycle will likely be clarified by a combination of cultivation-based approaches and molecular ecological techniques (Hayatsu *et al*., 2008). The composition of denitrifier communities in an acidic peat land soil provide a source and sink for N_2O (Palmer and Horn, 2012), although an acidic pH lead to a higher N_2O emission (Bakken *et al*., 2012; Bergaust *et al*., 2010). These acidic peatland soils represent reservoirs of diverse acidic tolerant denitrifiers (Palmer *et al*., 2012; Palmer and Horn, 2012).

Since community composition alone could not explain change in denitrification, it was assumed that instead the abundance of denitrifiers was more important for the function of a microbial community (Hallin *et al*., 2009). But this hypothesis is also controversial. While several studies observed a positive correlation between abundance and function (Hallin *et al.*, 2009; Morales *et al.*, 2010; Szukics *et al.*, 2010; Throbäck *et al.*, 2007), others found no correlation (Dandie *et al.*, 2008; Miller *et al.*, 2008; 2009; Morales *et al.*, 2010). Alternatively the ratio of N_2O producers (*nirS* + *nirK*) and N_2O reducers (*nosZ*) might be more suited to explain higher or lower N_2O emission (Billings and Tiemann, 2014; Čuhel *et al*., 2010; Morales *et al*., 2010; Philippot *et al*., 2011; Müller *et al*., 2014). However, again the

correlation seemed to be dependent on habitat and environmental conditions (Deslippe *et al*., 2014; Morales *et al*., 2010; Philippot *et al*., 2011).

However, only few studies focusing on the other important microbial groups involved in the N-cycle. For instance, an increase in the diversity of *amoA* is associated with a N_2O emission event (Smith *et al*., 2010). Also the abundance of nitrifiers plays a role in nitrification and the emission of N_2O by nitrification (Hallin *et al*., 2009). Sometimes AOA abundance exerts a key influence on nitrification (Yao *et al*., 2011) while in other studies the AOB are more important (Di *et al*., 2009, 2010; Shen *et al*., 2008; Wertz *et al*., 2012; 2013). Most studies confirm the observed results for denitrifiers that no general trend for the influence either from the structure or the abundance of the microbial communities exists. As stated before it is more of a mutual influence of all of this factors that lead to changes in the N turnover in soils.

All above mentioned parameters are responsible for the different N-turnover rates in different habitats (Fig. 1.9), but it is strongly habitat/environment dependent in which direction the process rates are altered. Future studies focusing on the combination of the microbial and the ecology data will allow to shed light into the unknown regulatory parameters of the N-cycle.

1.4. Free-Air Carbon dioxide Enrichment (FACE)

Free-Air Carbon dioxide Enrichment (FACE) is a field method in which the concentration of CO_2 for a specified site can be altered to a certain value. This allows studying the influence of higher CO_2 concentrations on various environments under near natural conditions. Since atmospheric CO_2 concentrations increased dramatically from 280 ppm to 400 ppm after the industrial revolution (Fig. 1.1) and increased even faster than previously calculated, it is important to understand the consequences for the environment (IPCC, 2013). Moreover, atmospheric CO_2 concentrations continue to rise by about 1% per year due to anthropogenic emissions and are expected to double in this coming century (IPCC, 2013). As CO_2 is an important greenhouse gas, an increase in CO_2 concentration in the atmosphere has a direct impact on the global warming at earth (IPCC, 2013). FACE facilities usually consist of at least one FACE ring fumigated with elevated atmospheric CO_2 (eCO_2) and an ambient control ring (aCO_2). These rings consist of pipes and vents positioned in a circle, with a diameter of 1-30 m surrounding the experimental sites. Through these pipes, vents and measurement equipment air with elevated CO_2 concentration flows inside of the rings and the CO_2 concentration can be adjusted according to wind speed. FACE rings are preferable to study the impact of increasing CO_2, because they have almost no influence on other environmental conditions (e.g. rain, wind, snow or sun light) compared to, e.g., Open Top-Chambers technique, which provide the CO_2 in plastic containers over the experimental site. In 2006 more than 32 FACE facilities existed worldwide (Nösberger *et al.*, 2006). One of the world-wide longest operating FACE facility is located in Gießen (GiFACE), which also provides the longest continuous trace gas emission (CO_2, CH_4, N_2O) data set (since 1998, and still ongoing).

Previously collected results on the effect of eCO_2 on the nitrogen cycle and the underlying microbial communities in soils

The increase in atmospheric CO_2-concentration can have a great impact on the N-transformation rates in soil. An increased availability of C via *e*CO_2 concentrations leads to an increased transfer of organic C from plants to the soil via rhizodeposition, thereby affecting N-transformation rates as well as microbial community dynamics (Denef *et al*., 2007). As stated before (**see 1.3.**), the interaction between C- and N-cycle is predominantly effected by interactions between plant and soil which determine whether ecosystems function as a carbon source or as a sink (Reich *et al*., 2006). Thereby, a higher demand for N under *e*CO_2 will increase the competition for available N between microbes and plants. It is likely that this correlation then affects plant and microbial community structures, N transformations and production of the important greenhouse gases CO_2, CH_4 and N_2O (Barnard *et al*., 2005; Freeman *et al*., 2004; Van Groeningen *et al*., 2011). Plant N-uptake may decrease the availability of N for microbes (Schimel and Bennett, 2004), which can then lead to a progressive N-limitation and to reduced ecosystem productivity in the long-term (Luo *et al*., 2004). The GiFACE facility observed a stimulation of the plant biomass production by ~ 10% from +20% *e*CO_2 along with a shift in the plant community structure (Kammann *et al*., 2005). Larger plant biomass also requires more N to support growth. This leads to change in the gross N-transformation rates, as shown by a ^{15}N tracing experiment (Müller *et al*., 2009). There, DNRA rates for instance, increased by 141%, caused by a change in the C/NO_3^- ratio, while the rate of heterotrophic nitrification (O_{Nrec}) decreased to almost zero. Furthermore, the total amount of nitrate was significantly lower under *e*CO_2 whereas the concentration of NH_4^+ increased by 17% (Müller *et al*., 2009; Rütting *et al*., 2010). The *e*CO_2 induced shift of available N towards NH_4^+ via increased DNRA is suggested to be an indicator of anoxic soil conditions and a typical feature of N-limited ecosystems to retain mineral N (Tiedje, 1988). One of the most dramatic observations was that an elevation of +20% CO_2 resulted in a more

than two-fold increase in N_2O emissions from a grassland site (Kammann *et al*., 2008). It was hypothesized that a higher N_2O:N_2 ratio during denitrification or enhanced fungal activities might be responsible for enhanced N_2O emissions (Denef *et al*., 2007; Regan *et al*., 2011). It was assumed that the changes in gross N-transformations and gaseous N emission rates mainly depended on the dynamics and the activity of the underlying microbial communities. These changes were thought to be a result of an indirect effect of the higher CO_2 concentration via the excretion of root exudates, since CO_2 concentrations in soils are naturally high (Gobat *et al*., 2004). The higher production of N_2O was predominantly originated from NO_3^- turnover rates (Müller *et al*., 2004), which led to the assumption that either denitrification or DNRA are likely to be responsible for this. However, this hypothesis could not be proven to date.

Several studies investigated the influence of *e*CO_2 on microorganisms, but the results are partially controversial, because the response of microbial communities depends on the plant-soil system and hence are most likely ecosystem dependent. As soil microorganisms are often C-limited, a plant mediated increase in C-supply under *e*CO_2 would be expected to result in growth of the microbial community and in increased microbial biomass. Additionally, this would promote the growth of microorganisms with faster carbon source utilization rates over slow growing ones (Tarnawski and Aragno, 2006). Even if no growth effect would be detectable at least the activity of the microorganisms should be affected. Several studies confirmed this hypothesis by reporting increased microbial growth and community dynamics under *e*CO_2 (Chung *et al*., 2007; Denef *et al*., 2007; Dijkstra *et al*., 2005; Drigo *et al*., 2008; 2009; He *et al*., 2010; Kassem *et al*., 2008). However, others did not find pronounced effects of *e*CO_2 on microbial abundance in soil (Haase *et al*., 2008; Marhan *et al*., 2011; Nelson *et al*., 2010) or even reported negative effects (Hodge *et al*., 1998; Lesaulnier *et al*., 2008). Metagenomic studies (GeoChip) showed that only the abundance of genes involved in the degradation of labile carbon compounds, the N_2-fixation marker gene

(*nifH*) and one of the two nitrite reduction genes (*nirS*) were influenced by *e*CO_2 of +50% (He *et al*., 2010; 2014; Xu *et al*., 2013). With respect to the composition of the microbial community involved in soil N-cycling the same inconsistent picture emerges. Nitrate reducers seem to be unaffected by *e*CO_2 (Deiglmayr *et al*., 2004), although cultivation studies showed that dissimilating *Pseudomonas* were overrepresented in the rhizosphere of a grassland under *e*CO_2 (Fromin *et al*., 2005; Roussel-Delif *et al*., 2005). Even when a community shift occurred for at least a part of the microorganisms involved in N-cycling (ammonium oxidizers) it was additionally related to other factors, such as precipitation and temperature but not exclusively caused by *e*CO_2 (Horz *et al*., 2004). Regan *et al*. (2011) found a similar trend, that soil parameters had stronger effects on a community than a continuous elevation of CO_2.

1.5. Aims of the dissertation

Increasing CO_2 concentrations or changes in the pH on agriculture fields due to anthropogenic influences often lead to changes in the N-transformation rates, along with an increase in N_2O emissions. Nevertheless, it is poorly understood so far how the underlying microbial communities are affected. Therefore, the main objective of this study was to shed light on the response of the overall and active microbial communities to pH shifts or to elevated CO_2 concentrations in soils. A short overview about the aims and the resulting major issues of each single project are given below:

Chapter II: pH-driven shifts in overall and transcriptionally active denitrifiers control gaseous product stoichiometry in growth experiments with extracted bacteria from soil

Understanding the influence of pH on denitrifier communities and their functioning is important, as acidic pH leads to higher N_2O/N_2 product ratios. Further, the composition and size of denitrifier communities in soil are affected by acidic pH. The underlying molecular mechanisms of direct pH control on N_2O emissions are not fully understood, but post-transcriptional impairment of nitrous oxide reductase (N_2OR) by pH < 6.1 has been suggested. *Do communities harbor species, which can process denitrification and N_2O reduction over a wide pH range? Do these communities consist of members with similar phenotype that are adapted to different pH ranges? Is the ability of a soil denitrifier community to reduce N_2O to N_2 entirely controlled by pH-impairment of N_2OR?*

Chapter III: Effect of eCO_2 on microbial communities involved in N cycling in soils

Elevated CO_2 concentrations led to an increase of N_2O emission from soil, but the source of this increase and the role of the microbial communities are not well understood.

Although GiFACE produced the longest continuous trace gas emission data set, detailed molecular analyzes of the microbes that are involved in N-cycling in soils under elevated CO2 are still missing. *Do eCO_2 or other soil parameters (soil moisture concentration, pH value, etc.) have an impact on the overall microbial community? Does knowledge on the composition and/ or abundance help to resolve the linkage between eCO_2 and increased N_2O emission rates?*

Chapter IV: Response to fertilization of transcriptionally active microbial communities involved in N-cycling in soils under eCO_2

The detailed analyses in ***Chapter III*** of the overall microbial community which is involved in N-transformation revealed almost no correlation with eCO_2. Since, the majority of FACE facilities are lacking a comprehensive study, monitoring gas and nutrient fluxes at the same time as the dynamics in the active microbial community, an experiment was constructed to analyse the transcriptionally active microorganisms under eCO_2 and an additional application of a ^{15}N labeled NH_4NO_3 fertilizer to follow the pathways of N_2O formation. *Does eCO_2 alter the active communities in soils compared to aCO_2 at least in part of the community? Which influence does fertilization with nitrogen exert in the microbial community? From which pathway the addition N_2O emission under eCO_2 originated?*

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Chapter II

pH-driven shifts in overall and transcriptionally active denitrifiers control gaseous product stoichiometry in growth experiments with extracted bacteria from soil

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2. pH-driven shifts in denitrifier community

2.1. Abstract

Soil pH is a strong regulator for activity as well as for size and composition of denitrifier communities. Low pH not only lowers overall denitrification rates but also influences denitrification kinetics and gaseous product stoichiometry. N_2O reductase is particularly sensitive to low pH which seems to impair its activity post-transcriptionally, leading to higher net N_2O production. Little is known about how complex soil denitrifier communities respond to pH change and whether their ability to maintain denitrification over a wider pH range relies on phenotypic redundancy. In the present study, we followed the abundance and composition of an overall and transcriptionally active denitrifier community extracted from a farmed organic soil in Sweden (pH_{H2O} = 7.1) when exposed to pH 5.4 and drifting back to pH 6.6. The soil was previously shown to retain much of its functioning (low N_2O/N_2 ratios) over a wide pH range, suggesting a high functional versatility of the underlying community. We found that denitrifier community composition, abundance and transcription changed throughout incubation concomitant with pH change in the medium, allowing for complete reduction of nitrate to N_2 with little accumulation of intermediates. When exposed to pH 5.4, the denitrifier community was able to grow but reduced N_2O to N_2 only when near-neutral pH was reestablished by the alkalizing metabolic activity of an acid-tolerant part of the community. The genotypes proliferating under these conditions differed from those dominant in the control experiment run at neutral pH. Denitrifiers of the *nirS*-type appeared to be severely suppressed by low pH and *nirK*-type and *nosZ*-containing denitrifiers showed strongly reduced transcriptional activity and growth, even after restoration of neutral pH. Our study suggests that low pH episodes alter transcriptionally active populations which shape denitrifier communities and determine their gas kinetics.

2.2. Introduction

Soil N_2O emissions from denitrification depend on environmental conditions that control the rates of denitrification and the N_2O/N_2 product ratio. Important soil and chemical factors are oxygen availability (as affected by soil moisture and respiration), temperature, nitrate availability and pH (Nömmik, 1956; Firestone, 1982; Wijler and Delwiche, 1954). Among these factors, soil pH is one of the most crucial ones, because it does not only affect overall denitrification rates, but more importantly seems to directly control the $N_2O/(N_2O+N_2)$ ratio of denitrification, and hence N_2O emission rates from soils (Šimek and Cooper, 2002; Liu *et al*., 2010; Bakken *et al*., 2012). Denitrification rates increase with higher pH, whereas $N_2O/(N_2O+N_2)$ ratios decrease (Wijler and Delwiche, 1954; Nömmik, 1956; Dörsch *et al*., 2012). Direct inhibition of N_2O reduction by low pH was demonstrated in laboratory experiments with *Paracoccus denitrificans* (Bergaust *et al*., 2010) and with soils from a long-term liming experiment in Norway (Liu *et al*., 2010) and may explain the negative correlation between soil pH and N_2O emission found in certain field studies (e.g. Weslien *et al*., 2009; Van den Heuvel *et al*., 2011).

It is well known that pH also affects the composition and size of denitrifier communities in soil. Acidic soils harbor smaller and less diverse 16S rRNA and denitrification gene pools than neutral soils (Čuhel *et al*., 2010; Fierer and Jackson, 2006; Braker *et al*., 2012). Acidity seems to be particularly detrimental to *nirS*-type denitrifiers, resulting in a strong decrease of *nirS*/16S rRNA gene ratios (Čuhel *et al*., 2010). Whether pH-induced changes in taxonomic denitrifier community composition translate into functional differences is unclear. Several studies have linked potential denitrification rates or kinetics to size and composition of denitrifier communities in soils differing in pH (Braker *et al*., 2012; Bru *et al*., 2010; Cavigelli and Robertson, 2001; Dandie *et al*., 2011), suggesting that pH controls soil denitrification and its product stoichiometry via taxonomic differences. In some

cases, the relative abundance of marker genes for N_2O reducers (*nosZ*) versus N_2O producers (*nirS*, *nirK*, *norB*) explained the (N_2O)/(N_2O+N_2) product ratio (Philippot *et al*., 2011; Morales *et al*., 2010; Billings and Tiemann, 2014), but this correlation seems to depend on habitat and environmental conditions (Morales *et al*., 2010; Philippot *et al*., 2011; Deslippe *et al*., 2014). In a recent study, Jones *et al*. (2014) proposed that soil pH controls the abundance of nitrite reductase genes as well as the abundance of the newly discovered *nos*Z Type II clade in soils with relevance to the soil's ability to reduce N_2O.

The direct effect of low pH on the transcription of denitrification genes has been studied in pure culture (Bergaust *et al*., 2010), soils (Liu *et al*., 2010) and cells extracted from soil (Liu *et al*., 2014). In general, low pH resulted in low numbers of transcripts encoding nitrite reductases (*nirS* and *nirK)* and N_2O reductase (*nosZ)* (Bergaust *et al*., 2010; Liu *et al*., 2010), but the *nosZ*/*nirK* transcript ratio did not change. Interestingly, transcription of *nirS* seemed to be more suppressed by acidity than of *nirK* (Liu *et al*., 2010), but it is unclear how this affects N_2O emissions. The underlying molecular mechanisms for direct pH control on N_2O emissions are not fully resolved, but post-transcriptional impairment of nitrous oxide reductase (N_2OR) by $pH < 6.1$ has been suggested (Liu *et al*., 2014).

Together, this raises three basic questions: i) is the ability of a soil denitrifier community to reduce N_2O to N_2 entirely controlled by pH-impairment of N_2OR? ii) do communities harbor organisms which can thrive over a wider pH range without losing N_2O reductase activity? or iii) are communities functionally redundant in that they contain distinct members with similar phenotypes adapted to different pH? In the present study, we approached these questions in a model community obtained by extracting microbial cells from a soil with neutral pH. The extracted cells were incubated in pH adjusted batch experiments and we followed the dynamics of denitrifying communities through the analysis of functional genes *nirK*, *nirS* and *nosZ* and their gene expression while monitoring gas kinetics at high

resolution. The community was extracted from a farmed organic soil in Sweden (SWE, native pH 7.1) which had been previously found to retain much of its functioning (low N_2O/N_2 ratios) in pH manipulation experiments (pH 5.4/7.1) (Dörsch *et al*., 2012). This finding was attributed to a species-rich denitrifier community, and hence to high functional diversity (Braker *et al*., 2012). Here, we revisited the pH manipulation experiment of Dörsch *et al*. (2012) and followed functional gene abundance and diversity of the overall denitrifier (ODC) and the transcriptionally active denitrifying community (TADC) throughout anoxic growth, covering a transient pH range from 5.4 to 7.1. We hypothesized that the inherent alkalization ensuing anoxic growth of denitrifiers induces a succession of taxonomically distinct but, in terms of pH adaptation, functionally redundant denitrifier populations, thus supporting complete denitrification to N_2 over a wide pH range. Since gene expression does not necessarily result in functional enzymes at low pH (e.g. Bergaust *et al*., 2010), we compared shifts in transcripts to those in DNA over time, hypothesizing that only taxa expressing functional enzymes would propagate in the growing culture. In this way we assessed whether sustained function (here: complete denitrification to N_2) would be linked to structural changes in the underlying community.

2.3. Materials and Methods

2.3.1. Soil sample

The soil was originally sampled from a Terric Histosol (FAO) in Sweden and has been used in several studies exploring functional characteristics of denitrification (Klemedtsson *et al*., 2009; Holtan-Hartwig *et al*., 2000; 2002; Dörsch and Bakken, 2004; Dörsch *et al*., 2012) and underlying denitrifier communities (Braker *et al*., 2012). The neutral pH of the organic

soil is due to inclusion of lacustrine limestone from a former lake bottom. Detailed soil characteristics are given in Dörsch *et al*. (2012). By the time of the present study, the soil had been stored moist at 4°C for 15 years.

2.3.2. Cell extraction and incubation conditions

Cell extraction was performed as described previously (Dörsch *et al.*, 2012) with the following modification: Instead of two portions of 50 g soils, four portions were used to recover a higher total cell number. Pellets with extracted cells were resuspended in a total volume of 75 mL filter-sterilized bi-distilled water and stirred aerobically for 0.5-1 h to inactivate any existing denitrification enzyme prior to inoculation into a He-washed hypoxic mineral medium (0.7 µM O_2; see below).

The mineral media contained (L^{-1}): 200 mg KH_2PO_4, 20 mg $CaCl_2$, 40 mg $MgSO_4$, 3.8 mg Fe-NaEDTA, 0.056 mg LiCl, 0.111 mg $CuSO_4$, 0.056 mg $SnCl_2$, 0.778 mg $MnCl_2$, 0.111 mg $NiSO_4$, 0.111 mg $Co(NO_3)_2$, 0.111 mg TiO_2, 0.056 mg KI, 0.056 mg KBr, 0.1 mg $NaMoO_4$. The medium was buffered with 25 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethane-sulfonic acid) and was supplemented with 3 mM of the electron acceptor KNO_3 and 3 mM Na-glutamate as carbon and nitrogen source. The medium had an initial pH of 5.1. Two aliquots of sterile autoclaved medium were adjusted to pH 5.4 and pH 7.1, respectively by adding 1 N NaOH to the medium. Two sets (15 each) of 120 mL-flasks were filled with 43 ml of medium of either pH 5.4 or pH 7.1, resulting in 30 sample flasks in total. Additional flasks were used as blanks without adding cells extracted from the soil. The serum flasks were crimp sealed with butyl septa and made near-anoxic (~ 0.7 µM O_2) by six cycles of evacuation and He-filling using an automated manifold while stirring the suspension with magnetic stirrers at 500 rpm (Molstad *et al.*, 2007).

2.3.3. Incubation, gas analyses and sampling

Denitrification activity was measured directly after inoculation with the cells by denitrification product accumulation. Thirty serum flasks, three blanks, three calibration standards and two flasks for NO_2^- measurements were placed on a submersible magnetic stirring board (Variomag HP 15; H+P Labortechnik GmbH, Oberschleissheim, Germany) in a 15°C water bath. The water bath is an integrated part of an automated incubation system for the quantification of O_2 consumption and CO_2, NO, N_2O and N_2 production in denitrifying cultures similar to that described by Molstad *et al.* (2007). After temperature equilibration, excess He was released by piercing the bottles with a syringe without plunger filled with 2 ml bi-destilled water to avoid entry of air. The bottles were inoculated with 2 mL of cell suspension, yielding approximate cell numbers of 2×10^9 cells per flask (4×10^7 mL^{-1}). The headspace concentrations of O_2, CO_2, NO, N_2O and N_2 were monitored every 5 h as described by Molstad *et al.* (2007) and Dörsch *et al.* (2012).

The incubation experiments were terminated after 210 h when NO_3^--N added to flasks was recovered as N_2-N. After 0, 12, 26, 48, 70, 96 and 206 h, two to three sample flasks of each pH treatment were sacrificed. Cell densities were determined by spectrophotometry (OD_{600}) and NO_2^- concentrations were measured by a spectrometer according to the international standard ISO 6777-1984 (E). The remaining suspension was centrifuged at 4°C and 8.400 × g and the cell pellet was immediately frozen in liquid nitrogen and stored at -80°C until further use. At each time point the pH in the supernatant was determined.

2.3.4. Extraction of nucleic acids

DNA and RNA were extracted from the frozen cell pellets (-80°C) collected at each sampling point. For this, one or two frozen cell pellets were resuspended in 400 µL sterile

water (Sigma-Aldrich, Taufkirchen, Germany). Nucleic acids were extracted using a modified SDS-based protocol (Pratscher *et al.*, 2011; Bürgmann *et al.*, 2003). In brief, the cells were disrupted in a FastPrep beat-beating system and nucleic acids were recovered from the supernatant using a phenol/chloroform/isoamyl alcohol extraction. Subsequently the nucleic acids were precipitated with polyethylene glycol (PEG) 6000 solution and redissolved in 100 µL of sterile (0.1 µm filtered) nuclease-free (DNase-, RNase-free) and protease-free bi-distilled (Sigma-Aldrich). An aliquot of 20 µL was stored at - 20°C for further DNA-based molecular analyses. The remaining 80 µL were treated with RNase-free DNase (Qiagen, Hilden, Germany) for removal of DNA. RNA was purified using the RNeasy Mini Kit (Qiagen), precipitated with 96% EtOH and resuspended in 15 µL nuclease-free water (Sigma-Aldrich) to increase the RNA concentration and stored at -80°C. The integrity of the RNA was checked on a 1.5% w/v agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and the concentration was determined by a NanoDrop1000 instrument (Thermo Fisher Scientific, Dreieich, Germany). The RNA was reverse transcribed with random hexamer primers (Roche, Mannheim, Germany) and M-MLV reverse transcriptase (Promega, Mannheim, Germany).

2.3.5. Analysis of the composition of *nirK*, *nirS* and *nosZ* genes and transcripts

The composition of the denitrifier community was determined by terminal restriction fragment length polymorphism (T-RFLP). The nitrite reductase genes *nirK* and *nirS* as well as the nitrous oxide reductase gene *nosZ* were amplified from cDNA and DNA using the primer pairs *nirK*1F-*nirK*5R (~ 516 bp), *nirS*1F-*nirS*6R (~ 890 bp), and Nos661F-Nos1773R (~ 1131 bp) and conditions described previously (Braker *et al.*, 1998; 2000; Scala and Kerkhof, 1998). Details on primers and procedures are given in Table S2.1. These primers were chosen to allow for comparison of the results obtained in this study to previous ones (Braker *et al.*,

2012), although different primers to target these genes have been published more recently (e.g. Green *et al.*, 2010; Verbaendert *et al.*, 2014). The forward *nirS* and *nosZ* primer and the reverse *nirK* primer were 5'-6-carboxyfluorescein labeled. The quantity and quality of the PCR product were analyzed by electrophoresis on a 1.5% w/v agarose gel after staining the gel with 3 × GelRed Nucleic Acid Stain (Biotium, Hayward, CA, USA). PCR products of the expected size were recovered from the gel using the DNA Wizard® SV Gel-and-PCR-Clean-up system (Promega). The PCR products of *nirK*, *nirS* and *nosZ* were digested using the restriction enzymes FastDigest *HaeIII*, FastDigest *MspI* and FastDigest *HinP1I* (Thermo Fisher Scientific), respectively, following the manufacturer's specifications. The purified fluorescently labeled restriction fragments were separated on an ABI PRISM 3100 Genetic Analyzer sequencer (Applera Deutschland GmbH, Darmstadt, Germany) and the lengths of fluorescently labeled terminal restriction fragments (T-RFs) were determined by comparison with the internal standard using GeneMapper software (Applied Biosystems). Peaks with fluorescence of > 1% of the total fluorescence of a sample and > 30 bp length were analyzed by aligning fragments to the internal DNA fragment length standard (X-Rhodamine MapMarker® 30-1000 bp; BioVentures, Murfreesboro, TN). Reproducibility of patterns was confirmed for repeated terminal restriction fragment length polymorphism (T-RFLP) analysis using the same DNA extracts. A difference of less than 2 base pairs in estimated length between different profiles was the basis for considering fragments identical. Peak heights from different samples were normalized to identical total fluorescence units by an iterative normalization procedure (Dunbar *et al.*, 2001).

2.3.6. Quantitative analysis of *nirK*, *nirS*, and *nosZ* genes and transcripts

The abundance of *nirK*, *nirS*, and *nosZ* genes and transcripts in the sample flasks was determined by qPCR using primers q*nirK*876-q*nirK*1040, qCd3af-qR3cd, and nosZ2F-

nosZ2R (Henry *et al*., 2004; 2006; Kandeler *et al*., 2006). Details on primers and procedures are given in Table S2.1. The reaction mixture contained 12.5 µL SyberGreen Jump-Start ReadyMix, 0.5 µM of each primer, 3-4.0 mM MgCl2, 1.0 µL template cDNA or DNA and 200 ng BSA mL^{-1} was added. All qPCR assays were performed in an iCycler (Applied Biosystem, Carlsbad CA, USA). Standard curves were obtained using serial 10-fold dilutions of a known amount of plasmid DNA containing the respective fragment of the *nirK-, nirS- and nosZ*-gene. Negative controls were always run with water instead of cDNA or DNA. PCR efficiencies for all assays were between 80-97% with r^2 values between 0.971 and 0.995.

2.3.7. Statistical analyses

All statistical analyses and graphics were done using R version 3.0.1 (R Development Core Team, 2013). Significant differences of *nirK*, *nirS*, *nosZ*, bacterial 16S rRNA gene and transcript abundance as well as the calculated ratios were assessed using ANOVA (P value < 0.05). All quantitative data were log-transformed prior to analysis to satisfy the assumptions of homoscedasticity and normally distributed residuals. The community composition changes in the overall and transcriptionally active denitrifier community by T-RFLP were analyzed using non-metric multidimensional scaling (NMDS) and overall differences were tested by ANOSIM (P < 0.05). Additionally, differences in the composition of transcriptionally active and overall denitrifier communities at a given time point were tested by ANOSIM (P < 0.05). An ANOSIM R value near +1 means that there is dissimilarity between the groups, while an R value near 0 indicates no significant dissimilarity between the groups (Clark, 1993). Non-metric multidimensional scaling (NMDS) analyses were performed with the Bray-Curtis similarity index (including presence and relative abundance of T-RF) which iteratively tries to plot the rank order of similarity of communities in a way that community point distances are exactly expressed on a two-dimensional sheet. The reliability of the test was calculated by a

stress-value. Stress > 0.05 provides an excellent representation in reduced dimensions, > 0.1 very good, > 0.2 good, and stress > 0.3 provides a poor representation. All community composition data were Hellinger-transformed before analysis, in order to reach normal distribution. ANOSIM, ANOVA and non-metric multidimensional scaling (NMDS) were done using package vegan version 2.0-5 (Oksanen *et al*., 2012).

2.4. Results and Discussion

2.4.1. Denitrification kinetics and shifts in abundance and composition of TADC and ODC at native pH 7.1

At native pH 7.1, residual O_2 after He-washing was depleted and all NO_3^- was stoichiometrically converted to N_2 within 96 h of incubation (Fig. 2.1A, B). Net accumulation of gaseous denitrification intermediates was low (< 0.2% of initially present NO_3^--N). Transcriptional activation of functional genes (Fig. 2.2A) and proliferation of denitrifiers containing *nirK* and *nosZ* (Fig. 2.3A, C) started instantly after the cells were transferred to the hypoxic medium. A maximum of relative transcription and community size was reached after 96 hours (Fig. 2.3A, C), ~ 40 h after the start of exponential product accumulation (CO_2, N_2) (Fig. 2.1A, B). The maximum relative transcriptional activity (cDNA/DNA ratio) was low with 0.077 for *nirK* (Fig. 2.3A) and 0.002 *nosZ* (Fig. 2.3C), but efficiently translated into denitrifier growth (Fig. 2.3A, C). The strongest growth occurred for *nosZ*-containing denitrifiers (16,500-fold) while denitrifiers of the *nirK*-type grew 400-fold (Table S2.2). In contrast, growth of *nirS*-type denitrifiers showed a lag-phase of 49 h (Fig. 2.2A, Table S2.2) after which they were transcriptionally activated (cDNA/DNA ratio of 0.11, Table S2.3) and increased in abundance, albeit only 50-fold (Fig. 2.3B). Ratios (*nosZ*/[*nirK* + *nirS*]) of > 50 after 96 h indicated a tendency of enhanced growth of *nosZ*-type denitrifiers compared to

nitrite reducers (Fig. 2.4, Table S2.4) which may explain the efficient conversion of N_2O to N_2 (Philippot *et al*., 2011). However, PCR-based analyses of genes and transcripts may be biased. The primers used do for instance neither target *nirK* genotypes from *Rhodanobacter* species (Green *et al*., 2010) nor thermophilic Gram-positive denitrifiers (Verbaendert *et al*., 2014). The recently postulated *nosZ* clade II (Jones *et al*., 2013; Sanford *et al*., 2012) was also not analyzed in this study. Hence, *nosZ*/(*nirK*+*nirS)* ratios and their response to pH must be taken with caution.

Community composition data indicated selective transcriptional activity, followed by growth of only a few organisms (Fig. S2.1A, S2.2A, S2.3A). Terminal restriction fragments (T-RFs) of 229 bp (representing *nirK* most closely related to *nirK* of *Alcaligenes xylosoxidans*) and of 37 bp length (38 bp *in silico* representing *nosZ* most closely related to *nosZ* of *Pseudomonas denitrificans*, *Ps. stutzeri* and *Ps. aeruginosa*), (Table S2.5) which were of little abundance in or absent from the inocula, respectively, dominated the transcriptionally active *nirK*- and *nosZ*-containing denitrifier communities (Fig. S2.1A, S2.3A). For *nirS*, a genotype most closely related to *nirS* of *Ps. migulae* (105-bp T-RF) was transcriptionally activated and proliferated that was not even detectable in the initial community (Fig. S2.2A). Still, the composition of the transcriptionally active (TADC) and overall denitrifier communities (ODC) converged throughout the first 96 h of incubation as indicated by multi-dimensional scaling of T-RFs (Fig. 2.5A-C; $ANOSIM_{26\text{-}49\ h}$: $P < 0.05$; R between 0.423-0.873; $ANOSIM_{70\text{-}96\ h}$: $P > 0.05$; R between 0.142-0.375). The shifts in denitrifier community composition and the decrease in denitrifier diversity (Shannon index, Fig. S2.1A-3A) did not result in impairment of function, i.e. gaseous intermediates were efficiently taken up and reduced to N_2 (Fig. 2.1A, B). This suggests that it was not the microbial diversity *per se* that mediated the community's functioning, but the specific metabolic capacities of the dominating denitrifying taxa. Transcription of denitrification genes decreased after all nitrogen oxides were depleted (Fig. 2.2A) and the number of transcripts relative to gene

copies became very low (Fig. 2.3A-C). Hence, the increase in diversity and shift in cDNA composition observed for *nirK* and *nosZ*-containing denitrifiers at 206 h was presumably the result of transcript degradation following starvation (Fig. S2.1A, S2.3A).

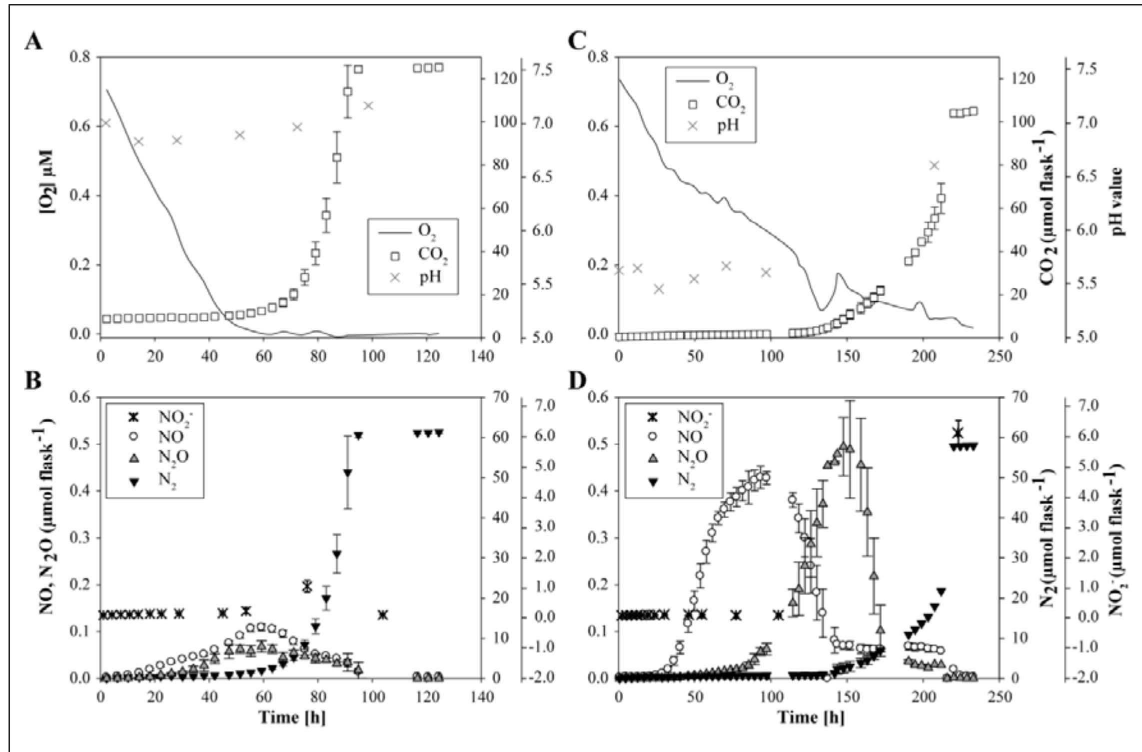

Figure 2.1. Accumulation of O_2, CO_2, NO, N_2O, N_2, NO_2^- and changes in pH value in suspensions of cells extracted from a soil from Sweden at initially pH 7.1 (left panels) and at initially pH 5.4 (right panels) during incubation (0-206 h). (A) O_2, CO_2 concentration and pH value at pH 7.1; (B) NO_2^-, NO, N_2O and N_2 concentration at pH 7.1; (C) O_2, CO_2 concentration and pH value at pH 5.4; (D) NO_2^-, NO, N_2O and N_2 concentration at pH 5.4.

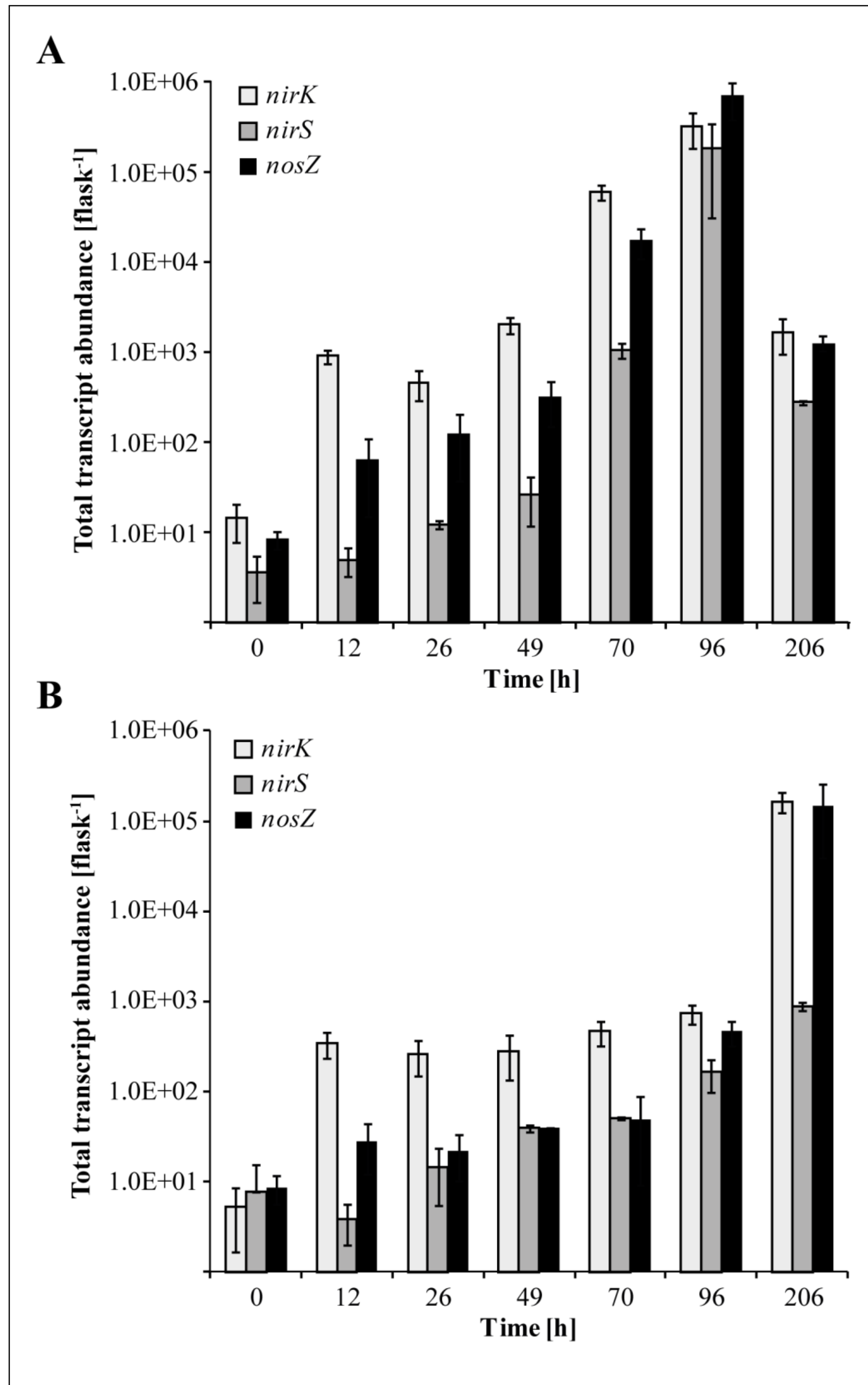

Figure 2.2. Transcript abundance of functional marker genes for denitrification (*nirK*, *nirS,* and *nosZ*) quantified by qPCR. (A) Transcript copy numbers of the incubation at pH 7.1; (B) Transcript copy numbers of the incubation at pH 5.4. (Mean ± SD, n=3).

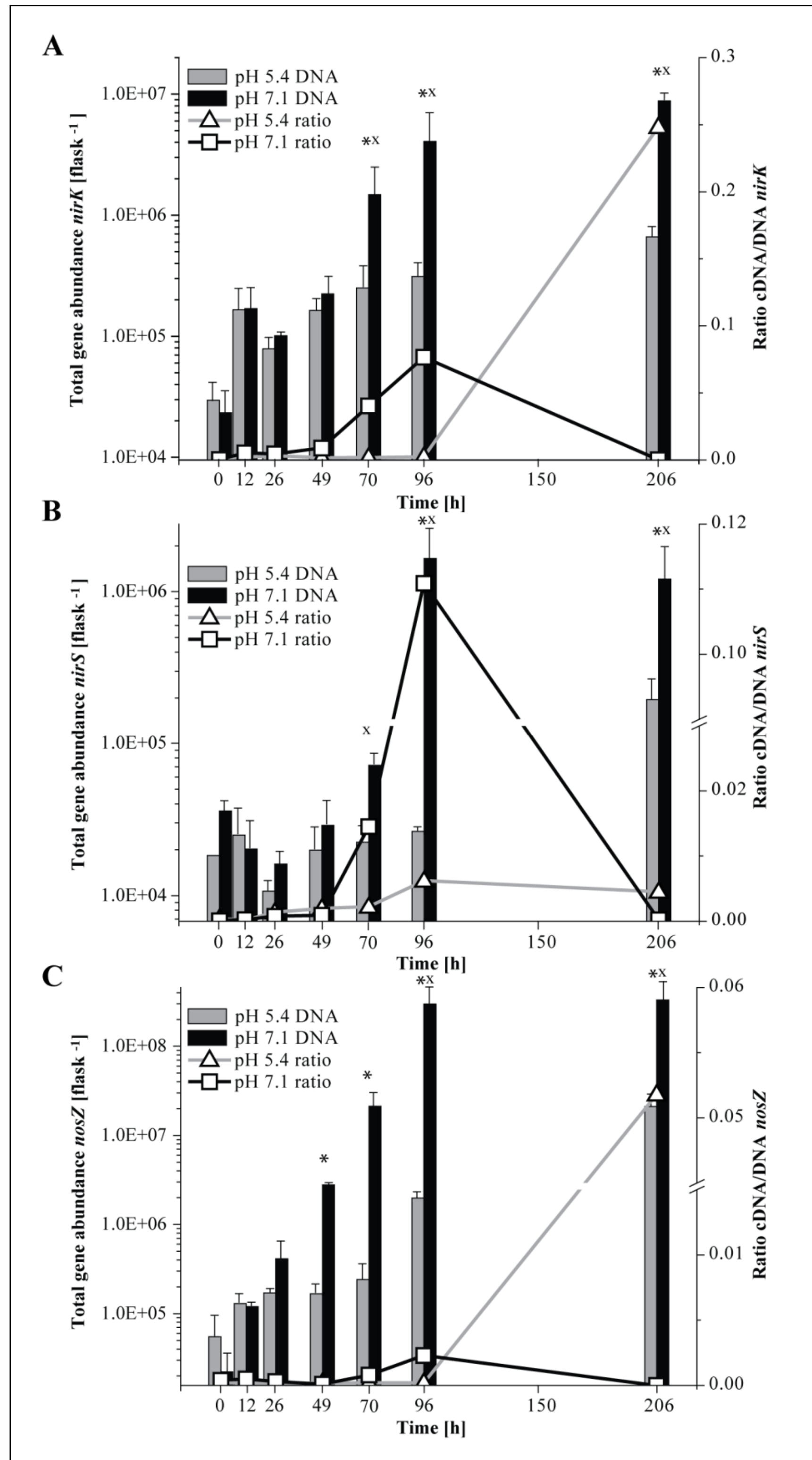

Figure 2.3. Abundance of functional marker genes for denitrification (*nirK*, *nirS,* and *nosZ*) quantified by qPCR and ratio of cDNA/DNA copy numbers. Left axis, total gene abundance and right axis, ratio of cDNA/DNA copy numbers. Bars indicate the total gene copy numbers and the line the cDNA/DNA ratio. An asterisk indicates significant differences in gene abundance, x indicates significant differences in the ratio of cDNA/DNA copy numbers between incubation at pH 5.4 and pH 7.1 at a given time point (ANOVA: $P < 0.05$). (A) *nirK*; (B) *nirS*; (C) *nosZ.* (Mean ± SD, n=3).

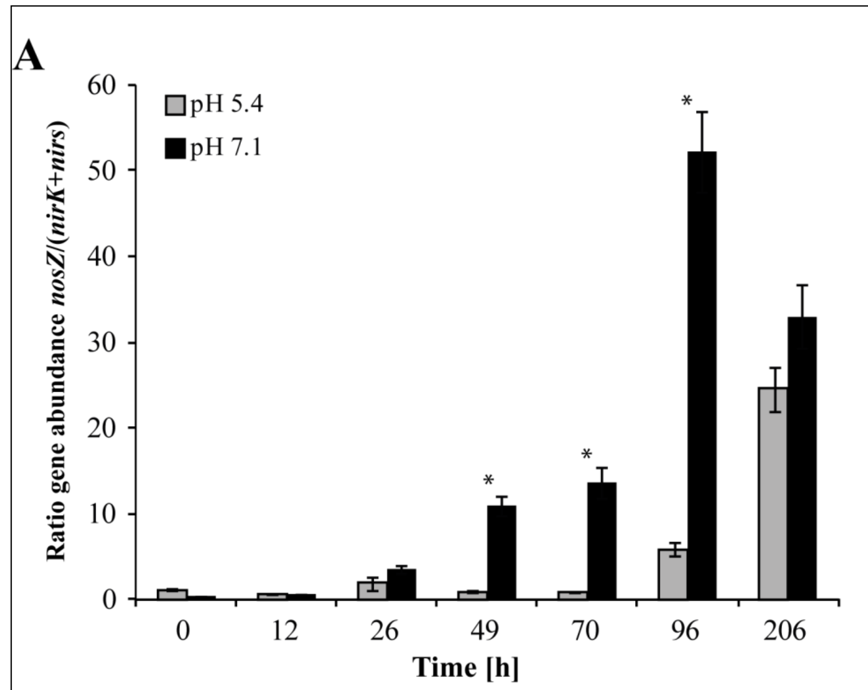

Figure 2.4. Ratio of *nosZ*/(*nirK*+*nirS*) gene and transcript copy numbers . An asterisk indicates significant differences in ratios between incubation at pH 5.4 and pH 7.1 at a given time point (ANOVA: $P < 0.05$). (Mean ± SD, n=3).

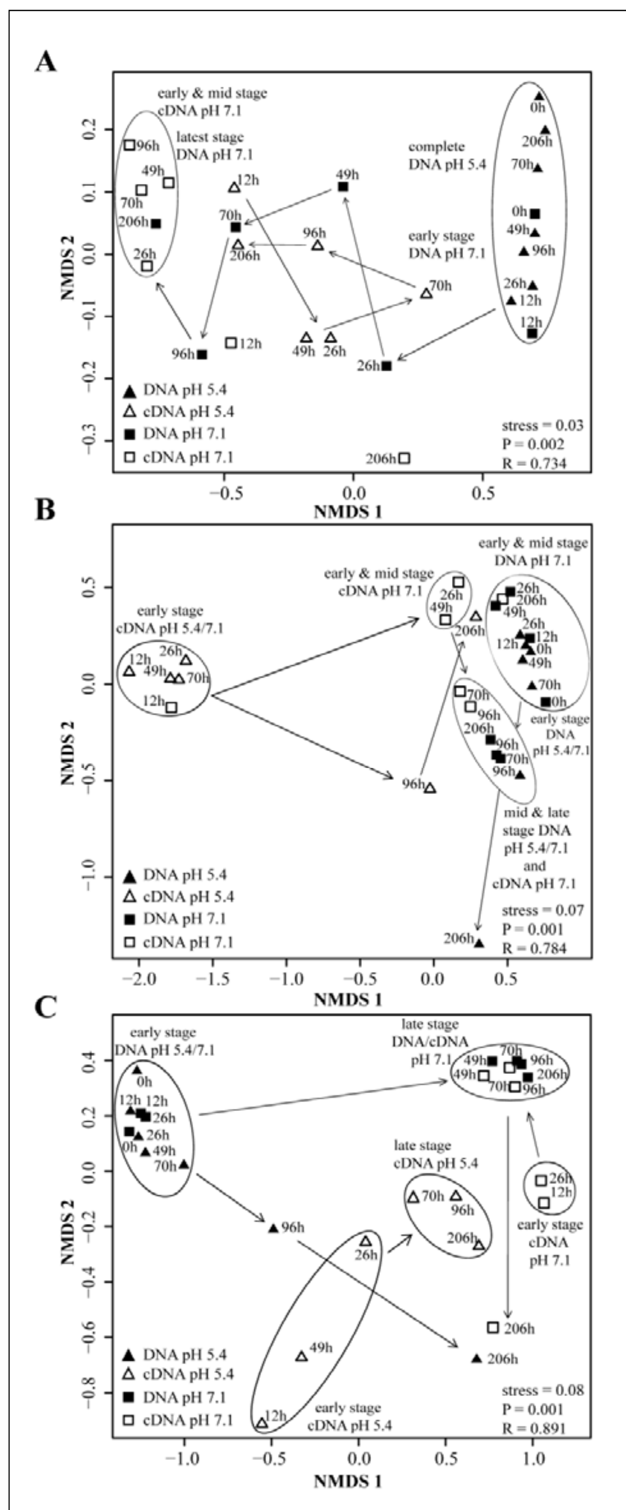

Figure 2.5. NMDS plots of denitrifier communities based on cDNA- and DNA-derived T-RFLP analysis of *nirK*, *nirS* and *nosZ* from three pooled samples. Data points represent averaged results of three replicate T-RFLP analyses. Community similarity was calculated by using the statistical program R and the Bray–Curtis similarity measurement, which includes presence and relative abundance of T-RF. Clusters and arrows were inserted manually to highlight clustering and community development. Significant differences in the composition of denitrifier communities at given time points were determined by ANOSIM ($P < 0.05$). (A) *nirK*; (B) *nirS*; (C) *nosZ*.

2.4.2. Denitrification kinetics and shifts in abundance and composition of TADC and ODC when exposed to low pH

2.4.2.1.Response of denitrification to incubation at acid pH

Exposing the extracted cells to pH 5.4 showed that most of the functionality in denitrification (low accumulation of denitrification intermediates) was retained (Fig. 2.1D). This was reported earlier for the denitrifying community of this soil (Dörsch *et al*. 2012). However, denitrification kinetics were clearly influenced by the initially low pH. Respiration activity (measured as CO_2 accumulation) at pH 5.4 was lower as compared to pH 7.1 (Fig. 2.1C) and NO and N_2O accumulation started approximately 15 h later (Fig. 2.1D). Net production of NO and N_2O was 4- and 9-fold higher, respectively, than at neutral pH and due to slower denitrification kinetics, the reduction of intermediates occurred sequentially. This is in line with previous studies, finding clear pH effects on the accumulation of intermediates in denitrification (Bergaust *et al*., 2010; Liu *et al*., 2010; 2014). For instance, transient accumulation of N_2O by *Paracoccus denitrificans* growing at pH 6.0 was 1,500-fold higher than at neutral pH (Bergaust *et al*., 2010). Liu *et al*. (2010) found that the production of N_2 declined to zero with decreasing pH when comparing soils from a long-term liming experiment with *in situ* pH ranging from pH 4.0 to 8.0. Cells extracted from one of the neutral soils and incubated at pH levels between 7.6 and 5.7 for up to 120 h showed a peculiar pH threshold of 6.1, below which no functional N_2O- reductase was produced (Liu *et al*., 2014). In our study, nitrate was stoichiometrically converted to N_2 with less than 1% net N_2O-N accumulation when incubated at initially pH 5.4 (Fig. 2.1D). However, complete N conversion coincided with a pH shift in the medium (from 5.4 to 6.6) which occurred between 150 and 206 hours of incubation (Fig. 2.1C, D). This shift was most likely driven by the strongly increasing denitrification activity during this period. Denitrification is an alkalizing reductive process, consuming 6 moles H^+ per mol NO_3^- reduced to N_2. CO_2 production was

clearly coupled to total N-gas production and came to a halt when all N-oxides were reduced to N_2 (Fig. 2.1C). This suggests that respiratory processes other than denitrification were absent and that the pH-threshold for N_2O reduction in the medium was overcome by growing denitrifiers which consumed [H^+] (Fig. 2.1C). This suggestion is further supported by the dominance (> 90%) of phylotypes closely related to known denitrifiers at the end of the incubation (Table S2.6). These findings, together with the transient accumulation of NO at pH 5.4, led us to the conclusion that acid tolerant denitrifiers present in the native community must have been metabolically active at pH 5.4, illustrating the high functional versatility of this community with respect to pH.

2.4.2.2.Response of nirK and *nosZ-containing denitrifier communities to incubation at low pH*

We studied how the denitrifier community responded to incubation at initially low pH in terms of growth and transcriptional activation of the functional denitrification genes *nirK*, *nirS* and *nosZ*. Unfortunately, although functional data were collected for the period when the pH shift occurred, due to limitations in the number of samples that could be processed, no community data are available for the period of rapid pH shift. In general, incubation at low pH retarded the transcriptional activation of the functional marker genes (compare Fig. 2.2A and B, Table S2.2). As long as the pH remained stable at about 5.4 (until 96 h), copy numbers of *nirK* and *nosZ* cDNA increased in a range similar to the initial phase of the incubation at pH 7.1 (until 49 h). Moreover, transcriptional activation of *nirK* and *nosZ* at pH 5.4 translated into growth of the communities albeit to a lesser extent than at neutral pH (Fig. 2.3A, C). During the pH shift to 6.6 (96–206 h), presumably concomitant with the exponential accumulation of the N_2, transcript abundances increased reaching their highest densities at the end of the incubation (Fig. 2.2B). However, the increase in denitrifier density was only 11-fold at most and hence less than at pH 7.1 (Table S2.2). Hence, although the relative

transcriptional activity (ratio of cDNA/DNA copies) of *nirK* and *nosZ* exceeded levels at pH 7.1, transcription seemed not to translate into growth as efficiently

2.4.3. Development of transcriptionally active and overall *nirK*-type denitrifier communities when exposed to low pH

Contrary to the incubation at pH 7.1, the composition of the growing ODC in the initially acid incubation changed only marginally and thus differed significantly between the two pH treatments at the end of the experiment. While the development of the ODC at the native pH of the soil (7.1) reflected the composition of the TADC within the first 96 hours (see above), this was not the case with initially acidic pH (Fig.2.5A, S2.1B). Here, TADC patterns clustered separate (ANOSIM: $P < 0.05$; R between 0.742-0.841) from those of the ODC throughout the experiment due to the continuous predominance of the terminal restriction fragment (T-RF) of 229 bp length in the TADC which was of constantly low relative abundance in the ODC (Fig. S2.1B). Thus, we conclude that transcriptional activation of the respective genotypes did not translate into denitrification activity and specific growth of these denitrifiers, suggesting regulation at the post-translational level. Such effects were previously suggested for *nosZ* gene expression in *P. denitrificans* by Bergaust *et al*. (2010) and confirmed by Liu *et al*. (2010; 2014) for soils and extracted cells. Bergaust *et al*. (2010) hypothesized that low pH (6.0) impairs the assembly of N_2O-reductase in *P. denitrificans*, leading to a dysfunctional enzyme and hence accumulation of N_2O.

2.4.4. Development of the transcriptionally active and overall *nosZ*-type denitrifier communities when exposed to low pH

Incubation at initially pH 5.4 altered the *nosZ*-TADC as well as the *nosZ*-ODC but they remained significantly different (Fig. 2.5C; ANOSIM: $P < 0.05$; R between 0.712-0.831). During the first phase of the incubation (up to 70 h) at low pH, growth was small. However, N_2O-reducers present at very low abundance in the native community seemed to be functional. T-RFLP analysis revealed that after a lag phase of 26 and 70 h, T-RFs of 37 bp and 40 bp, respectively, that were present at undetectable levels in the ODC, became transcriptionally activated and increased in relative abundance (Fig. S2.3B). After 96 hours of incubation, the initial community started to be outcompeted by transcriptionally active *nosZ*-containing organisms. While N_2O-reducers (40 bp T-RF) were transcriptionally active in the low pH incubation only and started proliferating in the ODC towards the end of the incubation, the T-RF of 37 bp was detected at both pH levels and even dominated the community at neutral pH. Existence of acid-tolerant denitrifiers containing *nosZ* was previously demonstrated for a nutrient poor acidic fen by Palmer *et al*. (2010) and a riparian ecosystem (Van den Heuvel *et al.*, 2011). Similar to pH 7.1, we observed a tendency of enhanced growth of *nosZ*-containing denitrifiers compared to nitrite reducers as reflected by a *nosZ*/(*nirK*+*nirS*) ratio > 25 after 206 h (Table S2.4) when N_2O was effectively reduced.

2.4.5. Transcriptional activity and development of transcriptionally active and overall *nirS*-type denitrifier communities when exposed to low pH

Transcription of *nirS* was not significantly inhibited by low pH and cDNA copy numbers increased slowly until 96 h (Fig. 2.2B). The response in transcription of the community to incubation resembled that during the first 49 h at neutral pH (Fig. 2.2A). When the pH started to shift back to near neutral (pH 6.6) and vigorous proliferation occurred (as

judged from N gas kinetics), transcription of *nirS* was further enhanced but the high absolute and relative transcription levels observed for *nirK* and *nosZ* were never reached (Fig. 2.2B, 2.3B). This contrasts a recently published study with cells extracted from soil (Liu *et al*., 2014). Liu *et al*. (2014) observed constantly lower *nirK* and slightly increasing *nirS* and *nosZ* transcript numbers during incubation at pH 5.7 and 6.1, as compared to pH 7.6 where transcripts of all three denitrification genes increased equally. However, in that study, starting conditions were different; the community had a native pH of 6.1 and was preincubated under oxic conditions for several hours. Our findings also contrast other results of Liu *et al*. (2014), who found stable, pH-independent cDNA/DNA ratios for *nirS* and *nosZ*, whereas for *nirK* the ratio declined due to efficient growth of the *nirK*-type denitrifier community but constant level of transcription at higher pH. We observed persistently reduced relative *nirS* transcription at low pH compared to pH 7.1 and the growth of *nirS*-type denitrifiers was severely inhibited by low pH during the first 96 h of incubation (Fig. 2.3). A previous pure culture study found that already at slightly acidic pH of 6.8, the *nirS*-type denitrifier *P. denitrificans* was unable to build up a functional denitrification pathway (Baumann *et al*., 1997). Although the nitrite reductase gene was properly induced, the enzyme could not be detected at sufficient amounts in the culture indicating that either translation was inhibited, or once synthesized, nitrite reductase was inactivated, possibly by high concentrations of nitrous acid (HNO_2). In our study, incubation at low pH did not increase NO_2^- until 96 h (Fig. 2.1D), and accumulation of NO was moderate within the nano-molar range (1 µmol NO in the bottle ~ 730 nM in liquid). Moreover, Baumann *et al*. (1997) demonstrated that a functional nitrite reductase assembled at pH 7.5 was still active if the culture was shifted to acidic pH. The cells exhibited a reduced overall denitrification activity, but neither nitrite nor any other denitrification intermediate accumulated which is in agreement with our findings (Fig. 2.1D). Despite the low levels of transcription, the *nirS* TADC shifted but only after 96 h of incubation and surprisingly, the ODC changed at the same time, although DNA copy numbers

did not increase which cannot be explained. Only with the pH upshift between 96 and 206 h, a slight growth (one order of magnitude) occurred but the community developed distinctly from the TADC (Fig. 2.5B; ANOSIM: $P < 0.05$; R between 0.671-0.912). Since the initial abundance of *nirK*- and *nirS*-type denitrifiers in the soil and hence in the inocula was equal, our results indicate a greater robustness of *nirK*-type versus *nirS*-type denitrifier communities to acidity.

2.4.6. Concluding discussion

In this study of a model community, we linked transcriptional activation of denitrification genes (*nirK*, *nirS,* and *nosZ*) and growth of the communities to conversion of nitrogen oxides to N_2. We found a pronounced succession of TADC and ODC in batch incubations even at neutral pH, suggesting a strong selective pressure on the extracted community. Exposure to low pH (5.4) resulted in i) sequential and slightly enhanced transient accumulation of denitrification intermediates (NO, N_2O), ii) lower and/ or retarded transcriptional activation of denitrification genes, together with selective activation of genotypes represented by certain T-RFs and iii) impaired translation into functional enzymes, with consequences for growth of denitrifier communities. However, since only < 1% of added N accumulated as N_2O and NO at low pH, and growth of nitrite- (*nirK*-type) and N_2O-reducers was observed, we conclude that acid-tolerant denitrifier species maintained the functionality of the community as a whole although full conversion of nitrate to N_2 required extended incubation periods. Experiments altering soil pH *in situ* or in laboratory experiments have repeatedly confirmed that denitrification rates and denitrifying enzyme activity are lower in acidic than in neutral or slightly alkaline soils (Simek and Cooper, 2002).

Overall, our results show that different mechanisms may determine the response to low pH of a soil denitrifier community adapted to neutral pH:

i) Activity and proliferation of *nirK*- and *nosZ*- but not of *nirS*-containing denitrifiers seemed to drive reduction of nitrogen oxides which in turn increased pH. Albeit not at the transcriptional level, growth of *nirS*-type denitrifiers was severely inhibited at low pH and occurred only during or after pH upshift. Acid pH has been repeatedly shown to impair nitrite and particularly N_2O reduction in certain denitrifiers (e.g. *P. denitrificans*) (Baumann *et al.*, 1997; Bergaust *et al.*, 2010), in soils (Liu *et al.*, 2014) and in cells extracted from soils (Liu *et al.*, 2010), presumably by impairing the assembly of denitrification enzymes post-transcriptionally (Bergaust *et al.*, 2010, Baumann *et al.*, 1997). Here, we could show that expression of *nirK* in some denitrifiers may be affected as well.

ii) These effects, however, might be compensated by acid-tolerant or acidophilic denitrifier species able to grow and actively denitrify at low pH. Denitrifiers of the *nirK*-type present in the native community of the soil seemed to tolerate a broad range of pH levels as the composition of the growing community remained unaltered during the incubation at low pH.

iii) Low pH prompted growth of *nosZ*-containing denitrifiers of minor abundance in the native community that were acid-tolerant or even acidophilic. At low pH these *nosZ*-containing denitrifiers seem capable of functionally substituting N_2O-reducers that were more prevalent in the native community. This agrees well with the functional redundancy hypothesis that distinct species perform similar roles in communities and ecosystems at different environmental conditions, and may therefore be substitutable with little impact on ecosystem processes (Rosenfeld, 2002).

Previous studies have shown that pH-dependent responses in denitrification product ratios in soils were related to the size and composition of the underlying denitrifier communities (Čuhel *et al.*, 2010; Braker *et al.*, 2012). Large variations have been found in the specific activity of e.g. nitrite reductases (50-fold) even between strains of the same species (Ka *et al.*, 1997). The higher susceptibility of *nirS*-type denitrifiers to environmental stressors

(e.g. low pH, low C-content) has been repeatedly reported in other studies (Bárta *et al*., 2009; Čuhel *et al*., 2010; He *et al*., 2010). The abundance of *nirS* was also most strongly affected when the pH of a grassland was lowered experimentally for about one year resulting in a high *nosZ*/*nirS* ratio while the *nosZ*/*nirK* ratio remained unaffected (Čuhel *et al*., 2010). Hence, long-term exposure to low pH in the natural environment will shape soil microbial communities and predetermine a dominance of either *nirK* or *nirS* (Chen *et al*., 2015). This strongly suggests that taxonomic composition matters for the capability of a soil denitrifier community to effectively denitrify. On the other hand, bulk soil pH is unlikely to be homogeneous in structured soils, probably providing a range of pH habitats distributed throughout the soil matrix. Thus, the occurrence of e.g. N_2O reduction in acidic soils can be explained by denitrification activity in neutral microsites as proposed by Liu *et al*. (2014) or by acid-tolerant denitrifiers being present in neutral soils. Consequently, soil denitrifier communities might be comprised of taxa differing in pH sensitivity, which jointly emulate the kinetic response of a soil to pH change.

2.5. Acknowledgements

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2.7. Supplementary Material

Table S2.1. Primer sets and PCR conditions used to amplify *nirK*, *nirS* and *nosZ* for T-RFLP analysis and cloning (top) and qPCR (bootom).

Gene	Primer sets	Forward primer/Reverse primer	Amplicon length (bp)	PCR conditions	References
nirK	*nirK*1F/ *nirK*5R-FAM	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA/ GCCTCGATCAG(A/G)TT(A/G)TGG	514	95 °C/5min, 10 cycles of (95°C/30sec, 56°C/40sec (-0.5°C every cycle), 72°C/40sec), 25 cycles (95°C/30sec, 54°C/40sec, 72°C/40sec), 72°C/7min.	Braker *et al*., 1998
nirS	*nirS*1F-FAM/ *nirS*6R	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T/ CGTTGAACTT(A/G)CCGGT	890	95 °C/5min, 10 cycles of (95°C/30sec, 56°C/40sec (-0.5°C every cycle), 72°C/40sec), 25 cycles (95°C/30sec, 54°C/40sec, 72°C/40sec) 72°C/7min.	Braker *et al*., 1998
nosZ	nosZ661F-FAM/ nosZ1773R	CGGCTGGGGGCTGACCAA/ ATRTCGATCARCTGBTCGTT	1100	95 °C 5min, 10 cycles of (95°C/30sec, 59°C/90sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 56°C/40sec, 72°C/2min) 72°C/10min.	Scala and Kerkhof, 1998
nirK	q*nirK*876/ q*nirK*1040	AT(C/T)GGCGG(A/C/G)A(C/T)GGCGA/ GCCTCGATCAG(A/G)TT(A/G)TGGTT	165	95 °C/15min, 6 cycles of (95°C/15sec, 63°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 58°C/30sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	Henry *et al*., 2004
nirS	qCd3af/ qR3cd	AACG(C/T)(G/C)AAGGA(A/G)AC(G/C)GG/ GA(G/C)TTCGG(A/G)TG(G/C)GTCTT(G/C)A(C/T)GAA	425	95 °C/15min, 6 cycles of (95°C/15sec, 63°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 58°C/30sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	Kandeler *et al*., 2006
nosZ	nosZ2F/ nosZ2R	CGC(A/G)ACGGCAA(G/C)AAGGT(G/C)(A/C)(G/C)(G/C)GT/ CA(G/T)(A/G)TGCA(G/T)(G/C)GC(A/G)TGGCAGAA	267	95 °C/15min, 6 cycles of (95°C/15sec, 65°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 60°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	Henry *et al*., 2006

Table S2.2. Abundance of functional marker genes for denitrification and of the respective reverse transcribed mRNA (cDNA). Analysis of variance (ANOVA) was performed to test for differences in copy numbers at different sampling times during the incubation at given pH.

Time [h]	*nirK* DNA pH 7.1	*nirK* cDNA pH 7.1	*nirS* DNA pH 7.1	*nirS* cDNA pH 7.1	*nosZ* DNA pH 7.1	*nosZ* cDNA pH 7.1	*nirK* DNA pH 5.4	*nirK* cDNA pH 5.4	*nirS* DNA pH 5.4	*nirS* cDNA pH 5.4	*nosZ* DNA pH 5.4	*nosZ* cDNA pH 5.4
0	2.3E+04^{A} ± 4.6E+04	1.4E+01^{A} ± 3.5E+00	3.6E+04^{A} ± 5.4E+03	5.0E+00^{AD} ± 8.0E+00	2.2E+04^{A} ± 1.3E+04	8.4E+00^{A} ± 3.1E+00	3.0E+04^{A} ± 1.2E+04	5.2E+00^{A} ± 2.4E+00	1.8E+04^{A} ± 9.7E+03	7.6E+00^{A} ± 8.0E+00	4.3E+04^{A} ± 3.9E+04	8.7E+00^{A} ± 2.2E+00
12	1.7E+05^{A} ± 6.0E+04	9.1E+02^{BCD} ± 1.1E+02	2.0E+04^{A} ± 9.0E+03	5.0E+00^{AD} ± 1.8E+00	1.2E+05^{B} ± 1.0E+04	6.30E+01^{AB} ± 1.5E+01	1.6E+05^{A} ± 7.7E+04	3.4E+02^{A} ± 1.1E+02	2.4E+04^{A} ± 2.1E+04	3.8E+00^{A} ± 1.8E+00	9.3E+04^{A} ± 6.5E+04	2.8E+01^{AB} ± 1.0E+01
26	1.3E+05^{A} ± 4.7E+04	4.6E+02^{C} ± 1.1E+02	1.6E+04^{A} ± 3.0E+03	9.8E+00^{A} ± 9.1E+00	4.1E+05^{BC} ± 2.2E+05	1.2E+02^{AB} ± 1.1E+01	7.9E+04^{A} ± 1.0E+04	2.6E+02^{A} ± 1.0E+02	1.0E+04^{A} ± 1.7E+03	1.4E+01^{A} ± 3.4E+01	1.5E+05^{A} ± 2.5E+04	2.1E+01^{A} ± 8.0E+00
49	2.2E+05^{B} ± 7.3E+04	2.0E+03^{D} ± 1.4E+02	2.9E+04^{A} ± 1.0E+04	2.6E+01^{A} ± 3.0E+00	2.8E+06^{C} ± 1.0E+06	3.1E+02^{B} ± 2.8E+01	1.6E+05^{A} ± 3.2E+04	2.7E+02^{A} ± 1.0E+02	1.9E+04^{A} ± 7.6E+03	3.7E+01^{A} ± 2.1E+01	1.6E+05^{A} ± 4.0E+04	4.0E+01^{A} ± 2.1E+01
70	1.5E+06^{B} ±6.1E+05	6.0E+04^{E} ± 1.4E+02	7.2E+04^{A} ± 1.2E+04	9.3E+02^{BD} ± 1.5E+00	2.1E+07^{D} ± 8.9E+06	1.7E+04^{C} ± 2.9E+03	2.5E+05^{A} ± 1.0E+05	4.6E+02^{A} ± 1.4E+02	2.2E+04^{A} ± 5.9E+03	5.0E+00^{A} ± 1.5E+00	2.4E+05^{B} ± 1.1E+05	4.5E+01^{AB} ± 1.0E+01
96	4.1E+06^{B} ± 1.3E+06	2.6E+05^{F} ± 1.7E+02	1.7E+06^{B} ± 5.6E+05	1.4E+05^{C} ± 6.6E+01	3.0E+08^{E} ± 1.8E+08	6.8E+05^{D} ± 1.0E+05	3.1E+05^{B} ± 8.4E+04	7.1E+02^{B} ± 1.2E+02	2.6E+04^{A} ± 1.3E+03	1.6E+02^{B} ± 8.8E+01	1.9E+06^{B} ± 3.0E+05	4.6E+02^{B} ± 1.4E+02
206	8.8E+06^{B} ± 1.0E+06	1.7E+03^{BD} ± 4.2E+04	1.2E+06^{B} ± 7.0E+05	1.9E+02^{D} ± 9.1E+01	3.3E+08^{E} ± 2.5E+08	1.2E+03^{C} ± 1.4E+02	6.6E+05^{B} ± 1.2E+05	1.6E+05^{B} ± 4.2E+04	1.9E+05^{A} ± 1.1E+05	8.8E+02^{B} ± 9.2E+01	2.1E+07^{C} ± 1.0E+07	1.4E+05^{C} ± 7.0E+04

^{ABCDEF} Identical letters behind numbers indicate non-significant differences in copy numbers ($P < 0.05$).

Table S2.3. Ratios of reverse transcribed mRNA (cDNA) to DNA copies. Analysis of variance (ANOVA) was performed to test for differences in copy numbers at different sampling times during the incubation at given pH.

Time [h]	Ratio *nirK* cDNA/DNA pH 7.1	Ratio *nirS* cDNA/DNA pH 7.1	Ratio *nosZ* cDNA/DNA pH 7.1	Ratio *nirK* cDNA/DNA pH 5.4	Ratio *nirS* cDNA/DNA pH 5.4	Ratio *nosZ* cDNA/DNA pH 5.4
0	0.0003^{A} ± 0.00020	0.0001^{A} ± 0.00005	0.0004^{A} ± 0.00013	0.0002^{A} ± 0.00005	0.0004^{A} ± 0.00033	0.0002^{A} ± 0.00010
12	0.0053^{BC} ± 0.00295	0.0003^{A} ± 0.00023	0.0005^{AB} ± 0.00051	0.0021^{B} ± 0.00104	0.0002^{A} ± 0.00020	0.0002^{A} ± 0.00732
26	0.0046^{B} ± 0.00245	0.0008^{AB} ± 0.00024	0.0003^{A} ± 0.00006	0.0033^{B} ± 0.00210	0.0014^{A} ± 0.00079	0.0001^{A} ± 0.00003
49	0.0090^{C} ± 0.00165	0.0009^{AB} ± 0.00052	0.0001^{A} ± 0.00012	0.0017^{B} ± 0.001053	0.0020^{AB} ± 0.00137	0.0002^{A} ± 0.00011
70	0.0403^{C} ± 0.03819	0.0150^{BC} ± 0.0041	0.0008^{B} ± 0.00106	0.0019^{B} ± 0.00130	0.0022^{A} ± 0.0004359	0.0002^{A} ± 0.00021
96	0.0770^{CD} ± 0.04991	0.1109^{C} ± 0.07318	0.0023^{B} ± 0.00118	0.0026^{B} ± 0.00115	0.0062^{AB} ± 0.00355	0.0002^{A} ± 0.00009
206	0.0002^{A} ± 0.00016	0.0002^{A} ± 0.00017	0.0001^{A} ± 0.00005	0.2483^{C} ± 0.12707	0.0045^{A} ± 0.03787	0.0518^{B} ± 0.00334

^{ABCDEF} Identical letters behind numbers indicate non-significant differences in copy numbers ($P < 0.05$).

Table S2.4. Ratios of DNA copies and copies of reverse transcribed mRNA (cDNA) of *nosZ/nirK+nirS*. Analysis of variance (ANOVA) was performed to test for differences in copy numbers at different sampling times during the incubation at given pH.

Time [h]	Ratio *nosZ*/(*nirK*+*nirS*) DNA pH 7.1	Ratio *nosZ*/(*nirK*+*nirS*) cDNA pH 7.1	Ratio *nosZ*/(*nirK*+*nirS*) DNA pH 5.4	Ratio *nosZ*/(*nirK*+*nirS*) cDNA pH 5.4
0	0.374^{A} ± 0.2351	0.469^{AB} ± 0.16563	1.147^{A} ± 0.5823	0.680^{A} ± 0.3271
12	0.632^{AB} ± 0.3080	0.069^{B} ± 0.0568	0.683^{A} ± 0.1913	0.080^{A} ± 0.3728
26	3.524^{BC} ± 2.0967	0.258^{AB} ± 0.0808	1.910^{AB} ± 0.9579	0.079^{A} ± 0.053865
49	11.007^{CD} ± 6.0650	0.153^{AB} ± 0.0152	0.911^{A} ± 0.0721	0.126^{A} ± 0.0360
70	13.630^{CDE} ± 3.4480	0.280^{AB} ± 0.6764	0.883^{AB} ± 0.7085	0.094^{A} ± 0.0611
96	52.220^{E} ± 11.7591	1.367^{C} ± 0.4061	5.860^{BC} ± 1.4262	0.519^{A} ± 0.2910
206	32.971^{DE} ± 4.4718	0.615^{AC} ± 0.6176	24.573^{C} ± 10.7139	0.883^{A} ± 0.5300

^{ABCDEF} Identical letters behind numbers indicate non-significant differences in copy numbers ($P < 0.05$).

Table S2.5. Assignment of cloned *nirK*, *nirS*, and *nosZ* gene and cDNA amplicons to terminal restriction fragments. Amplicons of *nirK*, *nirS*, (Braker *et al*., 1998), and *nosZ* (Scala and Kerkhof, 1998) from cDNA and DNA after 206 h of incubation were cloned into the pGEM-T vector and used to transform *Escherichia coli* JM109 competent cells (Promega, Mannheim, Germany). After blue-white selection inserts were sequenced (LGC Genomics, Berlin, Germany) and the nearest neighbor was determined by reconstructing phylogenetic gene trees using the ARB software package (Ludwig *et al*., 2004).

Gene	T-RF length (bp)	pH 5.4		pH 7.1		Organisms with most similar gene sequence and respective restriction site
		cDNA	DNA	cDNA	DNA	
nirK	65	-	+	+	+	*Alcaligenes faecalis*
	106	+	+	-	-	*Pseudomonas entomophila*
	153	-	+	-	-	*Mesorhizobium* sp.
		-	+	-	+	*Pseudomonas* sp. G-179
		-	+	-	-	*Rhodobacter* sp.
		-	+	-	-	*Rhodobacter sphaeroides*
	188	+	+	+	+	*Mesorhizobium* sp.
	229	+	+	+	+	*Alcaligenes xylosoxidans*
nirS	95	+	+	+	+	*Herbaspirillum* sp.
	99	-	+	+	-	*Pseudomonas fluorescens*
		-	+	+	+	*Pseudomonas migulea*
	105	+	+	+	+	*Ps. migulea*
	141	+	+	-	+	*Ps. migulea*
nosZ	38	-	+	-	+	*Brachymonas denitrificans*
		+	+	+	+	*Pseudomonas stutzeri*, *Pseudomonas aeruginosa*
	40	-	+	+	-	*B. denitrificans*
		-	+	+	-	*Ps. stutzeri*, *Ps. aeruginosa*
	148	-	+	+	-	*Ps. fluorescens*
		+	-	-	-	*Ps. stutzeri*, *Ps. aeruginosa*

bp, base pairs
+, sequence with restriction site corresponding to terminal restriction fragment (T-RF)
-, T-RF not detected

Table S2.6. Relative abundance of cloned bacterial 16S rRNA cDNA and DNA sequences after 206 h of incubation. Amplicons of 16S rRNA (primers Eub8-27F-Eub1392-1407R; Amann *et al*., 1995) from cDNA and DNA were cloned into the pGEM-T vector and used to transform *Escherichia coli* JM109 competent cells (Promega). After blue-white selection inserts were sequenced (LGC Genomics) and the nearest neighbor was determined by reconstructing phylogenetic gene trees using the ARB software package (Ludwig *et al*., 2004).

Gene	pH 5.4		pH 7.1		Organisms with most similar gene sequence and respective restriction site
	cDNA	DNA	cDNA	DNA	
Bacterial 16S rRNA gene	-	55%	-	17%	*Pseudomonas frederiksbergensis, Pseudomonas syringae*
	12%	29%	4%	21%	*Ps. fluorescens, Pseudomonas meridiana*
	12%	-	-	-	*Pseudomonas veronii, Ps. fluorescens*
	10%	4%	-	-	*Pseudomonas tolaasii, Ps. fluorescens*
	-	-	-	13%	*Aquaspirillium arcticum*
	62%	4%	96%	4%	*Herminiimonas glaciei, Herbaspirillum* sp.
	-	-	-	21%	*Paenibacillus wynni, P. borealis*
	-	-	-	8%	*Sphingobacterium* sp.
	4%	-	-		*A. xylosoxidans*
	-	8%	-	16%	others

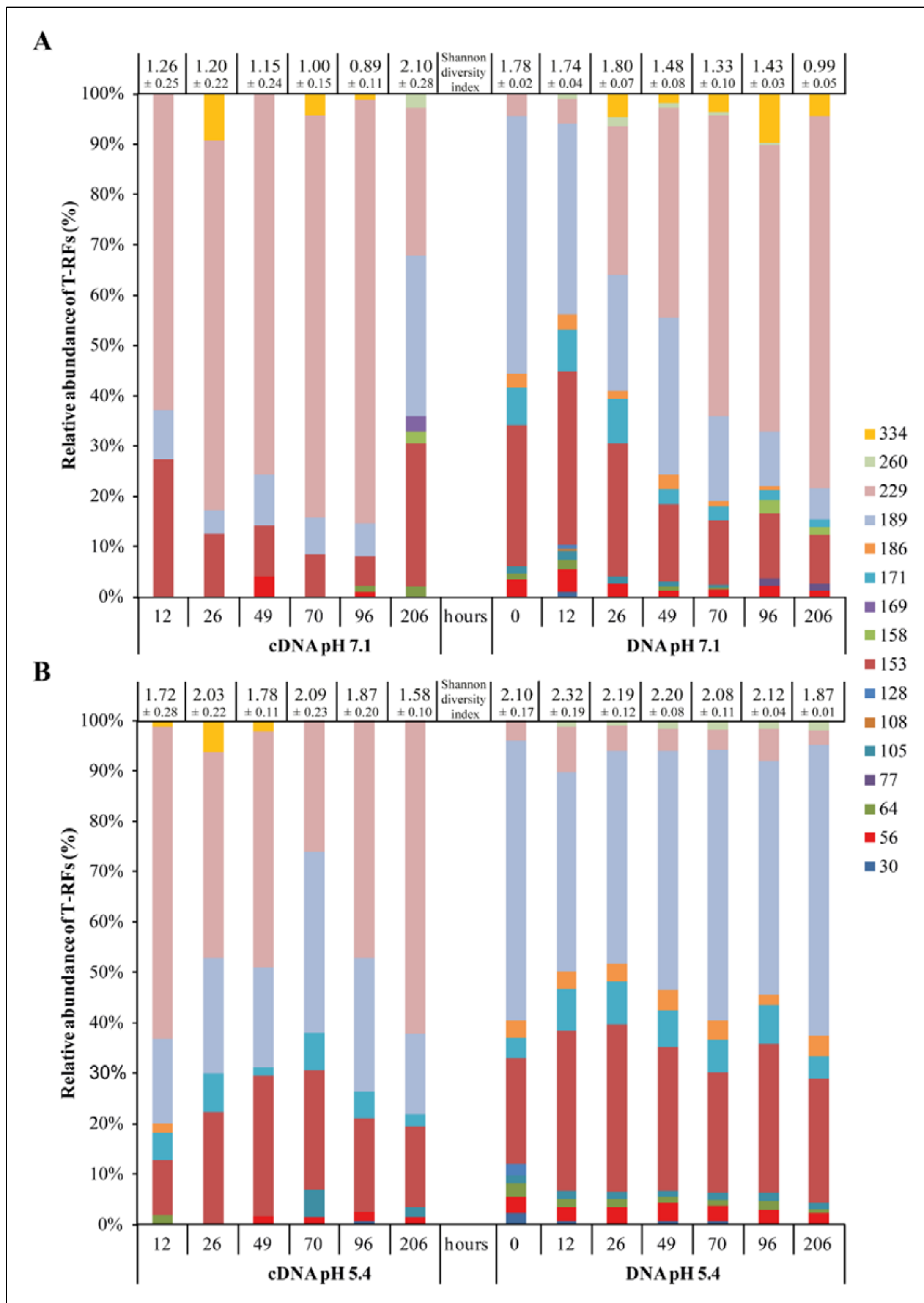

Figure S2.1. T-RFLP profiles (*nirK*) during incubation at (A) pH 7.1 and (B) pH 5.4. Left part of a panel, TADC; right part, ODC. Colors of the bars indicate relative abundance of T-RFs. Shannon diversity index is shown above each T-RFLP profile. T-RFs with minimum 1% relative abundance in at least one sample are plotted. (n=3). Numbers in the figure legend indicate lengths of the T-RFs in base pairs.

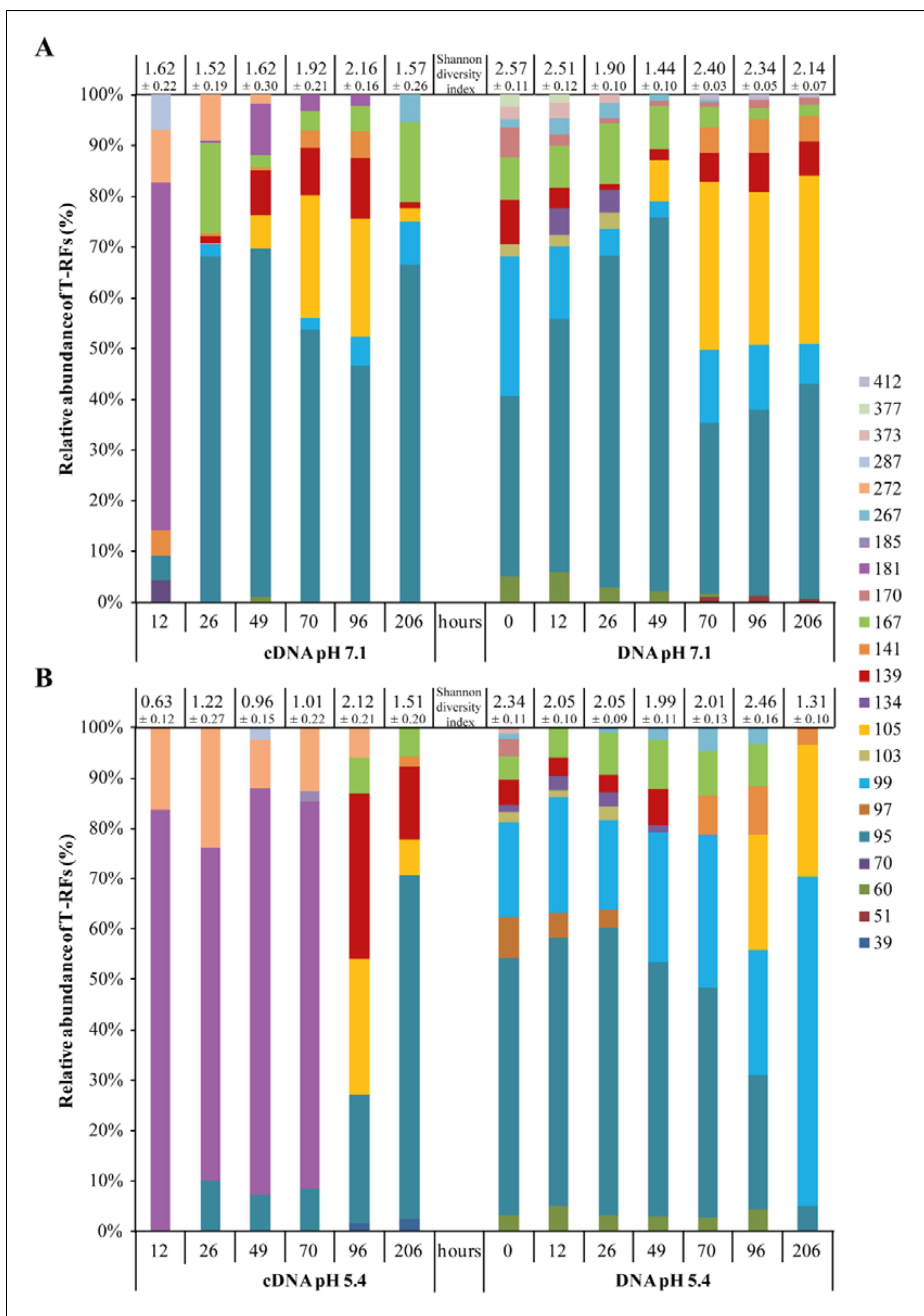

Figure S2.2. T-RFLP profiles (*nirS*) during incubation at (A) pH 7.1 and (B) pH 5.4. Left part of a panel, TADC; right part, ODC. Colors of the bars indicate relative abundance of the T-RFs. Shannon diversity index is shown above each T-RFLP profile. T-RFs with minimum 1% relative abundance in at least one sample are plotted. (n=3). Numbers in the figure legend indicate lengths of the T-RFs in base pairs.

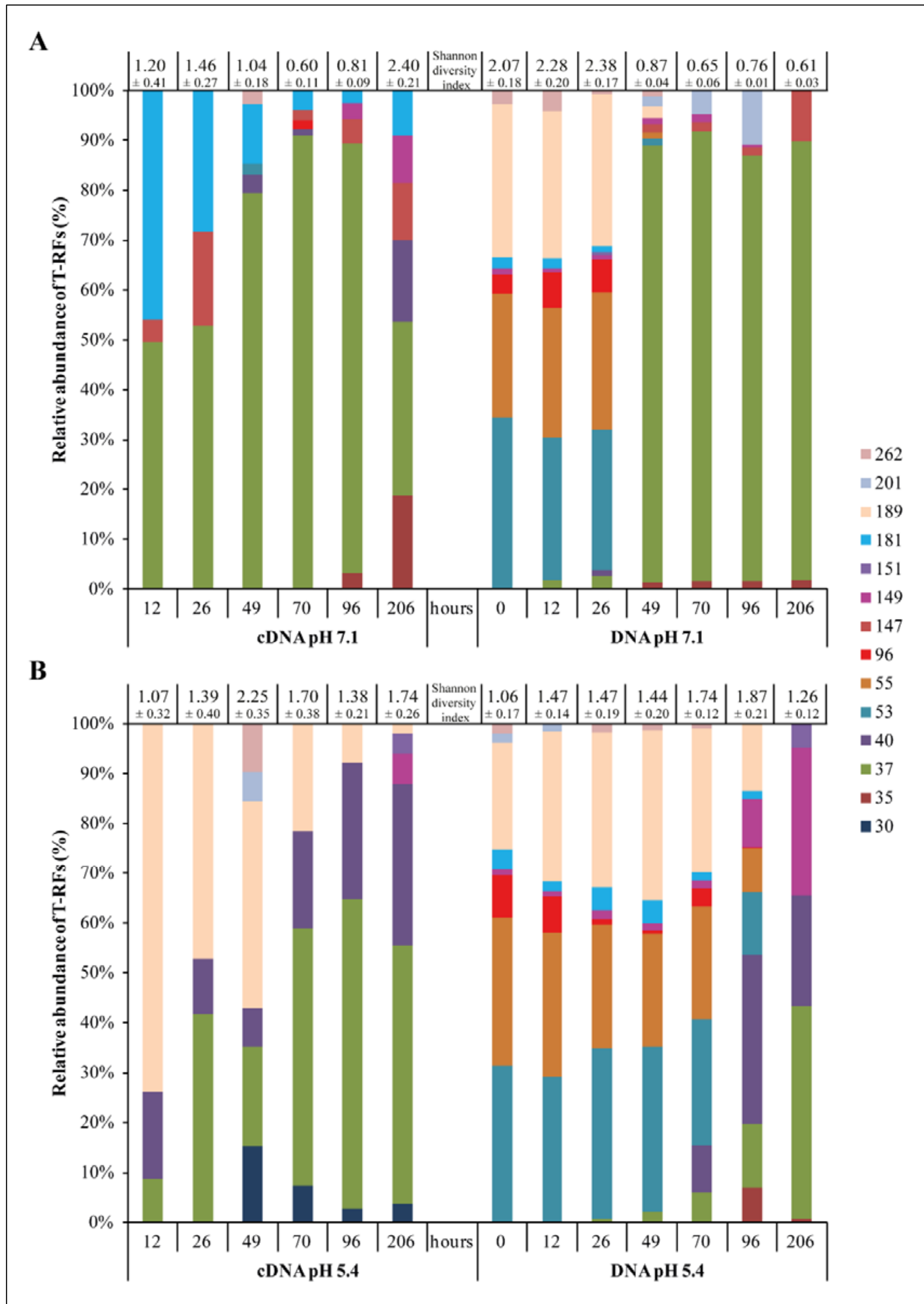

Figure S2.3. T-RFLP profiles (*nosZ*) during incubation at (A) pH 7.1 and (B) pH 5.4. Left part of a panel, TADC; right part, ODC. Colors of the bars indicate relative abundance of the T-RFs Shannon diversity index is shown above each T-RFLP profile. T-RFs with minimum 1% relative abundance in at least one sample are plotted. (n=3). Numbers in the figure legend indicate lengths of the T-RFs in base pairs.

Chapter III

Effect of *e*CO_2 on microbial communities involved in N cycling in soils

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Contributions:

K.B. designed the study, performed the field sampling, performed all lab work (nucleic-acid extractions, T-RFLP analysis, qPCR analysis, preparation of 454 pyrosequencing), performed statistical analysis, evaluated the data and wrote the manuscript.

K.P. performed analysis of 454 pyrosequencing data and wrote the manuscript.

M.H. performed analysis of 454 pyrosequencing data and wrote the manuscript.

G.M. designed the study and wrote the manuscript.

A.G. designed the study.

C.K. designed the gas analyses study and performed the sampling

C.M. designed the study and wrote the manuscript.

G.B. designed the study, evaluated the data and wrote the manuscript.

3 Influence of *e*CO_2 on microbial communities

3.1. Abstract

Continuously rising atmospheric CO_2 concentrations may lead to an increased transfer of organic C from plants to the soil through rhizodeposition and may affect the interaction between the C- and N-cycle. For instance, fumigation of soils with elevated CO_2 (*e*CO_2) concentrations (20% compared to current atmospheric concentrations) at the Giessen Free-Air Carbon Dioxide Enrichment (FACE) sites, resulted in a more than two fold increase of long-term N_2O emissions under *e*CO_2 compared to ambient CO_2 (*a*CO_2). However, the underlying mechanisms are not fully resolved yet. It is particularly unknown how the microbial communities which are responsible for N-transformations in the soil and hence for gaseous N emissions and their activity are affected by *e*CO_2. To get a better understanding of the impact of *e*CO_2 on soil microbial communities, we applied a molecular approach targeting several microbial groups involved in soil N-cycling (N-fixers, denitrifiers, archaeal and bacterial ammonia oxidizers, and dissimilatory nitrate reducers to ammonia). Remarkably soil parameters, overall microbial community abundance and composition in the top soil under *e*CO_2 differed only slightly from soil under *a*CO_2. Wherever differences in microbial community abundance and composition were detected, they were not linked to CO_2 level but rather determined by differences in soil parameters determined by the location of the FACE rings in the experimental field. We concluded that +20% *e*CO_2 had little to no effect on the overall microbial community involved in N-cycling in the soil but that spatial heterogeneity over extended periods had shaped microbial communities at a particular site in the field. Hence, microbial community composition and abundance alone cannot explain the functional differences leading to higher N_2O emissions under *e*CO_2 and future studies should consider the active members of the soil microbial community.

3.2. Introduction

Due to anthropogenic emissions, atmospheric CO_2 concentrations are rising by about 1% per year and are expected to double in this century (IPCC 2013) causing well known-climatic effects. Observations from the world-wide longest lasting CO_2 enrichment study, the Giessen Free Air Carbon Dioxide Enrichment (GiFACE since 1998, ongoing), showed that elevated atmospheric CO_2 (*e*CO_2) concentrations also exerts several impacts on soil communities. For instance, plant biomass was stimulated by approximately 10% and caused a plant community shift towards a dominance of grass species (Kammann *et al*. 2005; unpublished results). This may lead to an increased transfer of organic C from plants to the soil through rhizodeposition and affect soil microbial communities with implications for the interaction between C- and N-cycling (Denef *et al*., 2007, Freeman *et al*., 2004). A meta-analysis of greenhouse gas emission data demonstrated that increased CO_2 generally stimulated emissions of another potent greenhouse gas, N_2O, from terrestrial ecosystems (van Groeningen *et al*., 2011). At GiFACE for instance, long-term N_2O emissions under *e*CO_2 increased more than two fold compared to ambient CO_2 (*a*CO_2), but the underlying mechanisms are not fully resolved yet (Kammann *et al*., 2008). In two ^{15}N tracing laboratory experiments with soils from FACE sites in Giessen and New Zealand gross N-transformations under *e*CO_2 shifted towards a higher importance of heterotrophic processes (Müller *et al.* 2009, Rütting *et al*. 2010). In addition, turnover of NH_4^+ (heterotrophic nitrification) and the rates of dissimilatory reduction of NO_3^- to NH_4^+ (DNRA) increased, while turnover of NO_3^- was reduced. At GiFACE, NH_4^+ concentrations under *e*CO_2 were on average 17% higher while NO_3^- concentrations were significantly lower (Müller *et al*. 2009).

Changes in gross N-transformations and gaseous N emissions are dependent on the dynamics and activity of microbial communities. Under *e*CO_2, microbial communities in the plant rhizosphere are affected by altered C substrate inputs (root turnover and exudation)

rather than by a direct CO_2 effect, because CO_2 concentrations in the soil atmosphere are naturally high (Gobat *et al*., 2004). As soil microorganisms are often C-limited, a plant mediated increase in C-supply at *e*CO_2 would be expected to result in growth of the microbial community and hence in increased microbial biomass production. Several studies confirmed this expectation (Chung *et al*., 2007; Dijkstra *et al*., 2005; He *et al*., 2010; Kassem *et al*., 2008) while others observed decreased microbial abundances (Hodge *et al*., 1998; Lesaulnier *et al*., 2008). Moreover, the composition of the overall soil microbial communities under *e*CO_2 differed profoundly from communities under *a*CO_2 (Denef *et al*., 2007; Drigo *et al*., 2008; 2009; He *et al*. 2010). This suggests concomitant alterations of potential functional activity and hence of ecosystem functioning (He *et al*., 2010). However, other studies found no effect of *e*CO_2 (Haase *et al*., 2008; Marhan *et al*., 2011; Nelson *et al*., 2010). Hence, reports on *e*CO_2 effects on the overall microbial community in soils are at least in part controversial and responses of rhizosphere microbial communities may depend on the plant-soil system and are probably ecosystem dependent (Kowalchuk *et al*., 2002; Miethling *et al*., 2000, Okubo *et al*., 2015).

In soils, N_2O is mainly produced by denitrifiers and nitrifiers (Conrad, 1996; Butterbach-Bahl *et al*., 2013) and alterations in the functioning of denitrifiers and ammonia oxidizers in soils exposed to *e*CO_2 were clearly discernable (e.g. Barnard *et al*., 2005; 2006). However, little information is available to date how these functional shifts may be related to shifts in the underlying microbial communities and the understanding of potential feedback effects resulting in higher N_2O emissions is still limited. Again, reports on the effects of *e*CO_2 on the abundance and composition of microbial communities involved in N-cycling in soils are controversial. Lesaulnier *et al*. (2008) found a significant decrease of nitrate reducers and crenarchaeal ammonia oxidizers with *e*CO_2, but in that and other studies other genes involved in denitrification, ammonia oxidation and DNRA remained unaffected (Deiglmayr *et al*., 2004; Haase *et al*., 2008; Marhan *et al*., 2011; Pujol Pereira *et al*., 2011). On the other hand,

field exposure of a grassland ecosystem to *e*CO_2 for ten years significantly increased the abundance of N-fixers and *nirS*-type denitrifiers (He *et al*. 2010). Interestingly, in two out of three replicate FACE rings studied at GiFACE the ratio of N_2O reducers to nitrite reducers was lower under *e*CO_2 (Regan *et al*., 2011) and may thus explain higher N_2O fluxes from the soil (Philippot *et al*., 2011). Two earlier cultivation based studies showed an enhanced abundance of nitrate dissimilating *Pseudomonas* in the rhizosphere of grasses at *e*CO_2 (Fromin *et al*., 2005; Roussel-Delif *et al*., 2005). In a California grassland, the structure and abundance of the ammonia oxidizing bacterial community was altered by *e*CO_2, strongly interacting with the factor precipitation (Horz *et al*., 2004). Horz et al. (2004) also showed that multifactorial global changes (*e*CO_2, temperature, precipitation, N-deposition) feeds back into the enrichment of a specific clade of ammonia oxidizers with higher potential for nitrification.

Morales *et al*. (2015) demonstrated that functional diversity of e.g. denitrifier communities was among the variables sufficient to predict the amount and type of N-gas emitted from soils. Thus, we hypothesized that understanding the functional potential of microbial communities involved in N-cycling at Giessen FACE can help explain shifts in N-transformations, particularly the increased N_2O emissions in response to *e*CO_2. To explore the functional diversity of microbial communities involved in N-cycling at GiFACE, we applied a molecular approach to study the abundance and composition based on functional marker genes for denitrification (*nirK*/*nirS*, *nosZ*), ammonia oxidation (bacterial and archaeal *amoA*), nitrogen fixation (*nifH*), dissimilatory nitrate reduction to ammonia (DNRA, *nrfA*) as well as archaeal and bacterial communities.

3.3. Materials and Methods

3.3.1 Site description and sampling

Soil samples were taken from the GiFACE experiment site (50°32'N and 8°43.3'E; 172 m a.s.l.) near Giessen, Germany. Within the GiFACE experiment CO_2 fumigation on an old grassland site (> 100 years) was started in May 1998 to study the response of a semi-natural grassland to long-term, moderate atmospheric CO_2 enrichment. The whole facility consists of six plots, each with 8 m internal diameter. Two plots build one set each with an ambient (*a*CO_2) and an elevated (*e*CO_2) CO_2 plot. The *a*CO_2 plots receive currently 400 ppm CO_2 and hence atmospheric CO_2 concentration of about 480 ppm, i.e. the *e*CO_2 plots are fumigated with CO_2 20% above ambient air. The three sets differ in soil moisture concentration and exhibit a moisture gradient, which is generated by the gradual terrain slope in the direction of the rivulet Lückebach as well as varying depths of the subsoil clay layer. In the following, the sets along the soil moisture gradient are referred to as blocks and are designated as A1 and E1, *a*CO_2 and *e*CO_2, respectively (DRY)), A3 and E3, *a*CO_2 and *e*CO_2, respectively (MED) and A2 and E2, *a*CO_2 and *e*CO_2, respectively (WET). The soil in the FACE-rings was classified as a Fluvic Gleysol and has a sandy clay loam texture on top of a clay layer. The soil was characterized by a mean C and N content of 4.5% and 0.45%, respectively, a pH of ~ 6.2, and a mean annual temperature of 9.4°C. Mean annual precipitation was 575 mm during the observation period from 1996 to 2003 (Jäger *et al*., 2003). Vegetation is dominated by 12 grass species, 2 legumes, and 15 non-leguminous herbs, and is characterized as an *Arrhenatheretum elatioris* Br. Bl. *Filipendula ulmaria* subcommunity. The grassland has not been ploughed for at least 100 years. It has been managed for several decades as a hay meadow with two cuts per year, and fertilized in mid-April with granular mineral calcium-ammonium-nitrate fertilizer at the rate of 40 kg N ha^{-1} yr^{-1} since 1996; before 1996, it was fertilized at 50–100 kg N ha^{-1} yr^{-1} (Kammann *et al*., 2008).

In July 2012 three replicate soil core samples were taken for each of the 6 plots in the depth of 0-7.5 cm and were stored at - 20°C till further analyses. The samples (18) were taken at random locations inside the rings in east, south and west direction.

3.3.2. Measurement of soil parameters

N_2O flux, soil moisture content and precipitation at the field site was measured as described by Kammann *et al*. (2008) and Regan *et al*. (2011). All parameters were continuously recorded since the start of the FACE facility in 1997. Part of the dataset (1997-2006, 2008) used in this study was published previously (Kammann *et al*., 2008; Regan *et al*., 2011), data for 2007 and 2009 were additionally included for this study. The complete dataset was searched for dates and events, when the N_2O flux reached more than 100 µg $(m^2 \times h)^{-1}$ in at least one ring. Flux data as well as soil moisture content and precipitation for all rings were then outlined for these dates.

From each soil core sample pH value, water content, nitrate (NO_3^-)-/ nitrite (NO_2^-)-/ ammonia (NH_4^+)-concentration and total carbon (C), hydrogen (H) and nitrogen (N) content in the soil was determined. Soil pH was measured by shaking a soil sample (10 g) in 25 mL $CaCl_2$ solution (0.01 M $CaCl_2 \times 2H_2O$; Merck, Germany) for 20 min. After settling for one hour in the dark at room temperature and resuspension pH was measured with an InLab® semi-micro electrode (Mettler-Toledo GmbH, Giessen, Germany). Water content (%) was determined after drying 1 g of homogenized soil for 3 days at 65°C in a drying oven (Memmert GmbH & Co. KG, Schwabach, Germany). Afterwards, dried samples were grinded after addition of liquid nitrogen and aliquots were analyzed at the Chemical Department of the Phillips-University Marburg (Germany) with a CHN-elemental analyzer to determine the total C/H/N percentage concentration of the soil.

Concentrations of NO_3^- and NO_2^- were analyzed by ion chromatography. (IC; Skyam GmbH Eresing, Germany; 70°C oven temperature) equipped with a LCA A14 column (Skyam GmbH, Eresing, Germany) using a 50 µL injection volume and Na_2CO_3 as eluent (flow of 1.5 mL min^{-1}). For this purpose 1 g of soil sample were resuspended in 1 mL of Nuclease-free H_2O and subsequently sterile-filtered with a disposable Filter Unit (0.2 µm; Whatman, MAGV, Germany). The concentration of NH_4^+ in the soil samples was measured fluorometrically in triplicates by microscale analysis (Murase *et al.*, 2006).

3.3.3. Nucleic acid extraction

DNA was extracted from 0.35 g soil using the NucleoSpin® Soil Kit (Machery-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer's protocol. Afterwards, the amount and purity of extracted DNA was determined with a NanDrop1000 Spectrophotometer (Thermo Scientific, Langenselbold, Germany). The concentration of DNA ranged from 100-120 ng/µL and $A_{260/280}$ ratios between 1.6-1.9 indicated a high purity of the extracted DNA with minimum contamination of humic acids.

3.3.4. Quantification of functional marker and 16S rRNA genes

Copy numbers of genes encoding the denitrification enzymes nitrite reductase (*nirK*/*nirS*) and nitrous oxide reductase (*nosZ),* dinitrogenase (*nifH)*, archaeal and bacterial ammonia monooxygenase (A*amoA*/B*amoA)*, nitrite reductase of the dissimilatory reduction of nitrate to ammonia (*nrfA)* and archaeal and bacterial 16S rRNA were quantified by qPCR as described in Table S3.1. A typical reaction mixture contained 12.5 µL of SybrGreen Jump-Start ReadyMix (Sigma-Aldrich, Taufkirchen, Germany), 0.5 µM of each primer, 3-4.0 mM $MgCl_2$, 2 µL of soil DNA except for amplification of *nosZ*, for which 3 µL of DNA were

used. For the amplification of functional marker genes involved in nitrogen cycling 200 ng BSA mL^{-1} were added. All assays were performed in an iCycler (Applied Biosystems, Darmstadt, Germany). Standard curves were obtained using serial 10-fold dilutions of a known amount of plasmid DNA (10^8 to 10^1 gene copies) containing the respective gene fragment. Negative controls were always run with water instead of template DNA. PCR efficiencies for all assays were between 80-97 % with r^2 values between 0.971 and 0.996.

3.3.5. Analysis of the composition of functional marker and 16S rRNA genes

The composition of PCR amplified gene fragments of *nirK*/*nirS*, *nosZ*, A*amoA*/B*amoA*, *nifH*, *nrfA* as well as archaeal and bacterial 16S rRNA genes (T-RFLP only) was explored by terminal restriction length polymorphism (T-RFLP) and barcode labeled 454 pyrosequencing analyses. Details on primers and conditions are given in Table S3.2. Quantity and quality of the PCR amplicons were analyzed by gel electrophoresis (1.5% w/v agarose) and staining the gel with 3 × GelRed Nucleic Acid Stain (Biotium, Köln, Deutschland). PCR products of the expected size were excised from the gel and purified using the DNA Wizard® SV Gel-and-PCR-Clean-up system (Promega, Mannheim, Germany).

For T-RFLP, forward or reverse primers were 5’-6-carboxyfluorescein labeled and amplicons were hydrolyzed by the restriction enzymes (FastDigest, Fermentas, St. Leon-Rot, Germany) *HaeIII (nirK*/*nirS*), *HhaI* (*nosZ*, *nifH*, *nrfA* and A*amoA*/B*amoA*) and *MspI* and *Taq1*, (archaeal and bacterial 16SrRNA, respectively). Afterwards, reaction products were purified using the SigmaSpin™ Sequencing Reaction Clean-up Columns (Sigma-Aldrich) according to the manufacturer’s instructions. Fluorescently labeled restriction fragments were separated on an ABI PRISM 3100 Genetic Analyzer sequencer (Applera Deutschland GmbH, Darmstadt, Germany) and the lengths of fluorescently labeled terminal restriction fragments

(T-RFs) were determined by comparison with the internal standard using GeneMapper software (Applied Biosystems). Peaks with fluorescence of > 1% of the total fluorescence of a sample and > 30 bp length were analyzed by aligning fragments to the internal DNA fragment length standard (X-Rhodamine MapMarker® 30-1000 bp; BioVentures, Murfreesboro, TN). Reproducibility of patterns was confirmed for repeated terminal restriction fragment length polymorphism (T-RFLP) analysis using the same DNA extracts of selected samples. A difference of less than 2 base pairs in estimated length between different profiles was the basis for considering fragments identical. Peak heights from different samples were normalized to identical total fluorescence units by an iterative normalization procedure (Dunbar *et al*., 2001).

For pyrosequencing, DNA extracts from the three replicate samples of each ring were pooled and PCR amplified using the primers used for T-RFLP but with barcode labels (6 bp) designed to differentiate between FACE rings (E1: ACACAC; E2: ATGTAT; E3: AGCAGC; A1: ATCATC; A2: AGACTA; A3: AGTCAT) and with small variation at the annealing temperature due to barcode tagging (Table S3.2). DNA concentration was determined from by a Qubit® 2.0 Fluorometer using the Quant-iT TM dsDNA BR Assay Kit (Invitrogen Darmstadt, Germany). Amplicons (200 ng each) of each gene from six FACE rings were pooled and libraries were built and subjected to barcode labeled 454 pyrosequencing (GATC, Köln, Germany).

3.3.6. Sequence analysis

Sequence processing and analysis was done in Qiime 1.3 (qiime.org). Pyrosequencing and PCR errors of the reads were corrected using the AmpliconNoise pipeline (Quince *et al*., 2011). Sequences of functional marker genes (*nirK*/*nirS, nosZ,* A*amoA/*B*amoA, nifH* and

nrfA) were clustered as described previously (Caporaso *et al*., 2010; Palmer and Horn, 2012) using threshold similarities of 92%, because this reflects the threshold value beyond which the number of OTUs stays stable (Palmer and Horn, 2012; Palmer *et al*., 2012). Archaeal 16S rRNA gene sequences were clustered at 95% threshold similarities. Representative sequences were determined for each OTU. For statistical comparison of gene diversity in the plots, alpha-diversity measures were calculated in Qiime from rarified OTU tables as described elsewhere (Palmer and Horn, 2012; Hughes *et al*., 2005). Rarified OTU tables were generated by randomly subsampling original OTU tables 100 times. A sampling depth of 400 sequences was chosen for A*amoA*, B*amoA*, *nifH, nirK, nirS, nosZ*, and *nrfA* to allow comparison of diversity between the different functional marker genes, as the number of sequences obtained exceeded 400 for all genes and soils. Rarified OTU tables of 16S rRNA gene sequences were generated at a sampling depth of 150 sequences, due to a lower number of sequences.

3.3.7. Statistical analyses of collected data

All statistical analyses were done using R version 3.0.1 (R Development Core Team, 2013). Significant differences in copy numbers of archaeal/bacterial 16S rRNA genes, A*amoA*, B*amoA*, *nirK*, *nirS*, *nosZ*, *nifH* and *nrfA* were assessed using ANOVA (P value < 0.05). All quantitative data were log-transformed prior to analysis to satisfy the assumptions of homoscedasticity and normally distributed residuals.

The effect of soil parameters on T-RFLP and on OTU based 454 pyrosequencing community profiles was explored by canonical correspondence analysis (CCA). Statistical significance of the CCA was assessed using permutation test (1000 iterations). The clustering of the OTUs from the barcode labeled 454 pyrosequencing was analyzed by a hierarchical

cluster analysis and Ward's minimum variance method. All community composition data were log-transformed before analysis, in order to reach normal distribution.

3.4. Results

3.4.1. N_2O flux and soil moisture content over a period of 12 years

The complete data set of N_2O flux measurements since the start of the GiFACE facility in 1998 revealed only 169 days on which N_2O flux events in at least one ring exceeded 100 µg $(m^2 \times h)^{-1}$ (Fig. 3.1). The highest frequency of these events occurred in the first three years after the start of the experiment. Mostly but not generally, a rain event which increased soil moisture content preceded higher N_2O fluxes (Fig. S3.1 and S3.2). Soil fumigated with *e*CO_2 generally produced more N_2O than at *a*CO_2.

3.4.2. Soil characteristics

Soil characteristics showed only minor differences and differed only between ring pairs but not between *e*CO_2 and *a*CO_2 (Tables 3.1, S3.3). The soil was slightly acidic with pH levels ranging from 5.45-6.1. Differences existed mostly between soil of the first ring pair and the two other ring pairs. In ring pair E1/A1 pH was lower (5.55) than in the two other ring pairs (E2/A2, 6.03; E3/A3, 5.96). NO_3^-concentration and C- as well as H-content were lower in E2/A2 and E3/A3, respectively than in E1/A1. N-content was lower at E1/A1, while NH_4^+-concentration was higher than at the two other ring pairs. Water-content was similar in all rings.

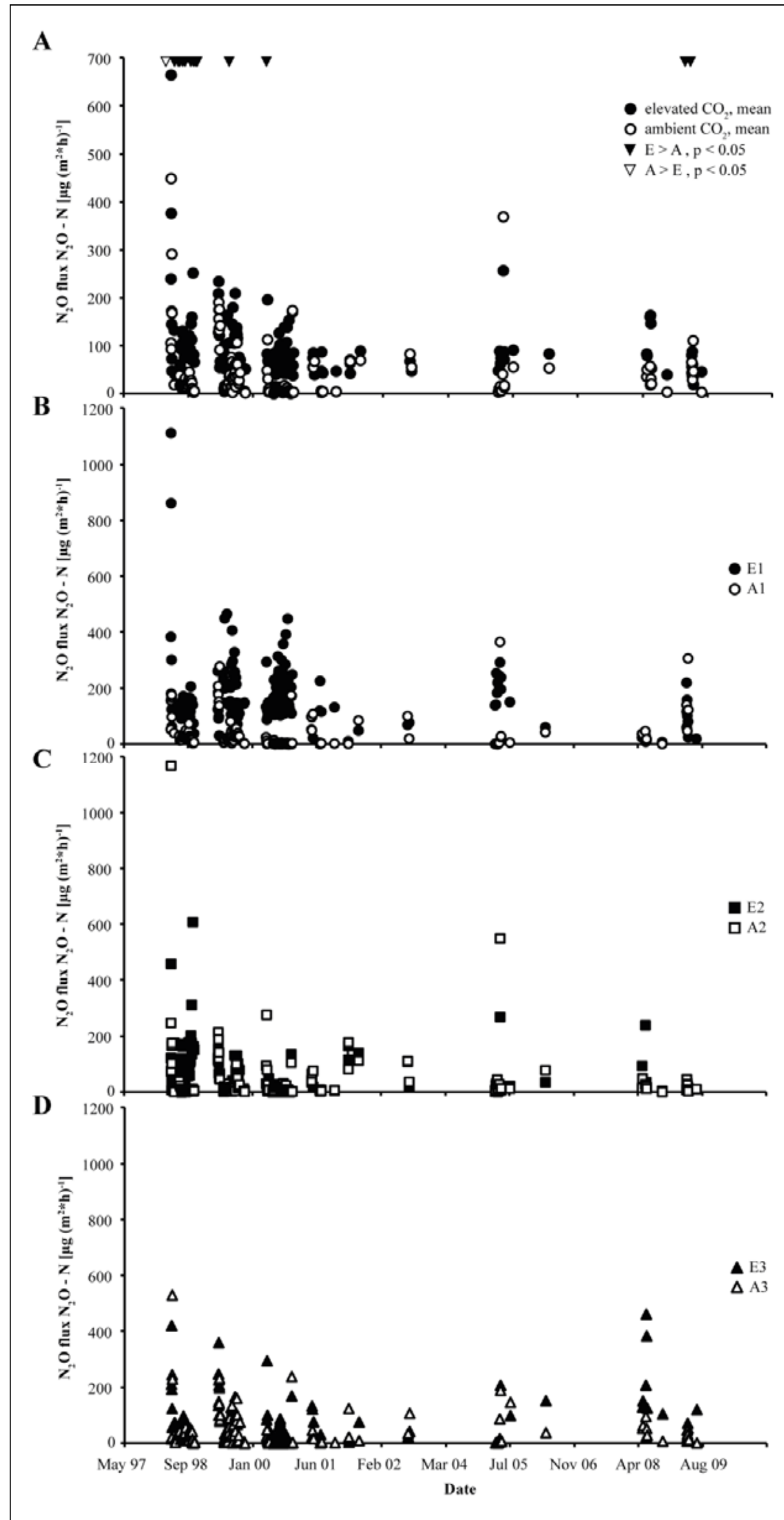

Figure 3.1. N_2O flux measurements for events in which the N_2O fluxes reached more than 100 µg $(m^2 \times h)^{-1}$ in at least one ring between the years 1997–2009: (A) Mean N_2O fluxes at elevated (E) and ambient (A) CO_2. Triangles mark occasions where N_2O fluxes from *e*CO_2 (E) rings were significantly greater than from *a*CO_2 (A) rings (black triangles) or the other way around (white triangles) at P > 0.05 tested by ANOVA. (B)–(D) N_2O fluxes in the three ring pairs (B, E1/A1; C, A3/E3; D, E2/A2).

Table 3.1. Soil parameters at GiFACE for ring pairs E1/A1, E2/A2 and E3/A3.

Ring	Soil characteristics							
	pH	NO_3^- [µM/g dw]	NH_4^+ [µM/g dw]	H_2O [%]	C [%]	H [%]	N [%]	C:N ratio
E1+A1	5.55^a ± 0.23	2.58^a ± 0.81	0.33^a ± 0.111	22.50^a ± 3.56	4.03^a ± 0.61	0.89^a ± 0.11	0.35^a ± 0.04	11.29^a ± 0.40
E2+A2	6.03^b ± 0.13	6.36^b ± 3.30	0.19^b ± 0.079	21.33^a ± 1.86	4.53^{ab} ± 0.71	1.04^{ab} ± 0.11	0.45^b ± 0.05	10.09^b ± 0.18
E3+A3	5.96^{ab} ± 0.24	5.33^{ab} ± 1.95	0.16^b ± 0.069	23.50^a ± 4.28	5.09^b ± 0.82	1.18^b ± 0.10	0.49^b ± 0.08	10.32^b ± 0.51

[ab] Identical letters indicate no significance differences ($P > 0.05$). Mean ±SD (n=6).

3.4.3. Abundance of microbial groups involved in soil nitrogen cycling

Total bacterial 16S rRNA gene copy numbers were in the order of 1×10^9 gdw^{-1}, while archaeal copy numbers ranged between 5×10^7 – 1×10^8 gdw^{-1} for all rings (Fig. 3.2). Abundance of bacteria and archaea did not differ between rings or ring pairs (Tables 3.2, S3.4). Absolute copy numbers of the functional marker genes *nirK*, *nosZ*, *nifH* and A*amoA* and numbers relative to total 16S rRNA gene copies (Bacteria + Archaea) were similarly high in all rings and did also not differ between ring pairs (Table S3.6). Genes *nirS* and B*amoA* were 5-10-fold and 100-fold less abundant than the other functional marker genes, respectively (Fig. 3.2). Relative copy numbers of *nirS* were higher in ring pair E2/A2 than in the other two ring pairs while relative numbers of B*amoA* were higher in ring pair E3/A3 than in E1/A1. Comparison of A*amoA* and 16S rRNA gene copy numbers indicated that a large fraction of Archaea harbored a copy of the *amoA* gene (ratios close to one, data not shown). The ratio of denitrification genes *nosZ*/*nirS* was generally higher than the ratio of *nosZ*/*nirK* which was constantly low in all rings (Fig. S3.3).

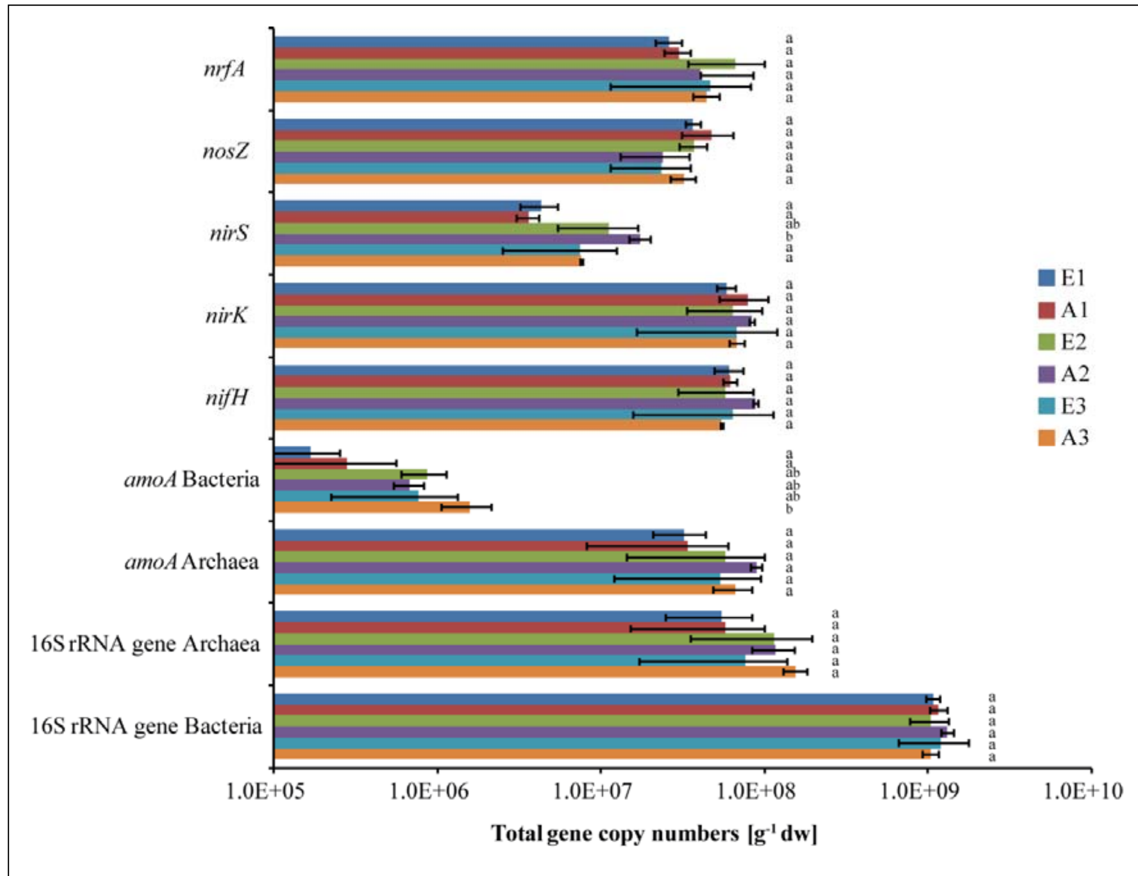

Figure 3.2. Abundance of denitrifiers, dissimilatory ammonia reducers, nitrogen fixers, ammonia oxidizers and total bacteria and archaea based on quantitative PCR of the functional marker genes *nrfA*, *nosZ*, *nirS*, *nirK*, *nifH*, A*amoA*, B*amoA*) as well as 16S rRNA genes, respectively. Bars indicate the total gene copy numbers. (Mean ± SD, n=3).

Table 3.2. Abundance of functional marker genes (A*amoA,* B*amoA nirK, nirS, nosZ, nrfA,* and *nifH*) relative to total 16S rRNA gene abundance (archaeal + bacterial) at GiFACE for ring pairs E1/A1, E2/A2 and E3/A3.

Ring	Ratio copy number of functional marker gene/total 16S rRNA genes						
	Denitrification			Nitrification		DNRA	N-fixation
	nirK	*nirS*	*nosZ*	Archaeal *amoA*	Bacterial *amoA*	*nrfA*	*nifH*
E1+A1	0.058^a ± 0.012	0.003^a ± 0.001	0.035^a ± 0.01	0.028^a ± 0.015	0.0002^a ± 0.0002	0.024^a ± 0.004	0.052^a ± 0.006
E2+A2	0.057^a ± 0.014	0.010^b ± 0.002	0.028^a ± 0.011	0.054^a ± 0.018	0.0007^{ab} ± 0.0004	0.042^a ±0.026	0.055^a ±0.011
E3+A3	0.052^a ± 0.017	0.006^a ± 0.002	0.022^a ± 0.006	0.048^a ± 0.021	0.0010^b ± 0.0005	0.034^a ± 0.013	0.044^a ± 0.016

[ab] Identical letters indicate no significant differences ($P > 0.05$). Mean ±SD (n=6).

3.4.4. Influence of soil characteristics on the composition of microbial communities involved in soil nitrogen cycling

We used Canonical Correspondence Analysis (CCA) based on T-RFLP and pyrosequencing data to explore whether differences in community composition were related to CO_2 levels or other soil parameters. CCA based on T-RFLP data clustered N-fixer communities (*nifH*) from single ring pairs according to the ring pair (Fig. 3.3). Communities of denitrifiers (*nirK*/*nirS*, *nosZ*), archaeal ammonia oxidizers (A*amoA*) and archaea in general (16S rRNA genes) were more distinct between ring pair E1/A1 and the other two ring pairs. No separation of communities from different ring pairs occurred for dissimilatory nitrate reducers (*nrfA*), for bacterial ammonia oxidizers (B*amoA*) as well as for bacteria in general (16S rRNA genes). CCA identified pH (16.4-29.6% of the variance; Table S3.5) and NH_4^+-concentration (12.9-30.7% of the variance; Table S3.5) as the most important soil parameters to shape the soil microbial communities because both exerted a significant impact on microbial community composition independent of the gene considered. Except for *nosZ*-containing denitrifiers and dissimilatory nitrate reducers, NO_3^--concentration (5.2-20.3% of the variance; Table S3.5) also determined the composition of the microbial communities.

Generally, lower pH and lower NO_3^- as well as higher NH_4^+ clearly separated communities of E1/A1 from those of the two other ring pairs (Table 3.1, Fig. 3.3). On the other hand, the level of CO_2, whether elevated or ambient influenced only the composition of dissimilatory nitrate reducer communities in all three rings while the other communities were unaffected (Table S3.5). Exploring whether CO_2 exerted an influence on community composition in single ring pairs showed that community composition of archaea in rings E1 and A1, of *nirS*-type denitrifiers and bacteria in the rings E2 and A2, and of dissimilatory nitrate reducers in ring E3 and A3 were influenced by the level of CO_2 (Table S3.6).

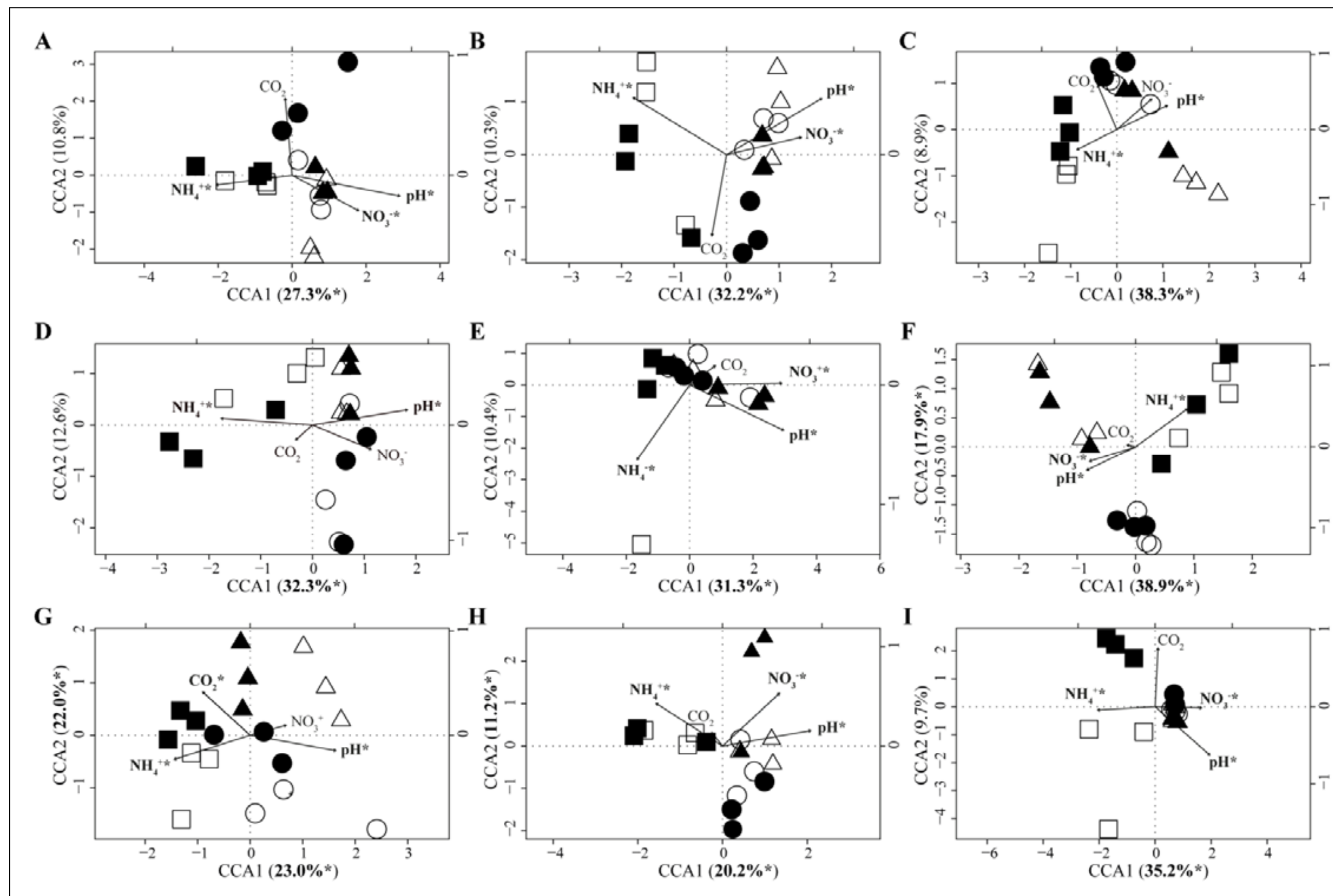

Figure 3.3. Canonical correspondence analysis (CCA) biplot based on T-RFLP community analyses of *nirK* (A), *nirS* (B), *nosZ* (C), A*amoA* (D), B*amoA* (E), *nifH* (F), *nrfA* (G), bacterial 16S rRNA genes (H) and archaeal 16S rRNA genes (I). Arrows indicate the direction and relative importance (arrow lengths) of soil parameters associated with the clustering of the communities. For each gene the most important environmental variables were displayed and highlighted in the graphic by an asterisk if significant in the model (ANOVA: P value < 0.05). Square, circle and triangle symbols represent ring pairs E1/A1, E3/A3 and E2/A2, respectively. Closed symbols represent fumigation with eCO_2 and open symbols the control ring at aCO_2. (n=3)

Since T-RFLP analysis does not necessarily separate different genotypes of functional marker genes, pyrosequencing analysis was performed additionally to provide in depth information on the composition of the communities. CCA analysis based on OTUs revealed that communities of nitrite reducers (*nirK*/*nirS)*, N_2O reducers (*nosZ),* N-fixers (*nifH)* and dissimilatory nitrate reducers to NH_4^+ (*nrfA)* clustered primarily according to ring pairs (Fig. 3.4). The archaeal and the archaeal ammonia oxidizer communities of ring pair E1/A1 were separated from the communities of the other two ring pairs (E2/A2, E3/A3). Communities of bacterial ammonia oxidizers did not cluster according to ring pairs. pH level and NH_4^+-concentration were the only soil parameters identified to have a significant impact on the composition of some microbial guilds. Archaeal ammonia oxidizers as well as the overall archaeal communities of the FACE rings were influenced by pH and NH_4^+-concentration, *nirK*-type nitrite reducers were solely influenced by pH, N-fixers and *nosZ*-containing denitrifiers by NH_4^+-concentration (Table S3.7). Communities of *nirS*-type denitrifiers and dissimilatory nitrate reducers were not influenced by any of the soil parameters determined. NO_3^--concentration and CO_2-level had no significant influence on the clustering of the microbial communities between ring pairs. However, communities of e.g. nitrite reducers exposed to *e*CO_2 were separated from those exposed to *a*CO_2 but whether the CO_2 level exerted a significant influence on the communities of single rings could not be tested due to the lack of replicates.

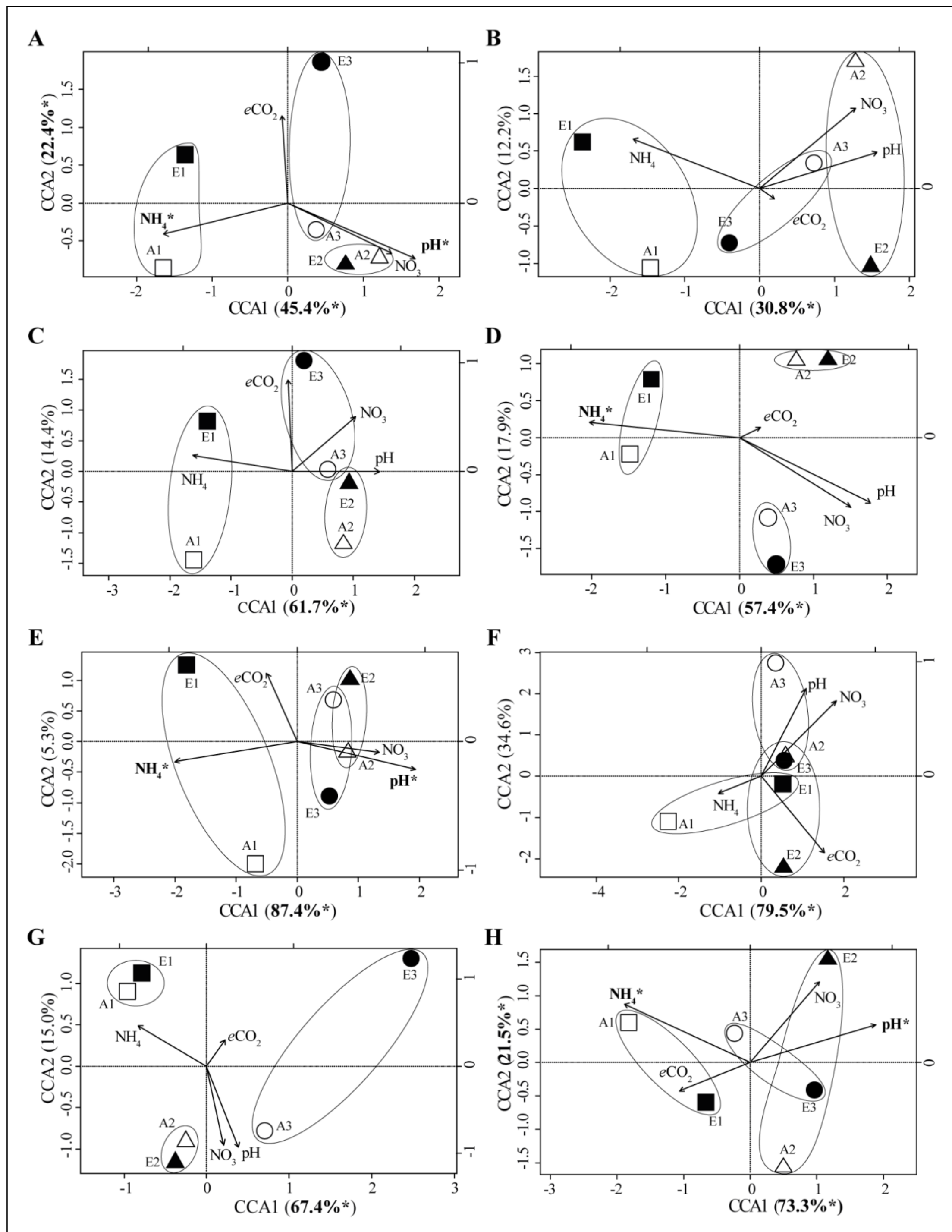

Figure 3.4. Canonical correspondence analysis (CCA) biplot based on OTUs from 454 barcode labeled pyrosequencing of *nirK* (A), *nirS* (B), *nosZ* (C), *nifH* (D), A*amoA* (E), B*amoA* (F), *nrfA* (G) and archaeal 16S rRNA gene (H). Arrows indicate the direction and relative importance (arrow lengths) of soil parameters associated with the clustering of the several communities. For each gene most important environmental variables were displayed and highlighted in the graphic by an asterisk, if significant in the model (ANOVA: P value < 0.05). Square, circle and triangle symbols, represent ring pairs E1/A1, E3/A3 and E2/A2, respectively. Closed symbols represent fumigation with eCO_2 and open symbols the control ring at aCO_2. Circular shape was added manually to highlight the ring pairs.

3.4.5. Composition of microbial communities involved in soil nitrogen cycling

Applying a threshold similarity of 92 % (95% for archaeal 16S rRNA genes) to sequences obtained from pyrosequencing, coverage of the libraries was in the range of 77.2-100% (Table S3.9). For *amoA* (archaeal and bacterial) and for archaeal 16S rRNA genes the number of operational taxonomic units (OTUs) was low with only 3-7 OTUs observed and 3-8 OTUs estimated. Hence diversity (Shannon Diversity index H_{AamoA}=0.90-1.59; H_{BamoA}=0.64-1.37; $H_{16S\ rRNA\ archaea}$=1.15-1.48) was also low. Evenness of the archaeal ammonia oxidizer community ranged from 0.47-0.64 and from 0.56-0.68 for the overall archaea communities since in all FACE rings they were dominated by only two *amoA* (OTU 1, 51.5-71.1 %; OTU 2, 26.6-35.4%) as well as two 16SrRNA (OTU 1, 63.8-66.1%; OTU 2, 20.7-29.4%) genotypes (Table S3.8). These genotypes were closely related to *amoA* and the 16S rRNA gene from Candidatus *Nitrosphaera gargensis* and Cand. *Nitrosphaera vienennensis*, respectively. Evenness (E=0.25-0.50) was even lower for bacterial ammonia oxidizers which were dominated by *BamoA* genotypes (OTU 1, 75.4-84.2%; OTU 2, 14.2-22.5%) closely related to *amoA* of *Nitrospira* spp..

OTU numbers ranged at least one order of magnitude higher for marker genes for N-fixation, denitrification and DNRA (Table S3.9). The communities were more diverse (H_{nifH}=4.53-5.25; H_{nirK}=4.11; H_{nirS}=2.80-3.75; H_{nosZ}=2.90-4.64; H_{nrfA}=1.72-6.53) but also more even (E_{nifH}=0.76-0.82; E_{nirK}=0.62-0.71; E_{nirS}=0.51-0.65; E_{nosZ}=0.53-0.76; E_{nrfA}=0.41-0.87). The lowest evenness levels of E=0.41 and 0.47 referred to DNRA communities of rings E3 and A3.

Generally, OTUs were most closely related to genes originating from as yet uncultured species but sequence identities of > 71% to genes from cultivated species known to be involved in N-cycling confirmed that these genes were indeed derived from organisms of the respective target group. OTUs representing species of *Bradyrhizobium* were most abundant

among N-fixers and nitrite reducers, while an OTU representing *Rhodopseudomonas palustris* dominated the *nosZ*-containing denitrifier communities. Communities of organisms capable of DNRA in ring pairs E1/A1 and E2/A2 were not dominated by single OTUs and sequences were most closely related to *nrfA* from *Bacteroides* spp., *Anaromyxobacter* spp., *Sorangium* spp. and *Geobacter* spp.

3.5. Discussion

The mechanisms for higher N_2O fluxes and other altered N transformation rates under elevated CO_2 (Kammann *et al*., 2008) at the GiFACE facility are still not fully resolved, particularly the response of the soil microbial communities to *e*CO_2. Therefore, the main goal of this work was to analyze the influence of *e*CO_2 on the abundance and composition of microbial communities involved in N-cycling. Remarkably, soil parameters of the FACE rings under *e*CO_2 did not differ significantly from those in rings under *a*CO_2 while soil parameters differed between FACE ring pairs. Hence, the location of the ring pairs at the GiFACE facility determined the prevalent soil parameters. Although in our study soil water content was similar in the upper soil layer in all rings, previous observations showed that the water level in the deeper soil layers differed between the ring pairs and was highest in ring pair E2/A2, intermediate in ring pair E3/A3 and lower in ring pair E1/A1 (Lenhart, 2008). Likewise microbial abundance and community composition was very similar in a given ring pair. Wherever differences were detectable in community composition, they were related to differences in soil parameters determined by the location of the ring pairs while exposure to elevated CO_2 for 18 years exerted almost no influence on the composition of the microbial communities in the soil.

Soil properties are known as the predominant factor driving the distribution of microorganisms and shaping communities (e.g. Zhou *et al.*, 2008). Spatial heterogeneity at scales similar to the experimental site at GiFACE was previously found to determine spatial variation in e.g. soil denitrifier (Enwall *et al.*, 2010) and ammonia oxidizer communities (Wessén *et al.*, 2011).

The impact of over 100 yr being under permanent grassland had presumably a more profound effect on the development of microbial communities than 18 years of moderate exposure to eCO_2. We assume that the cultivation before fumigation with eCO_2 led to the development of a microbial diversity adapted to prevailing soil conditions but which seems resilient to higher CO_2 levels. Additionally, the increase from CO_2 concentration from ~ 300 ppm to 400 ppm in the last 100 yr is larger than the additional 20% increase under eCO_2. In all rings a large fraction of sequences belonged to only a few OTUs which hence may represent the well-adapted key players of N-cycling in the soil. They occurred in almost identical relative abundance under eCO_2 and aCO_2 and differed only between the ring pairs. The stability of the microbial communities towards fumigation with elevated CO_2 is in agreement with previous studies on different FACE facilities (Haase *et al.*, 2008; Marhan *et al.*, 2011; Nelson *et al.*, 2010; Regan *et al.*, 2011). Regan *et al.* (2011) also found stronger influence of the location of the FACE ring pairs in Giessen or the soil depth on the abundance of *amoA*, *nirK*, *nirS* and *nosZ* than of eCO_2. Likewise, Marhan *et al.* (2011) observed a similar trend and that temporal variation and soil depth had a greater effect on the abundance of nitrate reducers and bacteria than eCO_2. Haase *et al.* (2008) attributed the lack of a response of microbial community abundance to unaltered C-flux from the whole root system into soil at eCO_2. At GiFACE, the additional C uptake also did not result in increased soil C sequestration. Instead, a loss of soil C, in together with the breakup of large macroaggregates, was detected and caused enhanced ecosystem respiration under eCO_2 (Lenhart, 2008). Influence of higher labile C input by the plant-root system may occur only directly at the root-

soil interface and would then be rapidly consumed by microorganisms attached or located around the root. It was also reported that fungal biomass was more strongly influenced by elevated CO_2 than bacterial biomass (Drigo *et al*., 2007; Jones *et al*., 1998), but other studies found a negligible effect on fungal communities by *e*CO_2 (Guenet *et al*., 2012; Lee *et al*., 2015). In our study, dissimilatory nitrate reducer communities were composed differently between *e*CO_2 and *a*CO_2 and varied with CO_2 level between rings as shown by CCA of T-RFLP fingerprinting data. This agrees well with the finding by Müller *et al*. (2009) that DNRA rates were increased by ~ 150% under *e*CO_2. Higher DNRA rates were supported under high labile C concentrations and low N (Nijburg *et al*., 1997; Tiedje, 1982). However, when using the higher resolution technique (454 pyrosequencing) differences in *nrfA* composition between *e*CO_2 and *a*CO_2 were again superimposed by variances between the ring pairs.

Differences between microbial communities in soils exposed to elevated and ambient CO_2 were also found by other studies (Deng *et al*., 2013; He *et al*., 2010; Xiong *et al*., 2015; Xu *et al*., 2013). Yet a comparison of results from different FACE facilities is ambiguous as the CO_2 concentration applied (to an overall concentration of +50%) varied which may have resulted in a higher C-input into the soil by plants. Moreover, other study sites were N-rich (e.g. BioCON experiment site: Reich *et al*., 2001) in contrast to GiFACE site, which is strongly N-limited. How much C is provided by the plants differs between 20-50% of total CO_2 uptake, depending on the plant population (Kuzyakov and Domanski, 2000) and only a small fraction can be used by the microorganisms for biomass production (van Veen *et al*., 1991). Changes in community composition and abundance were also observed in rice root samples, but again a much higher elevation of CO_2 was applied (Okubo *et al*., 2015). Thus, it remains unclear whether an elevation of CO_2 by +20% per plot suffices for increased C-inputs into the rhizosphere. Events of large N_2O fluxes were rare during 16 years of fumigation with *e*CO_2. Only during the first two years after establishing the GiFACE facility a series of events

with N_2O fluxes of more than 100 ng occurred in at least one ring and these events were related to a high N-status of the soil after fertilization and plant growth in spring.

3.6. Conclusion

Our results lead to the conclusion that +20% *e*CO_2 has little to no effect on the abundance and composition of microbial communities involved in N-cycling in soil. The main soil N_2O-fluxes from the FACE rings in Giessen occurred concomitant with N fertilization and plant growth. Hence, future studies should investigate in more detail how elevated CO_2 in conjunction with massive N inputs during fertilization impact microbial communities in the soil and whether this leads to a short-term activation of microbial groups involved in N-cycling and hence higher production of N_2O.

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3.9. Supplementary Material

Table S3.1. Primers and PCR conditions for amplifying functional marker genes *nirK*, *nirS*, *nosZ*, *nifH*, *amoA* Archaea, *amoA* Bacteria, *nrfA*, archaeal and bacterial 16S rRNA gene for qPCR.

Gene	Primer sets	Forward primer	Reverse primer	PCR conditions	PCR product length (bp)	References
nifH	PolF/ PolR	TGCGA(C/T)CC(G/C)A ARGC(C/G/T)GACTC	AT(G/C)GCCATCAT(C/T) TC(A/G)CCGGA	95 °C/15min, 6 cycles of (95°C/15sec, 60°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 55°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	360	Poly *et al*., 2001
nirK	qnirK876/ qnirK1040	AT(C/T)GGCGG(A/C/G) A(C/T)GGCGA	GCCTCGATCAG(A/G)TT (A/G)TGGTT	95 °C/15min, 6 cycles of (95°C/15sec, 63°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 58°C/30sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	165	Henry *et al*., 2004
nirS	qCd3af/ qR3cd	AACG(C/T)(G/C)AAGG A(A/G)AC(G/C)GG	GA(G/C)TTCGG(A/G)TG (G/C)GTCTT(G/C)A(C/T)G AA	95 °C/15min, 6 cycles of (95°C/15sec, 63°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 58°C/30sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	425	Kandeler *et al*., 2006
nosZ	nosZ2F/ nosZ2R	CGC(A/G)ACGGCAA (G/C)AAGGT(G/C) (A/C)(G/C)(G/C)GT	CA(G/T)(A/G)TGCA(G/T) (G/C)GC(A/G)TGGCAGA A	95 °C/15min, 6 cycles of (95°C/15sec, 65°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 60°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	267	Henry *et al*., 2006
nrfA	nrfA2aw/ nrfAR1	CA(A/G)TG(C/T)CA (C/T)GT(C/G/T)GA (A/G)TA	T(A/T)(A/C/G/T)GGCAT (A/G)TG(A/G)CA(A/G)TC	95 °C/15min, 6 cycles of (95°C/15sec, 58°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 53°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	269	Welsh *et al*., 2014; Mohan *et al*., 2004
Archaeal *amoA*	Arch-amoAF/ Arch-amoAR	(G/C)TAATGGTCTGGC TTAGACG	GCGGCCATCCATCTGTA TGT	95 °C/15min, 6 cycles of (95°C/15sec, 58°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 53°C/20sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	635	Francis *et al*., 2005
Bacterial *amoA*	amoA-1F/ amoA2R	GGGGTTTCTACTGGT GGT	CCCCTC(G/T)G(G/C)AAA GCCTTCTTC	95 °C/15min, 6 cycles of (95°C/15sec, 65°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 60°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	491	Rotthauwe *et al*., 1997
Archaeal 16S rRNA gene	Ar364f/ Ar934br	CGGGG(C/T)GCA(G/C) CAGGCGCGAA	GTGCTCCCCCGCCAATT CCT	95 °C/15min, 6 cycles of (95°C/15sec, 56°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 52°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	570	Burggraf *et al*., 1997; Großkopf *et al*., 1998
Bacterial 16S rRNA gene	Ba519f/ Ba907r	CAGC(A/C)GCCGCGG TAA(A/C/G/T)(A/T)C	CCGTCAATTC(A/C)TTT (A/G)AGTT	95 °C/15min, 6 cycles of (95°C/15sec, 54°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 49°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	388	Lane, 1991

Table S3.2. Primers and PCR conditions for amplifying functional marker genes *nirK*, *nirS*, *nosZ*, *nifH*, *amoA* Archaea, *amoA* Bacteria, *nrfA*, archaeal and bacterial 16S rRNA gene for T-RFLP and 454 pyrosequencing (without FAM label). For 454 pyrosequencing the annealing temperature was increased by +2°C.

Gene	Primer sets	Forward primer	Reverse primer	PCR conditions	PCR product length (bp)	References
nifH	PolF-FAM/ PolR	TGCGA(C/T)CC(G/C) AARGC(C/G/T)GAC TC	AT(G/C)GCCATCAT (C/T)TC(A/G)CCGGA	95 °C 5min, 10 cycles of (95°C/30sec, 60°C/40sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 55°C/40sec, 72°C/2min) 72°C/10min.	360	Poly *et al*., 2001
nirK	nirK1F/ nirK5R-FAM	GG(A/C)ATGGT (G/T)CC(C/G)TGGC A	GCCTCGATCAG(A/G) TT(A/G)TGG	95 °C/5min, 10 cycles of (95°C/30sec, 56°C/40sec (-0.5°C every cycle), 72°C/40sec), 25 cycles (95°C/30sec, 54°C/40sec, 72°C/40sec), 72°C/7min.	514	Braker *et al*., 1998
nirS	cd3aF-FAM/ R3cd	GT(C/G)AACGT (C/G)AAGGA(A/G)A C(C/G)GG	GA(C/G)TTCGG(A/G) TG(C/G)GTCTTG	95 °C/5min, 10 cycles of (95°C/30sec, 56°C/40sec (-0.5°C every cycle), 72°C/40sec), 25 cycles (95°C/30sec, 54°C/40sec, 72°C/40sec) 72°C/7min.	425	Throbäck *et al*., 2004
nosZ	NosF-FAM/ NosR	CG(C/T)TGTTC(A/C) TCGACAGCCAG	CATGTGCAG (A/C/G/T)GC(A/G)TG GCAGAA	95 °C 5min, 10 cycles of (95°C/30sec, 59°C/90sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 56°C/40sec, 72°C/2min) 72°C/10min.	700	Klooset *al*., 2001
nrfA	nrfA2aw-FAM/ nrfAR1	CA(A/G)TG(C/T)CA (C/T)GT(C/G/T)GA (A/G)TA	T(A/T)(A/C/G/T)GGC AT(A/G)TG(A/G)CA (A/G)TC	95 °C 5min, 10 cycles of (95°C/30sec, 57°C/40sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 53°C/30sec, 72°C/2min) 72°C/10min.	269	Welsh *et al*., 2014; Mohan *et al*., 2004
Archaeal *amoA*	Arch-amoAF-FAM/ Arch-amoAR	(G/C)TAATGGTCTG GCTTAGACG	GCGGCCATCCATCT GTATGT	95 °C 5min, 10 cycles of (95°C/30sec, 57°C/90sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 53°C/50sec, 72°C/2min) 72°C/10min.	635	Francis *et al*., 2005
Bacterial *amoA*	amoA-1F-FAM/ amoA2R	GGGGTTTCTACTG GTGGT	CCCCTC(G/T)G(G/C) AAAGCCTTCTTC	95 °C 5min, 10 cycles of (95°C/30sec, 65°C/40sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 60°C/40sec, 72°C/2min) 72°C/10min.	491	Rotthauwe *et al*., 1997
Archaeal 16S rRNA gene	Ar109f/ Ar912r-FAM	AC(G/T)GCTCAGTA ACACGT	GTGCTCCCCCGCCA ATTCCT	95 °C 5min, 10 cycles of (95°C/30sec, 58°C/60sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 53°C/60sec, 72°C/2min) 72°C/10min.	803	Großkopf *et al*., 1998; Lueders and Friedrich, 2000
Bacterial 16S rRNA gene	Ba27f-FAM/ Ba907r	GAGTTTG((A/C)TCC TGGCTCAG	CCGTCAATTC(A/C)T TT(A/G)AGTT	95 °C 5min, 10 cycles of (95°C/30sec, 49°C/60sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 44°C/30sec, 72°C/2min) 72°C/10min.	898	Weisburg *et al*., 1991; Lane, 1991

Table S3.3. Soil parameters of each FACE ring at GiFACE.

Ring	Soil characteristics							
	pH	**NO_3^- [µM/g dw]**	**NH_4^+ [µM/g dw]**	**H_2O [%]**	**C [%]**	**H [%]**	**N [%]**	**C:N ratio**
E1	5.45^{a} ± 0.10	3.02^{ab} ± 0.32	0.37^{a} ± 0.076	25.00^{a} ± 2.00	4.50^{a} ± 0.24	0.98^{ab} ± 0.02	0.39^{ab} ± 0.02	11.43^{a} ± 0.10
A1	5.66^{ab} ± 0.30	2.14^{a} ± 0.99	0.30^{ab} ± 0.144	20.00^{a} ± 3.00	3.56^{a} ± 0.45	0.80^{b} ± 0.09	0.32^{b} ± 0.04	11.14^{a} ± 0.57
E2	6.04^{b} ± 0.12	8.02^{b} ± 3.75	0.23^{ab} ± 0.081	20.00^{a} ± 1.73	4.50^{a} ± 0.75	1.05^{ab} ± 0.09	0.44^{ab} ± 0.07	10.14^{b} ± 0.09
A2	6.02^{b} ± 0.16	4.71^{ab} ± 2.23	0.16^{ab} ± 0.002	22.67^{a} ± 0.58	4.56^{a} ± 0.83	1.03^{ab} ± 0.15	0.45^{ab} ± 0.07	10.04^{b} ± 0.25
E3	5.81^{ab} ± 0.27	3.77^{ab} ± 0.53	0.12^{b} ± 0.027	23.33^{a} ± 3.06	4.83^{a} ± 1.04	1.17^{a} ± 0.13	0.48^{ab} ± 0.09	10.10^{b} ± 0.19
A3	6.11^{b} ± 0.09	6.88^{ab} ± 1.40	0.20^{ab} ± 0.079	23.67^{a} ± 6.03	5.35^{a} ± 0.63	1.18^{a} ± 0.09	0.51^{a} ± 0.08	10.52^{ab} ± 0.69

[ab] Identical letters indicate no significance differences ($P > 0.05$). Mean±SD (n=3).

Table S3.4. Abundance of functional marker genes relative to total bacterial and archaeal copy numbers in soil of single FACE rings at GiFACE.

Ring	Ratio copy number of functional marker gene/total 16S rRNA genes						
	Denitrification			Nitrification		DNRA	N-fixation
	nirK	*nirS*	*nosZ*	Archaeal *amoA*	Bacterial *amoA*	*nrfA*	*nifH*
E1	0.059[a] ± 0.014	0.004[a] ± 0.001	0.032[a] ± 0.002	0.029[a] ± 0.111	0.0002[a] ± 0.0001	0.023[a] ± 0.006	0.054[a] ± 0.008
A1	0.078[a] ± 0.010	0.003[a] ± 0.000	0.039[a] ± 0.016	0.028[a] ± 0.067	0.0003[a] ± 0.0003	0.024[a] ± 0.002	0.050[a] ±0.004
E2	0.071[a] ± 0.018	0.009[bc] ± 0.003	0.034[a] ± 0.012	0.047[a] ± 0.114	0.0009[ab] ± 0.0005	0.055[a] ±0.015	0.047[a] ±0.015
A2	0.058[a] ± 0.007	0.012[c] ± 0.003	0.021[a] ± 0.002	0.062[a] ± 0.332	0.0005[ab] ± 0.0001	0.028[a] ± 0.031	0.062[a] ± 0.005
E3	0.047[a] ± 0.022	0.005[ab] ± 0.003	0.018[a] ± 0.002	0.039[a] ± 0.112	0.0006[ab] ± 0.0004	0.031[a] ± 0.019	0.043[a] ± 0.027
A3	0.057[a] ±0.011	0.006[ab] ± 0.001	0.027[a] ± 0.005	0.056[a] ± 0.085	0.0013[b] ± 0.0004	0.037[a] ± 0.006	0.046[a] ± 0.006

[ab] Identical letters indicate no significance differences ($P > 0.05$). Mean±SD (n=3).

Table S3.5. Canonical correspondence analysis to determine the variance explained (percentage of total variation) in T-RFLP data based on the marker genes of the nitrogen cycle (*nifH, nirK*, *nirS*, *nosZ*, *amoA* of Archaea and Bacteria and *nrfA*) as well as of the archaeal and bacterial community by environmental variables for based on T-RFLP.

Community	Variable	% Variance explained	P-value
nifH	pH value	17.3	**0.010***
	NO_3^- concentration	14.5	**0.023***
	NH_4^+ concentration	20.6	**0.010***
	Elevated or ambient CO_2	4.6	0.632
nirK	pH value	23.0	**0.005***
	NO_3^- concentration	11.5	**0.031***
	NH_4^+ concentration	14.0	**0.015***
	Elevated or ambient CO_2	6.8	0.231
nirS	pH value	21.2	**0.005***
	NO_3^- concentration	13.0	**0.030***
	NH_4^+ concentration	20.1	**0.015***
	Elevated or ambient CO_2	6.1	0.354
nosZ	pH value	20.3	**0.010***
	NO_3^- concentration	9.5	0.093
	NH_4^+ concentration	12.9	**0.046***
	Elevated or ambient CO_2	6.3	0.372
nrfA	pH value	16.4	**0.010***
	NO_3^- concentration	5.2	0.539
	NH_4^+ concentration	14.5	**0.017***
	Elevated or ambient CO_2	12.7	**0.020***
Archaeal *amoA*	pH value	24.5	**0.005***
	NO_3^- concentration	11.9	0.056
	NH_4^+ concentration	22.5	**0.005***
	Elevated or ambient CO_2	3.9	0.816
Bacterial *amoA*	pH value	26.7	**0.005***
	NO_3^- concentration	16.8	**0.018***
	NH_4^+ concentration	18.3	**0.007***
	Elevated or ambient CO_2	5.9	0.424
Bacterial 16S rRNA gene	pH value	19.4	**0.005***
	NO_3^- concentration	12.5	**0.017***
	NH_4^+ concentration	13.5	**0.010***
	Elevated or ambient CO_2	6.4	0.343
Archaeal 16S rRNA gene	pH value	29.7	**0.005***
	NO_3^- concentration	20.3	**0.017***
	NH_4^+ concentration	30.7	**0.005***
	Elevated or ambient CO_2	10.2	0.145

*****: significant. Significance was tested by ANOVA (P value < 0.05).

Table S3.6. Influence of elevated atmospheric CO_2 analyzed by CCA for the marker genes of the nitrogen cycle (*nifH, nirK*, *nirS*, *nosZ*, *nrfA amoA* of Archaea and Bacteria) as well as of archaeal and bacterial 16S rRNA gene community based on T-RFLP. The samples are divided by the organization in the FACE facility.

Marker gene	***FACE ring pair***		
	E1/A1	***E3/A3***	***E2/A2***
nirK	0.700	0.197	0.401
nirS	0.201	0.082	**0.033***
nosZ	0.193	0.401	0.100
nifH	0.087	0.600	0.100
nrfA	0.151	**0.010***	0.125
Archaeal *amoA*	0.600	0.401	0.801
Bacterial *amoA*	0.418	0.056	0.084
Bacterial 16S rRNA gene	0.533	0.100	0**.001***
Archaeal 16S rRNA gene	**0.043***	0.053	0.415

*: significant. Significance was tested by ANOVA (P value < 0.05).

Table S3.7. Proportion of variance explained (percentage of total variation) by environmental variables determined by CCA for the marker genes of the nitrogen cycle (*nifH, nirK*, *nirS*, *nosZ*, *amoA* of Archaea and Bacteria and *nrfA*) as well as of archaeal and bacterial 16S rRNA gene community based on pyrosequencing.

Community	Variable	% Variance explained	P-value
nifH	pH value	37.1	0.104
	NO_3^- concentration	30.3	0.165
	NH_4^+ concentration	47.0	**0.026***
	Elevated/ ambient CO_2	6.1	0.900
nirK	pH value	40.7	**0.043***
	NO_3^- concentration	30.1	0.154
	NH_4^+ concentration	14.0	0.600
	Elevated/ ambient CO_2	15.9	0.231
nirS	pH value	23.7	0.278
	NO_3^- concentration	19.9	0.347
	NH_4^+ concentration	26.8	0.239
	Elevated/ ambient CO_2	6.4	1.000
nosZ	pH value	40.4	0.083
	NO_3^- concentration	26.2	0.226
	NH_4^+ concentration	51.6	**0.015***
	Elevated/ ambient CO_2	11.7	0.600
nrfA	pH value	20.7	0.360
	NO_3^- concentration	14.5	0.513
	NH_4^+ concentration	40.9	0.075
	Elevated/ ambient CO_2	6.6	0.800
Archaeal *amoA*	pH value	73.5	**0.022***
	NO_3^- concentration	35.3	0.203
	NH_4^+ concentration	78.4	**0.007***
	Elevated/ ambient CO_2	7.8	0.800
Bacterial *amoA*	pH value	13.9	0.550
	NO_3^- concentration	34.8	0.217
	NH_4^+ concentration	10.5	0.635
	Elevated/ ambient CO_2	25.0	0.300
Archaeal 16S rRNA gene	pH value	57.8	**0.022***
	NO_3^- concentration	23.7	0.316
	NH_4^+ concentration	59.8	**0.017***
	Elevated/ ambient CO_2	18.4	0.500

*****: significant. Significance was tested by ANOVA (P value < 0.05)

Table S3.8. Amino acid identities of *in silico* translated OTU representatives of nitrogen cycle associated genes retrieved from FACE facility to closely related sequences.

Gene	OTU (No.)	Relative abundance of OTUs per ring pair (1/2/3 in %)	Closest relative (accession No.)	Identity (%)	Closest cultured relative (accession No.)	Identity (%)
nifH	17	15.6/18.8/15.2	Uncult. bact. (KF847701)	92	*Bradyrhizobium denitrificans* LMG 8443 (AP012279)	88
	8	9.3/16.0/22.8	Uncult. bact. (HQ335832)	94	*Azospirillum brasilense* AWC8 (GQ161227)	86
	28	11.7/5.2/3.3	Uncult. bact. (KF847733)	99	*Halorhodospira halophila* DSM 244 (EF199951)	87
	7	1.6/6.1/9.8	Uncult. bact. (JX268406)	99	*Azospirillum brasilense* AWB4 (GQ161231)	89
	1	8.1/2.5/1.8	Uncult. bact. (KC667514)	99	*Mesorhizobium huakuii* (KF800056)	85
	3	7.8/2.4/1.7	*Azospirillum brasilense* Gr58 (FR745919)	90	*Azospirillum brasilense* Gr58 (FR745919)	90
	18	3.8/4.4/3.9	Uncult. bact. (JX865930)	90	*Desulfovibrio magneticus* RS-1 (AP010904)	84
	12	5.7/4.5/1.5	Uncult. soil bact. (DQ776436)	99	*Gluconacetobacter diazotrophicus* (AF030414)	90
	2	1.3/0.8/2.6	Uncult. bact. (AY601063)	93	*Methylobact.* sp. 4-46 (CP000943)	89
	6	1.0/1.9/3.6	Uncult. bact. (AY601063)	97	*Azospirillum brasilense* Sp245 (HE577327)	91
nirK	8	23.7/23.1/23.9	Uncult. bact. (DQ783977)	99	*Bradyrhizobium japonicum* USDA 6 (AP012206)	85
	27	19.1/19.0/16.7	Uncult. bact. (DQ783979)	99	*Bradyrhizobium* sp. ORS278 (CU234118)	85
	19	5.3/13.1/8.2	Uncult. bact. (DQ304355)	100	*Azospirillum lipoferum* A5 (HQ288913)	94
	3	10.2/3.7/7.5	Uncult. bact. (DQ784024)	100	*Bradyrhizobium japonicum* SEMIA 5079 (CP007569)	81
	2	7.3/3.7/5.2	Uncult. bact. (DQ783839)	100	*Mesorhizobium ciceri* WSM1271 (CP002448)	85
	34	1.8/1.2/6.1	Uncult. bact. (DQ783865)	100	*Rhodopseudomonas palustris* TIE-1 (CP001096)	86
	4	2.2/3.3/1.9	Uncult. bact. (DQ783332)	99	*Bradyrhizobium japonicum* SEMIA 5079 (CP007569)	89
	25	2.0/2.2/2.5	Uncult. bact. (EF645006)	100	*Bradyrhizobium* sp. GSM-471 (FN600571)	83
	66	1.1/2.3/2.8	Uncult. bact. (AY249359)	99	*Sinorhizobium fredii* HH103 (HE616890)	82
	79	0.5/3.5/2.1	Uncult. bact. (DQ783944)	96	*Rhodopseudomonas palustris* TIE-1 (CP001096)	84
nirS	2	53.8/32.6/42.3	Uncult. bact. (KC468992)	99	*Bradyrhizobium oligotrophicum* S58 (AP012603)	87
	6	14.1/13.9/11.3	Uncult. bact. (KC010976)	98	*Thiobacillus denitrificans* ATCC 25259 (CP000116)	80
	9	4.7/10.1/12.7	Uncult. bact. (AY583422)	95	*Rubrivivax gelatinosus* IL144 (AP012320)	79
	12	1.6/11.0/9.8	Uncult. bact. (HE818699)	88	*Azoarcus aromaticum* EbN1 (CR555306)	79
	3	0.7/3.3/1.9	Uncult. bact. (HE995561)	100	*Bradyrhizobium oligotrophicum* S58 (AP012603)	85
	4	3.0/0.7/0.7	Uncult. bact. (JN179277)	95	*Rhodanobacter* sp. D206a (AB480490)	92
	25	0.5/1.7/1.7	Uncult. bact. (KC010985)	94	*Thiobacillus denitrificans* ATCC 25259 (CP000116)	76
	80	0.3/2.5/0.8	Uncult. bact. (JN179307)	92	*Pseudomonas stutzeri* ATCC 17588 (CP002881)	76

	22	0.2/1.4/2.1	Uncult. bact. (GU393213)	94	*Rubrivivax gelatinosus* IL144 (AP012320)	80
	28	0.3/1.2/1.9	Uncult. bact. (EU650311)	94	*Azospirillum* sp. TSO28-1 (AB545704)	83
nosZ	1	58.5/38.1/42.6	Uncult. bact. (FN859706)	99	*Rhodopseudomonas palustris* HaA2 (CP000250)	91
	7	17.1/12.4/14.3	Uncult. bact. (FM993387)	99	*Bradyrhizobium* sp. GSM-467 (FN600633)	96
	8	2.2/5.6/4.8	Uncult. bact. (FN859751)	99	*Bradyrhizobium japonicum* USDA 110 (BA000040)	88
	2	0.9/3.4/5.3	Uncult. bact. (AY325632)	90	*Bradyrhizobium japonicum* USDA 110 (BA000040)	82
	5	2.8/3.4/2.2	Uncult. bact. (FN295856)	99	*Bradyrhizobiaceae* bact. D195a (AB480505)	96
	22	1.1/3.2/3.4	Uncult. bact. (FN859742)	99	*Rhodopseudomonas palustris* HaA2 (CP000250)	85
	23	0.4/3.8/2.7	Uncult. bact. (JQ038940)	93	*Oligotropha carboxidovorans* OM5 (CP002825)	83
	12	4.0/1.5/1.4	Uncult. bact. (FN295926)	94	*Azospirillum* sp. TSH10 (AB542250)	91
	3	1.2/2.4/2.3	Uncult. bact. (FN859774)	99	*Oligotropha carboxidovorans* OM5 (CP002825)	87
	28	0.4/3.8/2.7	Uncult. bact. (FN859905)	99	*Bradyrhizobium japonicum* USDA 110 (BA000040)	87
nrfA	22	2.4/14.7/54.0	*Bacteroides fragilis* 638R (FQ312004)	73	*Bacteroides fragilis* 638R (FQ312004)	73
	19	0.5/1.4/28.0	Uncult. bact. (JX293771)	75	*Anaeromyxobacter dehalogenans* 2CP-1 (CP001359)	74
	30	9.4/12.3/0.9	Uncult. bact. (JX293735)	77	*Anaeromyxobacter dehalogenans* 2CP-1 (CP001359)	73
	23	12.2/2.8/0.4	Uncult. bact. (JX293808)	88	*Sorangium cellulosum* So ce 56 (AM746676)	80
	41	0.5/7.3/0.7	Uncult. bact. (JX293737)	88	*Anaeromyxobacter dehalogenans* 2CP-1 (CP001359)	77
	48	2.5/5.2/0.8	*Geobacter* sp. M18 (CP002479)	71	*Geobacter* sp. M18 (CP002479)	71
	7	4.6/1.4/0.3	Uncult. bact. (JX293797)	88	*Anaeromyxobacter* sp. Fw109-5 (CP000769)	79
	6	4.8/0.6/0.7	Uncult. bact. (JX293771)	80	*Anaeromyxobacter* sp. Fw109-5 (CP000769)	72
	16	0.8/4.2/0.5	Uncult. bact. (JX293798)	89	*Sorangium cellulosum* So ce 56 (AM746676)	80
	277	0.5/2.3/0.3	Uncult. bact. (JX293810)	89	*Anaeromyxobacter dehalogenans* 2CP-1 (CP001359)	79
Archaeal	2	51.5/71.7/68.4	Uncult. arch. (JQ750224)	100	Cand. *Nitrososphaera gargensis* Ga9.2 (CP002408)	80
amoA	3	35.4/26.6/30.0	Uncult. bact. (KJ645270)	100	Cand. *Nitrososphaera gargensis* Ga9.2 (CP002408)	81
	1	11.1/0.0/0.1	Uncult. Thaumarchaeote (KC962900)	100	Cand. *Nitrososphaera gargensis* Ga9.2 (CP002408)	91
	6	0.2/1.5/1.4	Uncult. Crenarchaeote (JF748278)	100	Cand. *Nitrososphaera evergladensis* SR1 (CP007174)	80
	7	0.8/0.0/0.1	Uncult. arch. (JQ750204)	99	Cand. *Nitrososphaera evergladensis* SR1 (CP007174)	79
	4	0.9/0.0/0.0	Uncult. arch. (KF709843)	100	Cand. *Nitrososphaera gargensis* Ga9.2 (CP002408)	81
Bacterial	2	75.4/84.2/76.7	Uncult. bact. (KC010733)	100	*Nitrosospira* sp. Wyke8 (EF175099)	99
amoA	1	16.4/14.2/22.5	Uncult. ammonia-oxidizing bact. (HQ638973)	100	*Nitrosospira* sp. Nsp12 (AY123823)	97
	15	5.1/0.4/0.3	Uncult. ammonia-oxidizing bact. (KC454074)	99	*Nitrosospira* sp. Nsp65 (AY123839)	93
	6	2.0/0.6/0.2	Uncult. ammonia-oxidizing bact.	100	*Nitrosospira* sp. CT2F (AY189143)	99

			(JF936483)			
	27	0.5/0.1/0.0	Uncult. bact. (KC010732)	100	*Nitrosolobus multiformis* (AF042171)	100
Archaeal 16S rRNA gene	1	64.3/66.1/63.8	Uncult. thaumarchaeote (KF276537)	99	*Nitrososphaera viennensis* EN76 (CP007536)	94
	2	29.4/20.7/25.2	Uncult. thaumarchaeote KF275841)	99	*Nitrososphaera viennensis* EN76 (CP007536)	96
	6	5.7/10.9/9.9	Uncult. arch. (EF023033)	99	Cand. *Nitrososphaera evergladensis* SR1 (CP007174)	96
	3	0.4/0.8/0.0	Uncult. arch. (KM273713)	97	Cand. *Nitrososphaera evergladensis* SR1 (CP007174)	100
	56	0.2/0.2/0.3	Uncult. soil archaeon (HM224540)	99	Cand. *Methanomethylophilus alvus* Mx1201 (CP004049)	83

Table S3.9. Analysis of *in silico*-translated amino-acid sequences of representatives of nitrogen cycle associated genes derived from GiFACE

Gene marker	Threshold similarity (%)	FACE ring	No. of sequences	Good's coverage (%)[a]	No. of OTUs observed	No. of OTUs estimated[b]	H[c]	E[d]
nifH	92	E1	521	93.9	66	106	4.84	0.80
		A1	576	89.6	80	208	5.05	0.80
		E2	756	92.5	83	164	5.07	0.80
		A2	733	91.8	85	179	5.25	0.82
		E3	431	92.8	61	122	4.53	0.76
		A3	778	93.2	67	153	4.55	0.75
nirK	92	E1	881	94.3	108	178	4.82	0.71
		A1	1196	94.8	99	180	4.11	0.62
		E2	1275	95.5	98	176	4.21	0.63
		A2	904	94.5	93	178	4.39	0.67
		E3	2075	95.8	103	215	4.61	0.69
		A3	2370	96.3	108	210	4.31	0.64
nirS	92	E1	607	96.7	46	80	2.96	0.54
		A1	1373	97.6	45	84	2.80	0.51
		E2	2004	97.5	50	117	3.65	0.65
		A2	1324	97.3	53	108	3.75	0.65
		E3	1909	98.4	43	92	2.81	0.52
		A3	2384	98.2	52	106	3.60	0.63
nosZ	92	E1	432	92.4	57	107	3.40	0.58
		A1	840	95.4	44	100	2.90	0.53
		E2	1247	95.3	70	137	4.64	0.76
		A2	1073	95.1	62	128	4.27	0.72
		E3	1196	95.6	57	121	3.96	0.68
		A3	1510	95.3	74	151	4.50	0.72
nrfA	92	E1	1147	77.2	182	559	6.53	0.87
		A1	4999	86.1	177	596	6.35	0.85
		E2	5015	88.5	154	570	5.85	0.80
		A2	4778	87.3	154	590	5.78	0.80
		E3	6928	98.8	19	60	1.72	0.41
		A3	6583	95.0	60	233	2.80	0.47
Archaeal amoA	92	E1	586	99.7	7	8	1.59	0.58
		A1	2269	100.0	5	5	1.31	0.58
		E2	2137	100.0	4	4	0.85	0.47
		A2	565	100.0	3	3	1.02	0.64
		E3	2966	99.9	4	4	0.90	0.52
		A3	3547	100.0	4	4	1.08	0.54
Bacterial amoA	92	E1	1119	99.9	4	4	0.79	0.42
		A1	1099	100.0	7	7	1.37	0.50
		E2	2170	100.0	5	6	0.93	0.43
		A2	2839	100.0	4	5	0.75	0.38
		E3	1044	99.9	5	6	0.80	0.35
		A3	815	99.6	6	8	0.64	0.25
Archaeal 16S rRNA gene	95	E1	827	99.8	3	3	1.15	0.68
		A1	2241	99.9	4	5	1.22	0.61
		E2	201	99.0	5	6	1.41	0.59
		A2	191	99.5	5	5	1.27	0.56
		E3	249	99.6	4	4	1.15	0.63
		A3	320	100.0	5	5	1.48	0.67

[a] Percent libaray coverage (Good's coverage): C = (1-ns/nt) × 100, where ns is the number of OTUs that occur only once and nt is the total number of sequences.
[b] Chao 1 richness.
[c] Shannon diversity index.
[d] Species evenness

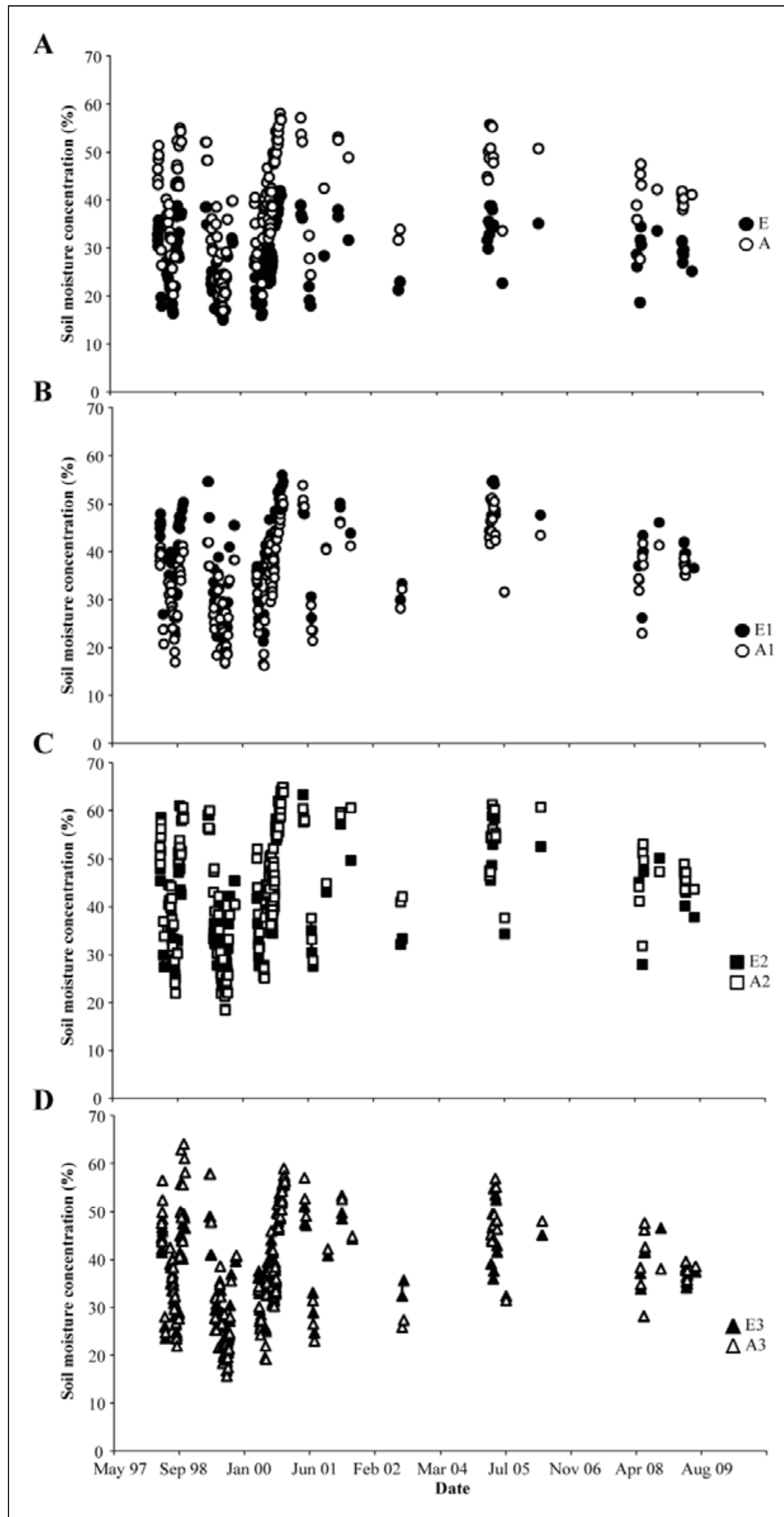

Figure S3.1. Soil moisture concentration for events in which the N_2O fluxes reached more than 100 µg $(m^2 \times h)^{-1}$ in at least one ring between the years 1997–2009: (A) Mean soil moisture concentrations at elevated (E) and ambient (A) CO_2. (B)–(D) in the three ring pairs (B, E1/A1; C, E3/A3; D, E2/A2) between 1997 to 2009.

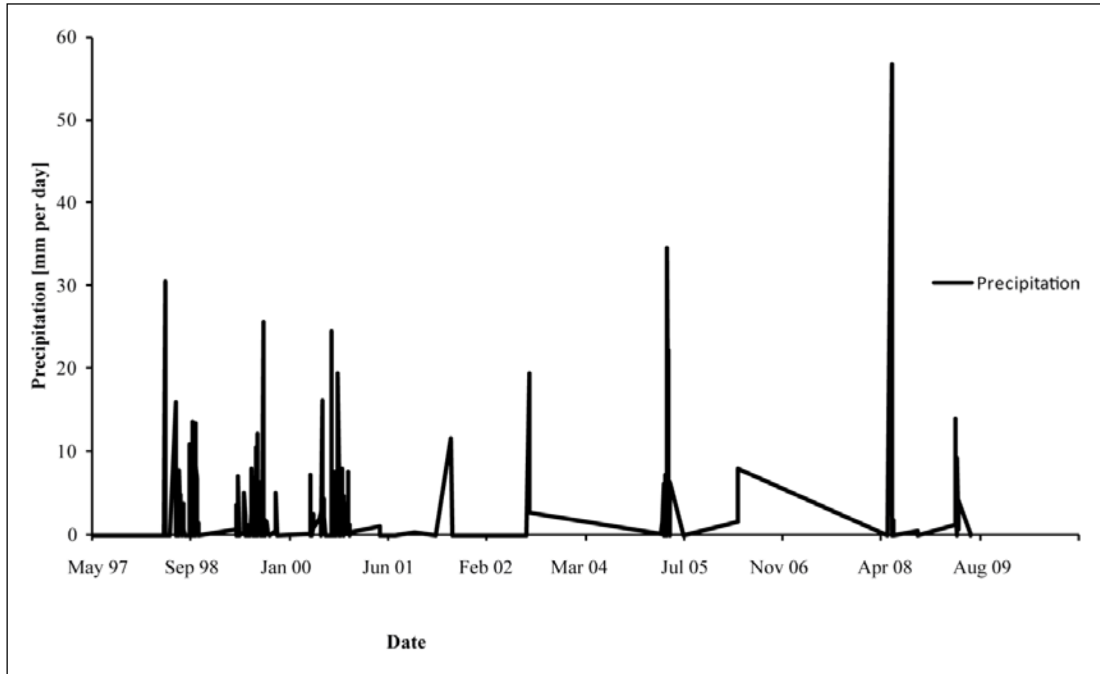

Figure S3.2. Precipitation at GiFACE for events in which the N_2O fluxes reached more than 100 µg $(m^2 \times h)^{-1}$ in at least one ring from 1997–2009.

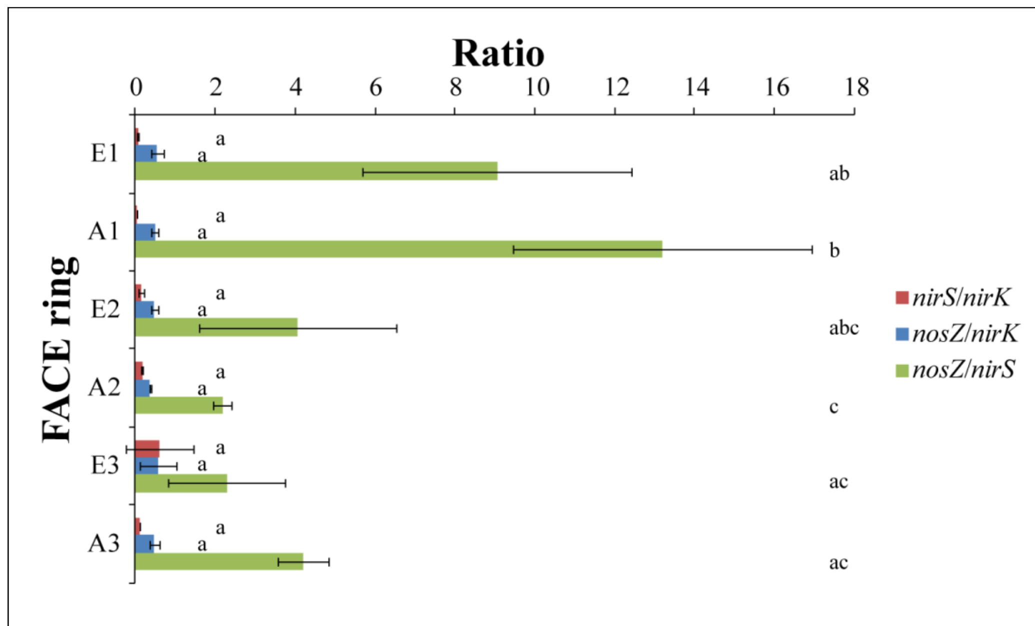

Figure S3.3. Ratios in copy numbers of denitrification genes *nirS*/*nirK*, *nosZ*/*nirS* and *nosZ*/*nirK* in single FACE rings. (mean ± SD, n=3).

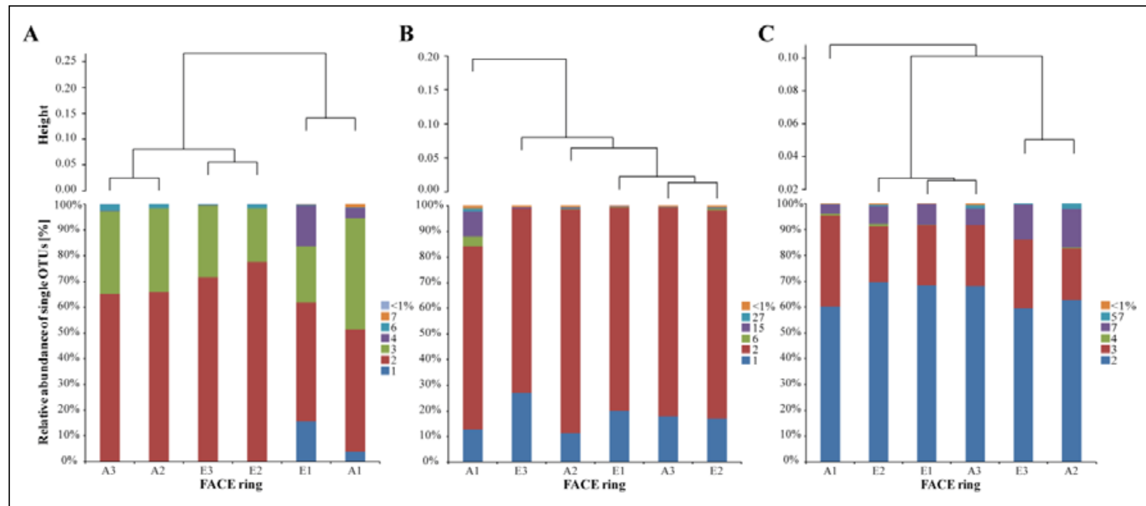

Figure S3.4. OTU based analyses of barcode labeled 454 pyrosequencing data of *amoA* Archaea (A), *amoA* Bacteria (B) and 16S rRNA gene of Archaea (C) retrieved from FACE facility soil samples as hierarchical cluster analysis by using the statistical program R and Ward's minimum variance method (upper part) and as relative abundance bar diagram (bottom part). OTUs are calculated with 8% threshold distances from rarified data sets. All OTUs that were displayed in the relative abundance graphic had at least a relative abundance of 1% in average, all other OTUs were gathered together and listed as < 1% in the graphic. Please note that the same color coding for different structural genes does not indicate whether or not such genes were derived from the same organisms.

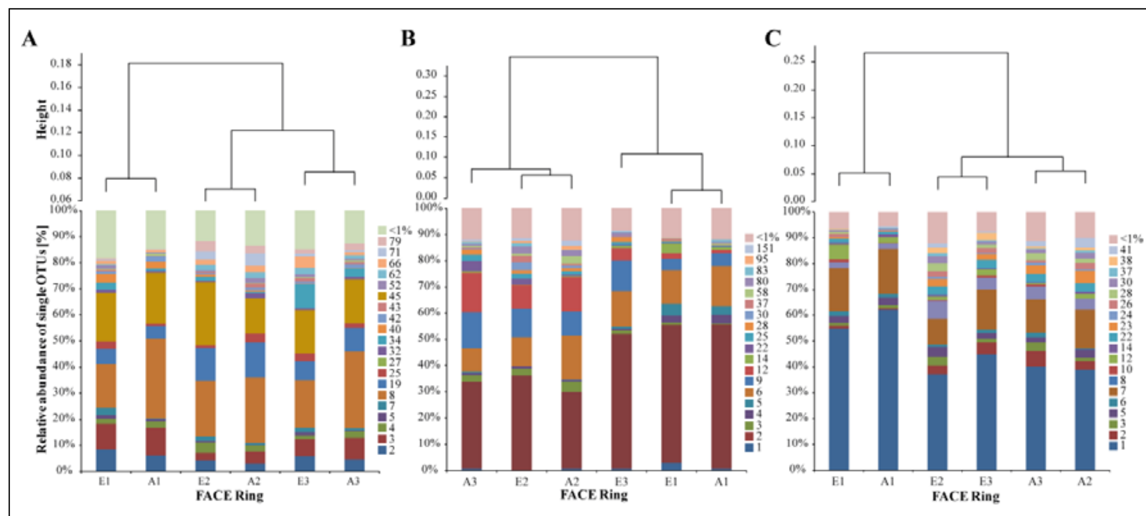

Figure S3.5. OTU based analyses of barcode labeled 454 pyrosequencing data of *nirK* (A), *nirS* (B) and *nosZ* (C) retrieved from FACE facility soil samples as hierarchical cluster analysis by using the statistical program R and Ward's minimum variance method (upper part) and as relative abundance bar diagram (bottom part). OTUs are calculated with 8% threshold distances from rarified data sets. All OTUs that were displayed in the relative abundance graphic had at least a relative abundance of 1% in average, all other OTUs were gathered together and listed as < 1% in the graphic. Please note that the same color coding for different structural genes does not indicate whether or not such genes were derived from the same organisms.

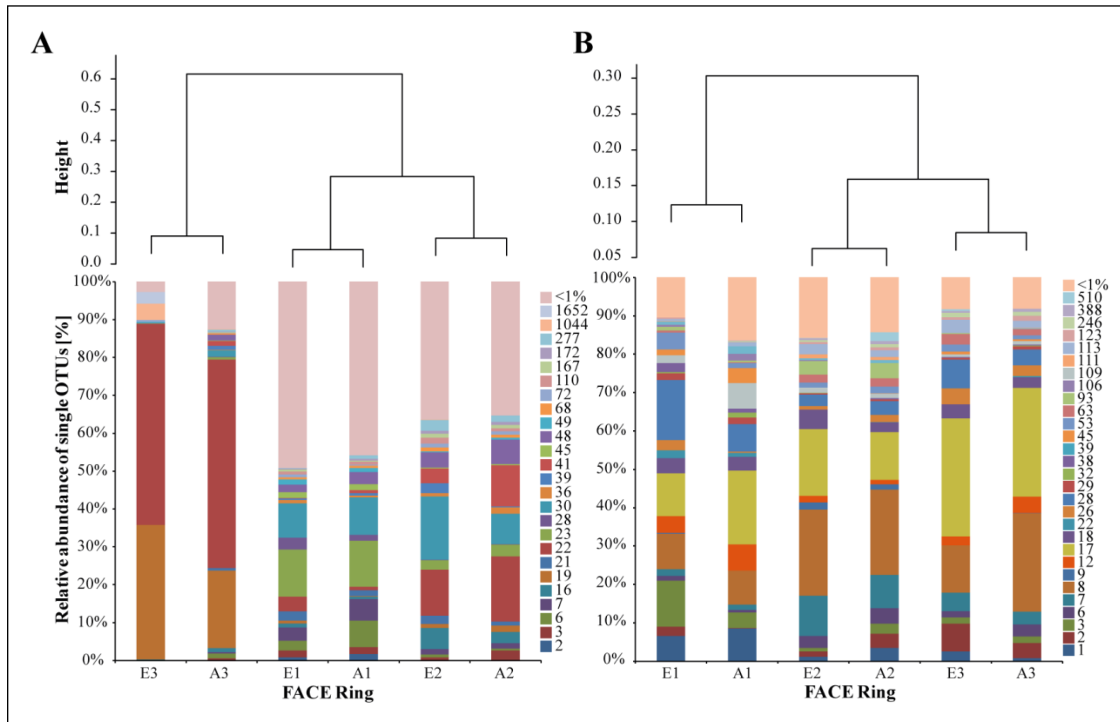

Figure S3.6. OTU based analyses of barcode labeled 454 pyrosequencing data of *nrfA* (A) and *nirS* (B) retrieved from FACE facility soil samples as hierarchical cluster analysis by using the statistical program R and Ward's minimum variance method (upper part) and as relative abundance bar diagram (bottom part). OTUs are calculated with 8% threshold distances from rarified data sets. All OTUs that were displayed in the relative abundance graphic had at least a relative abundance of 1% in average, all other OTUs were gathered together and listed as < 1% in the graphic. Please note that the same color coding for different structural genes does not indicate whether or not such genes were derived from the same organisms.

Chapter IV

Response to fertilization of transcriptionally active microbial communities involved in N-cycling in soils under eCO_2

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Contributions:

K.B. designed the study, performed the field experiment and did the sampling, performed all lab work (nucleic-acid extractions, T-RFLP analysis, qPCR analysis), performed statistical analysis, evaluated the data and wrote the manuscript.

G.M. designed the study, performed the field experiment and wrote the manuscript.

A.G. designed the study and performed the field experiment

M.S. performed the field experiment and did the sampling

L.K. designed the study

C.M. designed the study, performed the field experiment and wrote the manuscript.

G.B. designed the study, evaluated the data and wrote the manuscript.

4. Microbial response to eCO_2 and N-fertilization

4.1. Abstract

Under elevated of atmospheric CO_2 (eCO_2) mixing ratios, N_2O emission increased more than two folds compared to ambient CO_2 (aCO_2). Highest N_2O fluxes occurred in spring during the plant growth period when N-fertilizer was applied. However, the underlying mechanisms are not fully resolved yet. Thus, identification and quantification of various interactions among soil C- and N-pools, plants and soil microbial communities is the prerequisite to understand the response of ecosystems to eCO_2. We performed a comprehensive study which aimed at linking N-transformation rates, nutrient fluxes, gaseous emission (CO_2, CH_4 and N_2O) to the transcriptional response of microbial communities involved in N-cycling. The study was conducted at the Free Air Carbon Enrichment site in Giessen (GiFACE) under field conditions. Higher N_2O fluxes under eCO_2 after fertilization were attributed to denitrification and correlated with the transcriptional activation of *nirS*, a functional marker gene for denitrification. This stimulation may be triggered by higher availability of labile C in the rhizosphere under eCO_2 due to increased plant biomass and photosynthesis. Hence, this minor part of the microbial communities involved in N-transformation seems to be sufficient to sustainably influencing the N_2O emission.

4.2. Introduction

Due to anthropogenic influences, atmospheric carbon dioxide (CO_2) concentration has increased dramatically from 280 to 400 ppmv since the industrial revolution. It will continue to rise by about 1% per year and is calculated to double in the coming century (IPCC, 2013) causing well-known climatic effects (IPCC, 2013). Direct effects of elevated concentrations of atmospheric CO_2 (eCO_2) on soil microbial communities can be excluded since CO_2 concentrations in soil atmosphere are naturally high (Gobat *et al*., 2004; He *et al*., 2012). However, since approximately 40% of photosynthetically fixed C by plants were estimated to be transferred to soil via rhizodeposition (Bais *et al*., 2006; Jones *et al*., 2009; van Veen *et al*., 1991). Altered soil carbon pools and higher C:N ratios in the soil (Nie *et al*., 2015) are in turn known to affect microbial community dynamics (Denef *et al*., 2007; He *et al*., 2012).

eCO_2 has significant impacts on N-transformation rates in soil (Kammann *et al*., 2008; Müller *et al*., 2009). Observations from Giessen Free Air Carbon Dioxide Enrichment (GiFACE) facility (since 1998, ongoing) with the worldwide longest continuous trace gas emission (CO_2, methane (CH_4), nitrous oxide (N_2O) data set, showed that eCO_2 (approx. 20% above ambient) influenced both C- and N-cycling in the soil leading to a more than two-fold increase of N_2O emissions under eCO_2 (Kammann *et al*., 2008). In soils, denitrifiers and ammonia oxidizers are the main producers of N_2O (Conrad, 1996, Butterbach-Bahl *et al*., 2013) and enhanced N_2O emissions may be due to an altered $N_2O:N_2$ ratio during denitrification (Regan *et al*., 2011) or due to differences in gross N-transformation rates between eCO_2 and an ambient atmospheric CO_2 (aCO_2) control (Müller *et al*., 2004; 2009; Rütting *et al*., 2010). Moreover, a ^{15}N-tracing model based on the dataset from GiFACE revealed that dissimilatory nitrate reduction to ammonium (DNRA) rates increased by 141% along with a decrease to almost zero of the rate of heterotrophic nitrification (O_{Nrec}) (Müller *et al*., 2009). Ammonium concentrations were 17% higher and the amount of nitrate was

significantly lower under *e*CO_2 as compared to *a*CO_2 (Müller *et al*., 2009). Since N-transformations reflect the activity of the underlying microbial communities, we hypothesized that *e*CO_2 altered the abundance and composition of microbial communities involved in N-cycling in soils and thus ecosystem functioning. However, previous studies exploring their potential functional activity are not consistent and *e*CO_2 affected the abundance and/ or community composition of ammonia oxidizers, denitrifiers, dissimilatory nitrate reducers (DNRA), and N-fixers only in some instances (He *et al*., 2010; 2012; 2014; Xu *et al*., 2013; Lesaulnier *et al*., 2008; Regan *et al*., 2011; Horz *et al*., 2004).

Previous results from GiFACE showed that in two out of three replicate FACE rings the ratio of N_2O reducers to nitrite reducers was lower under *e*CO_2 (Regan *et al*., 2011) and the composition of the dissimilatory nitrate reducer (DNRA) community was altered under *e*CO_2 (Brenzinger *et al*., in preparation) which may have implications on N_2O emissions (Philippot *et al*., 2011) and N-transformations (Müller *et al*., 2009), respectively. However, soil microbial communities involved in N-cycling at GiFACE were mainly shaped by differences in soil physical and chemical factors rather than by *e*CO_2 (Brenzinger *et al*., in preparation) which cannot explain the elevated N_2O fluxes and altered N-transformations under *e*CO_2. Since the highest N_2O fluxes at GiFACE occurred mainly in spring during the plant growth period when the soil was fertilized (Kammann *et al*., 2008) we hypothesized that prompted by a close linkage between C- and N-cycling short-term responses of an overall stable microbial community accounted for the pronounced temporary alterations in N-cycling under *e*CO_2. We used a comprehensive approach to study N-transformation rates, nutrient and gas fluxes (CO_2, CH_4 and N_2O) including the dynamics of the transcriptionally active microbial community involved in N-cycling. We assumed that studying transcriptional activation of functional marker genes provides a link to the activity of the enzymes encoded helping to elucidate the response of microbial communities to *e*CO_2. Shedding light on the

various interactions among soil C- and N-cycling, among plants and soil microbial communities will be crucial to understand the response of ecosystems to *e*CO_2.

4.3. Materials & Methods

4.3.1. Site description and sampling

Soil samples were collected at the GiFACE experimental site (50°32'N and 8°43.3'E; 172 m a.s.l.) near Giessen, Germany. CO_2 fumigation at GiFACE started on a grassland site in May 1998 (> 100 years) to study the response of a semi-natural grassland to long-term, moderate atmospheric CO_2 enrichment. The whole facility consists of six plots, each with 8 m internal diameter. Two plots build one set each with an ambient (*a*CO_2) and an elevated (*e*CO_2) CO_2 plot. The *a*CO_2 plots receive 400 ppm CO_2 and the *e*CO_2 plots are fumigated with CO_2 20% above ambient air to 480 ppm. The three sets differ in soil moisture concentration and exhibit a moisture gradient, which is generated by the gradual terrain slope in the direction of the rivulet Lückebach as well as varying depths of the subsoil clay layer. In the following, the sets along the soil moisture gradient are referred to as blocks and are designated as A1 and E1, *a*CO_2 and *e*CO_2, respectively (DRY), A3 and E3, *a*CO_2 and *e*CO_2, respectively (MED) and A2 and E2, *a*CO_2 and *e*CO_2, respectively (WET). For at least 100 years, the grassland has not been ploughed. Since several decades, it was managed as a hay meadow with two cuts per year, and fertilized in mid-April with granular mineral calcium-ammonium-nitrate fertilizer at the rate of 40 kg N ha^{-1} yr^{-1}. Before 1996, fertilizer was applied at a rate of 50–100 kg N ha^{-1} yr^{-1} (Kammann *et al.*, 2008). A more detailed description of the FACE facility can be found in several publications (Jäger *et al.*, 2003; Kammann *et al.*, 2008; Regan *et al.*, 2011).

In March 2013 in each of the six ring plots two subplots for ^{15}N labelling (60×90 cm) and one overlapping subplot for ^{13}C labelling were installed for a pulse labelling experiment (see also Moser *et al*., in preparation). Each 60×90 cm big subplot contained an area to be able to take 10 times plant and soil samples, a metal frame (38×38 cm) inserted about 8 cm into the ground for static chamber (40×40×20 cm) gas flux measurements. Within the 70×70 cm subplot remained an area where no ^{15}N labelled fertilizer but pure NH_4NO_3 was applied later on, which was used to take samples to analyse the microbial community and activity during the labelling experiment.

Gas samples (CO_2, CH_4 and N_2O) as well as soil samples (0-7.5 cm depth and 4 cm diameter) were taken directly before the $^{13}CO_2$ pulse labelling started. At 7 am on 7th of May 2013 transparent fumigation chambers (70×70×40 cm) were put over one of the subplots per ring and fumigated for 6 hours with ^{13}C-labeled CO_2 in air. The CO_2 concentration was set to 390 ppm for A rings and 470 ppm for E rings (CK Special Gases Ltd, Leicester, UK). Directly after the 6 hours of fumigation, emptying the gas cylinders the ^{15}N labelling started which equalled the annual fertilization of 40 kg N ha^{-2} yr^{-1}. During application it was taken care that the labelled fertilizer solution was only applied between 0-10 cm aboveground, so that no ^{15}N could sprinkle to plant leaves at more than 10 cm above the soil surface. The first subplot, which was in parts ^{13}C labelled, was then labelled with $NH_4{}^{15}NO_3$ and the second with $^{15}NH_4NO_3$ solution. When the application of ^{15}N fertilizer was finished in all rings, one sample of soil and plants per subplot was taken. After plant sampling a soil auger with 8 cm diameter and 7.5 cm deep were taken within the 10×10 cm square using a soil auger including soil sampling rings (Eijkelkamp Agrisearch Equipment, Giesbeek, Netherlands). The soil core was divided in the field into a 2.5 cm top soil disk and a 5 cm deep core that was transferred to the lab within the metal ring used with the corer.

For molecular analyses, rhizosphere samples were taken from each FACE ring from the 10×60 cm area to which pure non-labeled NH_4NO_3 solution was added. Sampling was repeated at eight time points, starting with the sample taken before start of fumigation with $^{13}CO_2$ (-11 h), then one sample was taken directly after the end of fumigation (0 h) before fertilizer was applied at dawn. Afterwards, samples were taken after 3, 6, 11, 23 and 46 h after the end of fumigation with labeled CO_2. Samples were homogenized, separated in portions of 0.35 g in 2 mL vials, directly deep-frozen in liquid N_2 at the nearby Justus-Liebig University Giessen and stored at -80°C to ensure RNA stability. For further microbial community abundance and composition analyses the three *e*CO_2 and *a*CO_2 FACE rings were used as biological replicates and all data is shown as average of these three samples.

A more detailed description of the experiment and the different sampling and measurements can be found in Moser *et al*. (in preparation).

4.3.2. Nucleic acid extraction

DNA and RNA were extracted soil using a modified SDS-based protocol (Breidenbach *et al*., 2015; Brenzinger *et al*., 2015; Bürgmann *et al*., 2003; Pratscher *et al.*, 2011). In brief, the cells were disrupted in a FastPrep®-24 Instrument (MP Biomedicals Germany GmbH, Eschwege, Germany) beat-beating system and nucleic acids were recovered from the supernatant using a phenol/chloroform/isoamyl (Sigma-Aldrich, Taufkirchen, Germany) alcohol extraction. Subsequently the nucleic acids were precipitated with polyethylene glycol (PEG) 6000 solution and redissolved in 100 µL of nuclease-free water (Thermo Fisher Scientific, Dreieich, Germany). An aliquot of 20 µL was stored at -20°C for further DNA-based molecular analyses. The remaining 80 µL were treated with RNase-free DNase (Qiagen, Hilden, Germany) to remove DNA. RNA was purified using the RNeasy

Mini Kit (Qiagen), precipitated with 96% EtOH and resuspended in 15 µL nuclease-free water to increase the RNA concentration and stored at -80°C. The integrity of the RNA was controlled via a 1.5% w/v agarose gel (Biozym Scientific GmbH, Hessisch Oldendorf, Germany) and RNA concentration was determined with NanoDrop1000 (Thermo Fisher Scientific). High quality RNA was reverse transcribed into cDNA using random hexamer primers (Roche, Mannheim, Germany) and M-MLV reverse transcriptase (Promega, Mannheim, Germany). For each sample RNA purity was verified by using a control reaction without reverse transcriptase. Finally, cDNAs and negative controls were stored at -20°C for further analyses.

4.3.3. Quantification of functional marker and 16S rRNA genes

Copy numbers of genes and transcripts encoding nitrite reductase (*nirK*/*nirS*), nitrous oxide reductase (*nosZ),* dinitrogenase (*nifH)*, archaeal and bacterial ammonia monooxygenase (A*amoA/*B*amoA)*, nitrite reductase of the dissimilatory reduction of nitrate to ammonia (*nrfA)* and archaeal and bacterial 16S rRNA were quantified by qPCR using primer pairs and temperature profiles shown in Table S4.1. A typical reaction mixture contained 12.5 µL of SybrGreen Jump-Start ReadyMix (Sigma-Aldrich), 0.5 µM of each primer, 3-4.0 mM $MgCl_2$, 2 µL of soil DNA or RNA except for amplification of *nosZ*, for which 3 µL of DNA/RNA was used. For the amplification of functional marker genes involved in nitrogen cycling 200 ng BSA mL^{-1} were added to the reaction. All assays were performed in an iCycler (Applied Biosystems, Darmstadt, Germany). Standard curves were obtained using serial 10-fold dilutions of a known amount of plasmid DNA (10^8 to 10^1 gene copies) containing the respective gene fragment. Negative controls contained water instead of template DNA or RNA. PCR efficiencies for all assays were between 80-97 % with r^2 values between 0.981 and 0.998. In order to compare copy numbers across samples, numbers were calculated relative to

g dry weight of soil. To determine dry weight of soil, small amounts of soil (1-5 g) were dried at 65°C for 3 days.

4.3.4. Analysis of community composition based on functional marker and 16S rRNA genes

Community composition based on PCR amplified gene fragments of *nirK*/*nirS*, *nosZ*, A*amoA*/B*amoA*, *nifH*, *nrfA* and on archaeal and bacterial 16S rRNA genes was analyzed by terminal restriction length polymorphism (T-RFLP). Details on primers and conditions are given in Table S4.2. Quantity and quality of the PCR amplicons were analyzed by gel electrophoresis (1.5% w/v agarose) stained with 3 × GelRed Nucleic Acid Stain (Biotium, Köln, Germany). PCR products of the correct size were excised from the gel and purified using the DNA Wizard® SV Gel-and-PCR-Clean-up system (Promega, Mannheim, Germany) following the manufacturer´s instructions. Forward or reverse primers were 5’-6-carboxyfluorescein labeled (Table S4.2) and amplicons were fragmented using specific restriction enzymes (FastDigest, Fermentas, St. Leon-Rot, Germany) *HaeIII (nirK*/*nirS*), *HhaI* (*nosZ*, *nifH*, *nrfA* and A*amoA*/B*amoA*) and *MspI* and *Taq1*, (archaeal and bacterial 16SrRNA, respectively). Afterwards, reaction products were purified using the SigmaSpin™ Sequencing Reaction Clean-up Columns (Sigma-Aldrich) according to the manufacturer’s instructions. Fluorescently labeled restriction fragments were separated on an ABI PRISM 3100 Genetic Analyzer sequencer (Applera Deutschland GmbH, Darmstadt, Germany) and the length of fluorescently labeled terminal restriction fragments (T-RFs) was determined by comparison with the internal standard (X-Rhodamine MapMarker® 30-1000 bp; BioVentures, Murfreesboro, TN) using GeneMapper software (Applied Biosystems). Peaks with fluorescence of > 1% of the total fluorescence of a sample and > 30 bp length were analyzed by aligning fragments to the internal DNA fragment length. A difference of less than two base

pairs in estimated length between different profiles was the basis for considering fragments identical. Peak heights from different samples were normalized to identical total fluorescence units by an iterative normalization procedure (Dunbar *et al*., 2001).

4.3.5. Statistical analyses of collected data

All statistical analyses and graphics were done using R version 3.0.1 (R Development Core Team, 2013). Significant differences of *nirK*, *nirS*, *nosZ*, *nifH*, *nrfA* and A*amoA*/B*amoA* and 16S rRNA gene and transcript abundance as well as the calculated ratios were assessed using ANOVA ($P < 0.05$). Differences/similarities in the composition of the transcriptionally active communities were analyzed using non-metric multidimensional scaling (NMDS). Non-metric multidimensional scaling (NMDS) analyses were performed with the Bray-Curtis similarity index (including presence and relative abundance of T-RF) which iteratively tries to plot the rank order of similarity of communities in a way that community point distances are exactly expressed on a two-dimensional sheet. The reliability of the test was calculated by a stress-value. Stress > 0.05 provides an excellent representation in reduced dimensions, > 0.1 very good, > 0.2 good, and stress > 0.3 provides a poor representation. All community composition data were Hellinger-transformed before analysis, in order to reach normal distribution. Differences in the composition of transcriptionally active and overall denitrifier communities at a given time point were tested by ANOSIM ($P < 0.05$). ANOSIM generates a value of R which is scaled to lie between -1 and +1, a value of zero representing the null hypothesis (no difference among a set of samples). In ANOSIM, comparison of pair-wise R values, measuring how separate groups are, on a scale of 0 (indistinguishable) to 1 (all similarities within groups are less than any similarity between groups) gives an interpretable number for the difference between groups. We interpreted R-values > 0.75 as well separated; R > 0.5 as overlapping, but clearly different and R < 0.25 as barely separable at all (Clarke,

2006). ANOVA, NMDS and ANOSIM) were done using package vegan version 2.0-5 (Oksanen *et al*., 2012). All data were log-transformed prior to analysis to satisfy the assumptions of homoscedasticity and normally distributed residuals.

4.4. Results

4.4.1. N_2O fluxes from soil under *e*CO_2 and *a*CO_2

N_2O fluxes were low in the range of 1.2-1.3 µg N_2O $m^{-3}*h^{-1}$ 24 h before the start of the experiment and increased up to 400-fold reaching a peak 22 h after addition of the fertilizer (Fig 4.1). Afterwards within 70 h after fertilization, N_2O fluxes decreased to the low rates measured prior to fertilizer application. During the first 64 h after addition of fertilizer fluxes were different between *e*CO_2 and *a*CO_2 (Fig 4.1). While the highest flux for soil under *a*CO_2 reached only 97 µg N_2O $m^{-3}*h^{-1}$, soils under *e*CO_2 reached N_2O fluxes from ~ 144 µg N_2O $m^{-3}*h^{-1}$ equal to an increase of approximately 48%. In addition, fluxes from soil under *e*CO_2 continued being higher compared to *a*CO_2 in the year after the start of this experiment (Gorenflo *et al*., in preparation). A detailed overview on N_2O, CO_2 and CH_4 flux data, data from the ^{15}N tracing model and an overall model of this experiment can be found in Gorenflo *et al*. (in preparation) and Moser *et al*. (in preparation).

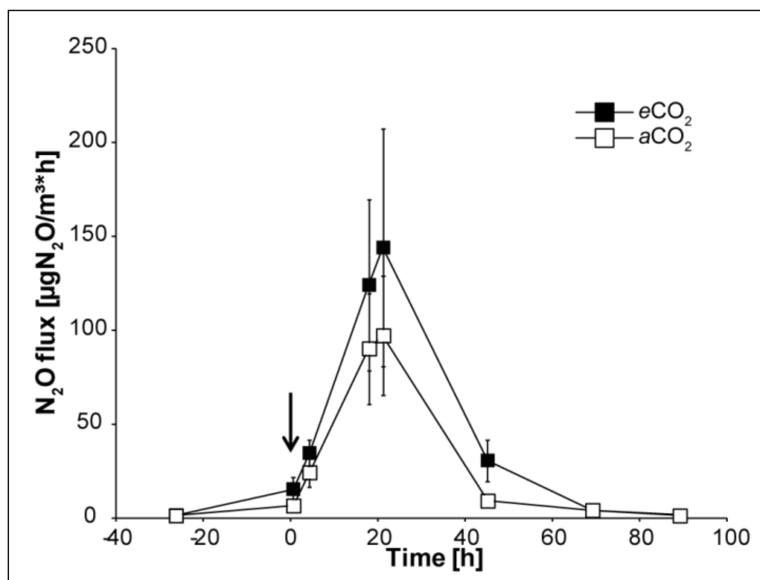

Figure 4.1. Average N_2O fluxes from soil of three of FACE rings each under *e*CO_2 and *a*CO_2 during the first 100 hours after addition of the NH_4NO_3 fertilizer (arrow indicates addition of fertilizer) and 24 h before the start of the experiment. (Mean ± SD, n=3).

4.4.2. Abundance of the overall and transcriptionally active microbial communities involved in nitrogen cycling

The size of the microbial communities remained rather stable during the experiment and if differences occurred such as for bacterial ammonia oxidizers (B*amoA*), dissimilatory nitrate reducers (*nrfA*) and N-fixers (*nifH*), they did not follow a clear trend (Fig. S4.1, Table S4.3). Community size was also similar between soils under *e*CO_2 and the control under *a*CO_2. Only the ratio of *nosZ*/(*nirK*+*nirS*) genes was higher under *a*CO_2 after 6 h of fertilizer application indicating a higher abundance of N_2O reducers relative to nitrite reducers (Fig. 4.2).

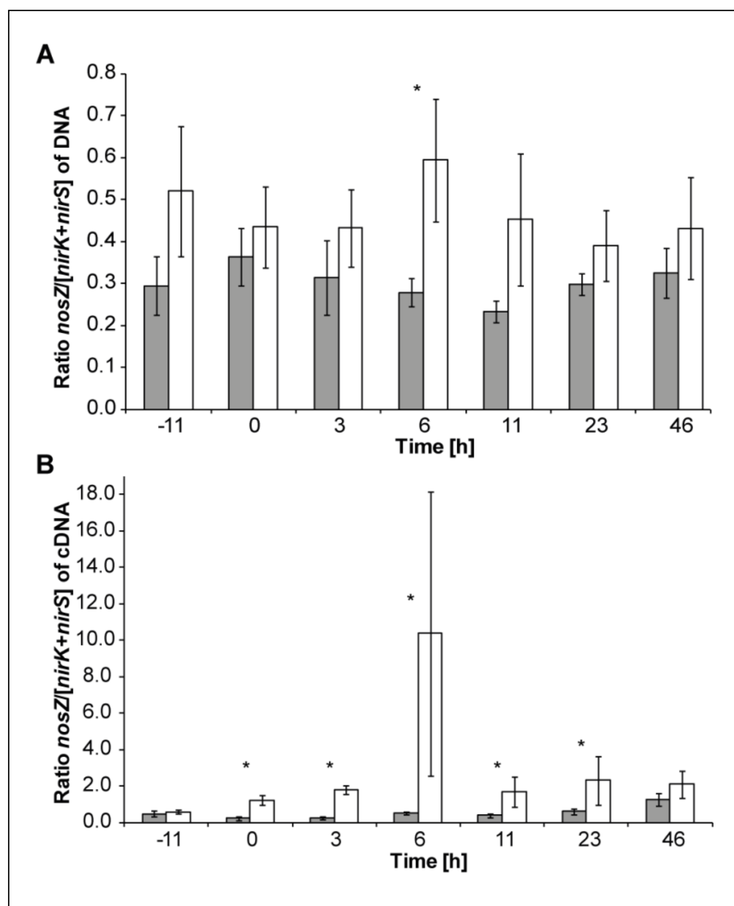

Figure 4.2. Ratios *nosZ*/(*nirK*+*nirS)* of DNA copy numbers (A) and ratios *nosZ*/(*nirK*+*nirS)* of cDNA (B). Asterisks indicate significant differences between eCO_2 and aCO_2 (ANOVA: P value < 0.05). Grey bars = eCO_2 plots; white bars = aCO_2 plots (Mean ± SD, n=3).

Transcriptional activation of all genes was in part affected by fertilization (*nirS*, *nosZ* and A*amoA*) with higher abundances towards the end of the experiment but again showing no clear trends if differences occurred (Table S4.4). Transcription of bacterial *amoA* was even below the detection limit. eCO_2 also had no effect except on the abundance of *nirS* (Fig. S4.2) which was significantly higher in soil under eCO_2 compared to aCO_2 at all sampling times, except for 23 h after fertilizer application. Copy numbers of *nirK* and *nosZ* cDNA were higher under eCO_2 but only before N-fertilizer were applied. Interestingly, the ratio of transcripts of *nosZ*/(*nirK*+*nirS*) was significantly higher at aCO_2 between 0 h-23 h hence suggesting a higher potential for N_2O reduction relative to nitrite reduction (Fig. 4.2). Relative transcriptional activation expressed as the ratio of cDNA/gene copy numbers confirmed these

results (Fig. 4.3). The level of relative transcriptional activity of *nifH* was almost one order of magnitude higher compared to the other genes. Despite high variability, cDNA copy numbers followed a trend of increased relative transcriptional activity beginning 6 h after fertilization. Relative transcriptional activity of *nirK* was initially higher under eCO$_2$ compared to aCO$_2$ and decreased upon fertilizer application. In contrast, relative transcriptional activity of *nirS* peaked 6 h after fertilization and was higher under eCO$_2$ between 3-23 h after fertilizer application.

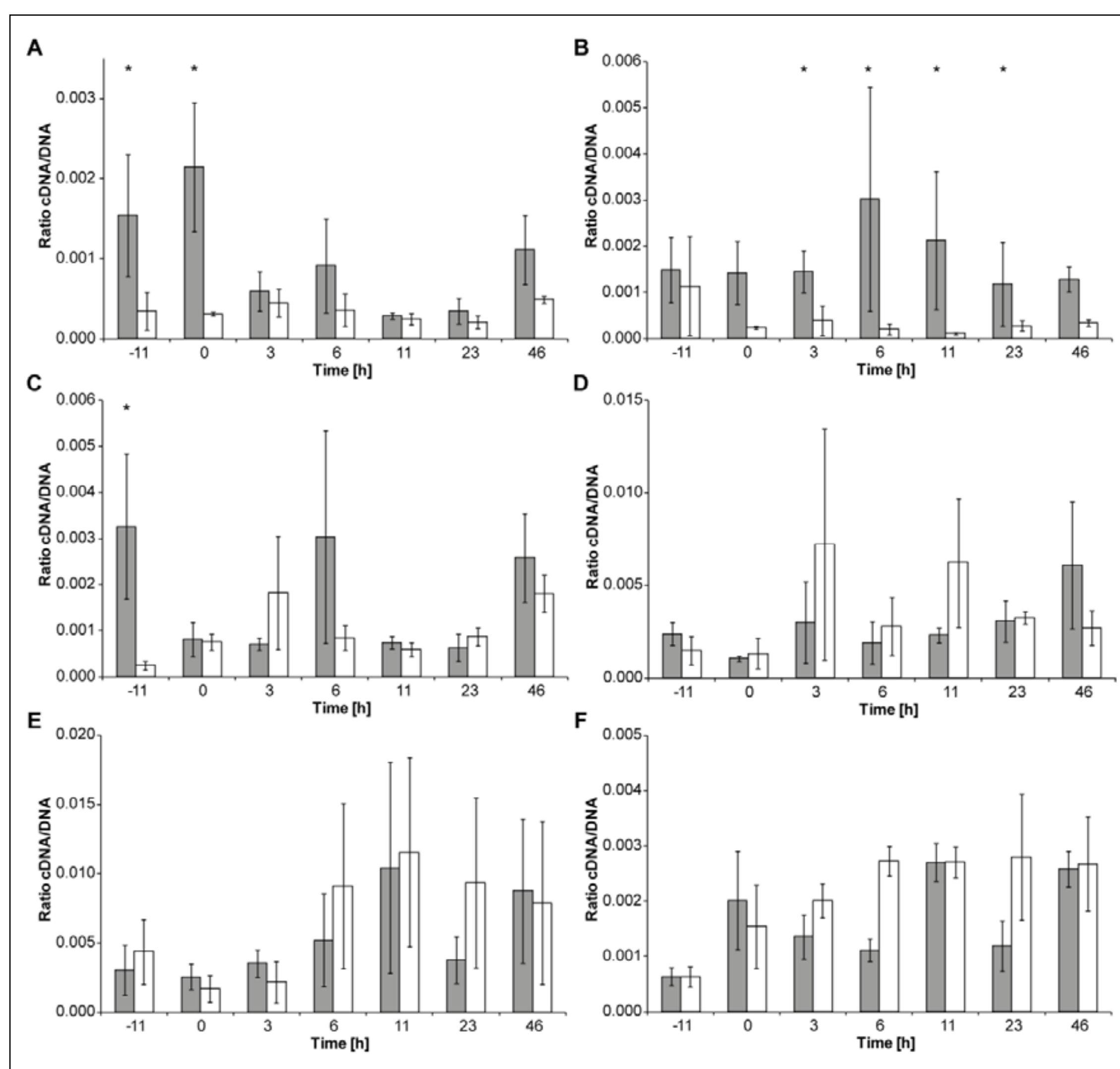

Figure 4.3. Ratios of cDNA/DNA copy numbers of functional marker genes involved in N-cycling. Bars indicate the total gene copy numbers. Asterisks indicate significant differences between eCO$_2$ and aCO$_2$ (ANOVA: P value < 0.05). (A) *nirK*; (B) *nirS*; (C) *nosZ*; (D) *nrfA*; (E) *nifH*; (F) archaeal *amoA*. Grey bars = eCO$_2$ plots; white bars = aCO$_2$ plots. (Mean ± SD, n=3).

4.4.3. Impact of *e*CO_2 and N-input on microbial community composition involved in N-cycling

Transcriptionally active microbial communities in soil from *e*CO_2 rings and the control rings at *a*CO_2 clustered separately except for *nosZ*-containing denitrifiers and N-fixers where no clear separation of the communities according to CO_2 level occurred (ANOSIM: $P < 0.05$) (Fig. 4.4). Except for dissimilatory nitrate reducers and archaea, transcriptionally active communities in the soil under both CO_2 levels clustered closely together before N-fertilization (-11 h) and then diverged. In most cases (exception *nosZ*-containing denitrifiers) the transcriptionally active community under *e*CO_2 showed a greater compositional variation over time than the community under *a*CO_2 but there was no linear trend of succession. Generally, the differences in community composition were small and depended mainly on changes in the relative abundance of dominant T-RFs or the presence/absence of less dominant fragments (Fig. S4.3).

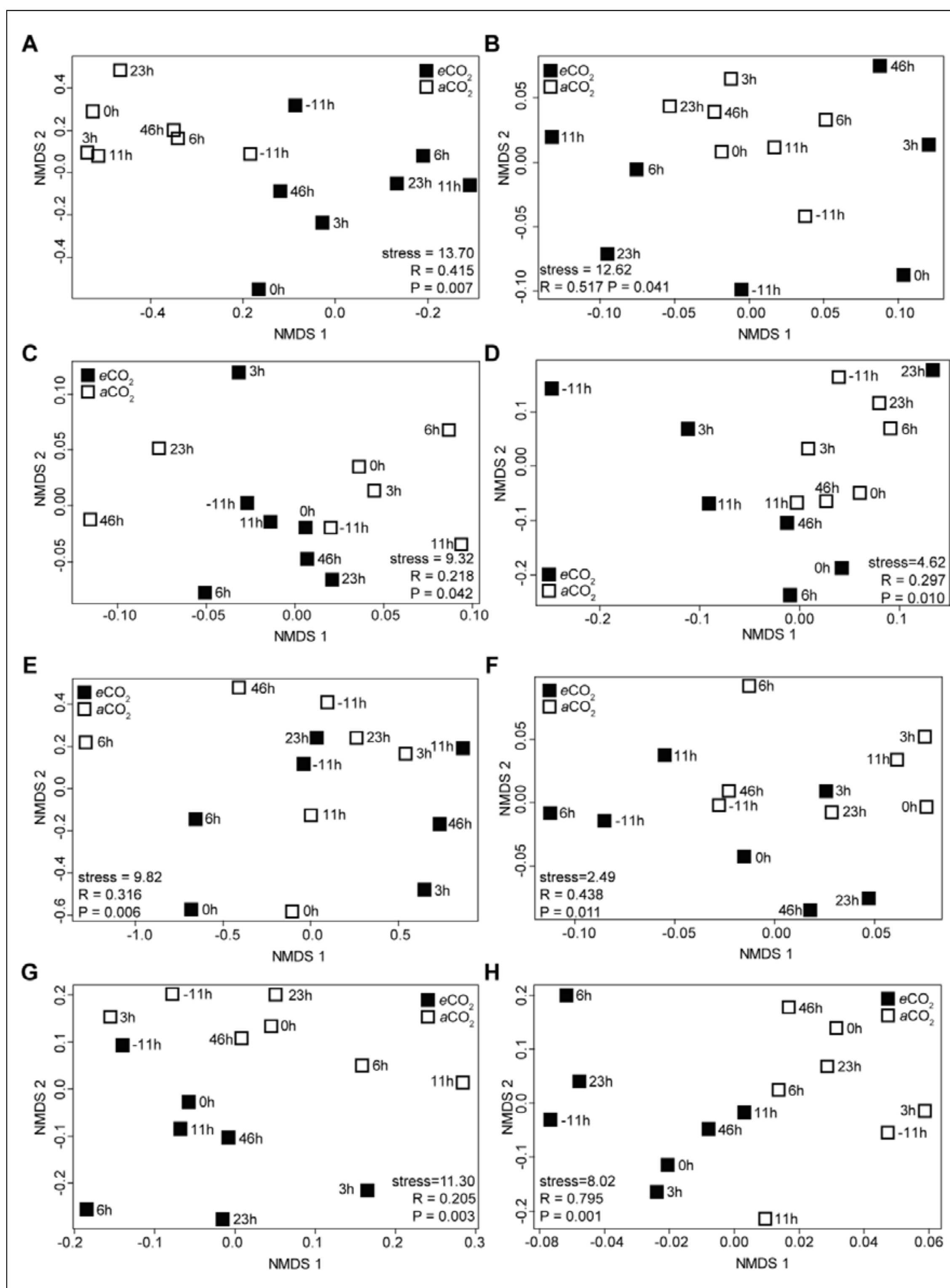

Figure 4.4. NMDS plots of microbial communities involved in N-cycling based on cDNA-derived T-RFLP analysis at different sampling times. Data points represent averaged results of three replicate T-RFLP analyses. Significant differences in the composition of transcriptionally active denitrifier communities at given time points were determined by ANOSIM (P < 0.05) and indicated in the graph with *R*- and *P*-values. (A) *nirK*; (B) *nirS*; (C) *nosZ*;(D) *nrfA*; (E) *nifH*; (F) archaeal *amoA*; (G) bacterial 16S rRNA; (H) archaeal 16S rRNA.

4.5. Discussion

The experiment was conducted at the Giessen FACE facility which allows studying the effects of *e*CO_2 on soil microbial communities under field conditions. Previous studies at GiFACE showed that *e*CO_2 and the concomitantly increased C-fluxes into the soil altered N-transformations and caused feedback effects resulting in enhanced N_2O fluxes (Kammann *et al.*, 2008; Müller *et al.*, 2009). N_2O fluxes from soil under *e*CO_2 were primarily increased during the plant growth period when the soil was fertilized in spring (Kammann *et al.*, 2008). Therefore, we conducted a fertilization experiment applying ^{15}N-labeled NH_4NO_3-fertilizer at the start of the growing season in May 2013 and aimed to link the functional response of the soil under *e*CO_2 (e.g. N_2O production, NO_3^--and NH_4^+-turnover) to the response of the soil microbial communities involved in these N-transformations. The size of the microbial communities involved in N-cycling (denitrification, DNRA, ammonia oxidation and N-fixation) and of the bacterial and archaeal communities was not affected by CO_2 and only in part by fertilization (Fig. S4.2, Table S4.4). Stability in community size was expected as the duration of the experiment (100 h) and rapid consumption by microorganisms and plants of the N-fertilizer applied (Moser *et al.*, in preparation) presumably did not promote substantial growth of the soil microbial communities. However, after 6 hours of fertilizer application the *nosZ/(nirK+nirS)* ratio was higher in soil under *a*CO_2 than under *e*CO_2 indicating that shifts in the abundance of N_2O reducers relative to nitrite reducers occurred (Fig. 4.2A). The *nosZ(nirK+nirS)* ratio is a good predictor for the potential of some soils to effectively reduce N_2O (Morales *et al.*, 2010; Philippot *et al.*, 2011) but we assumed that addressing those parts of the microbial communities that responded to fertilization by activating gene expression would provide a more direct link to ecosystem functioning. Therefore, we studied transcripts (cDNA) of functional marker genes involved in N-cycling and observed that the level of atmospheric CO_2 influenced the ratio of transcriptionally active N_2O reducers relative to N_2O producers (Fig. 4.3B) as well as the structure of the transcriptionally active soil microbial

communities (Fig. 4.4). Generally, addition of N-fertilizer had little effect on the abundance of transcripts of functional marker genes involved in N-cycling but the relative transcriptional activity (ratio of cDNA/DNA copy numbers) of denitrifiers was affected (Fig. 4.3). This agrees well with the results of the ^{15}N-tracing model calculated based on the results of the present experiment (Gorenflo *et al*., in preparation; Moser *et al*., in preparation). The model revealed that N_2O emissions as well as increased N_2O fluxes at *e*CO_2 originated primarily from denitrification processes in the soil. Application of fertilizer to soil *e*CO_2 on one hand stimulated the relative transcriptional activity of *nirS* compared to *a*CO_2 while on the other hand lowering relative transcriptional activity of *nirK* and *nosZ* to levels observed for *a*CO_2. This was also reflected by lower numbers of transcriptionally active N_2O reducers relative to N_2O producers in soil under *e*CO_2 and may hence explain the higher increase of N_2O emission after the addition of N-fertilizer (Gorenflo *et al*., in preparation; Kammann *et al*., 2008; Müller *et al*., 2009;). Ratios of *nosZ*/(*nirK*+*nirS*) explained N_2O fluxes in some studies (Billings and Tiemann, 2014; Čuhel *et al*., 2010; Morales *et al*., 2010; Philippot *et al*., 2011) but were dependent on habitat and environmental conditions (Morales *et al*., 2010; Philippot *et al*., 2011; Deslippe *et al*., 2014). Slightly increased *nosZ/*(*nirK*+*nirS*) ratios at *a*CO_2 occurred already before fertilizer was applied (0 h) which may be due to photosynthetic activity upon sampling. While the sample collected 11 h before N-fertilization was taken at dawn limiting photosynthesis to almost zero, the sample taken shortly before N-fertilization (0 h) was collected under direct sunlight. Thus, transcription of *nirS* may have been stimulated through increased carbon input into the soil via increased plant photosynthesis and CO_2 uptake at daylight (Gorenflo *et al*., in preparation) and was then further enhanced by N-inputs and peaked 6 h after the fertilizer was applied.

Why *e*CO_2 stimulates transcription of *nirS* but not of *nirK* and *nosZ* is not resolved yet but responses to *e*CO_2 in *nirS*-type denitrifier abundance were reported previously while *nirK*- and *nosZ*-containing denitrifiers remained unaffected (He *et al*., 2010; 2012; Xu *et al*., 2013).

We speculate that due to increased N-availability along with rising labile C at the root-soil interface from *e*CO_2, lead to a lower competitor situation between *nirK*- and *nirS*-type denitrifiers. Another hypothesis would be that the higher plant and root biomass lead possibly to more neutral/alkaline pH zones in the rhizosphere. This has been demonstrated in biofilms and by nondenitrifying colonies in agar (Li and Bishop, 2003; Mazoch and Kucera, 2002). In soils this phenomenon was not studied yet. However, during active uptake of NO_3^- by plants and microbes an alkalinization (1 to 2 pH units) of soil close to roots has been demonstrated (Nye, 1981). Maybe, also bacteria which can consume oxygen are stimulated by increasing labile C input and thus lead to more anoxic zones in the soil. The presence of NO_2^- and low oxygen partial pressure are the predominant exogenous signals that induce the activation of the denitrification system (van Spanning *et al*., 2007). Unfortunately, oxygen uptake was not measured during our experiment. Nevertheless, it is conceivable that a higher plant biomass and *e*CO_2 correlates with an increased oxygen uptake rate. Both scenarios would provide habitats/niches for the less abundant *nirS*-type denitrifiers to be metabolically active.

Recent studies of different FACE facilities world-wide observed effects on almost all functional marker genes involved in N-transformations (He *et al*., 2014; Lee *et al*., 2015; Okubo *et al*., 2015; Xiong *et al*., 2015). It is hypothesized that *e*CO_2 indirectly affects microbial communities through increased root growth and changes in the quality and quantity of root exudates (Denef *et al*., 2007; Freeman *et al*., 2009; Rogers *et al*., 1998). Such plant effects were shown previously to shape the genetic makeup of microbial communities in experimental grassland sites (Bais *et al*., 2006; Baudoin *et al*., 2003; Bremer *et al*., 2009; Bürgmann *et al*., 2005) and were linked to functional differences in potential N_2O emissions (Bremer *et al*., 2009). Our data showed that fertilization of the soil at distinct atmospheric CO_2 levels activated transcription of parts of the denitrifier, DNRA, ammonia oxidizer and N-fixer communities distinct from those at *a*CO_2. However, only the transcription of *nirS*, *nosZ* and archaeal *amoA* was enhanced but not of the other genes studied which is surprising.

Transcriptional activation of archaeal *amoA* was enhanced through fertilization but not influenced by CO_2 levels but the communities developed differently at distinct CO_2 levels suggesting that *e*CO_2 had an effect. Pratscher *et al*. (2011) demonstrated that ammonia-oxidizing archaea in soil contributed significantly to ammonia oxidation and CO_2 assimilation suggesting that nitrification may increase in soils under *e*CO_2. On the other hand, a mixo- or heterotrophic lifestyle was also suggested for archaeal ammonia oxidizers (Nicole and Schleper, 2006). Hence, they may be also able to respond to plant-mediated indirect CO_2 effects rather than to *e*CO_2 as CO_2 concentrations in the soil atmosphere are naturally high (Gobat *et al*., 2004). Another surprising finding was that transcriptional activation of *nrfA* (composition of transcripts) responded differently to distinct CO_2 levels though not by increased transcript abundance. However, the increase in DNRA rates by 141% in soil under *e*CO_2 compared to *a*CO_2 (Müller *et al*., 2009) suggests high levels of gene expression which could not be confirmed. Similarly we found no enhanced transcription of *nifH* upon fertilization despite differences in the composition in the transcriptionally active communities.

A preceding study demonstrated that communities involved in N-cycling in the soil at GiFACE were mainly shaped by the prevalent soil parameters and only marginally by the level of CO_2 (Brenzinger *et al*., in preparation). Here we could show that long-term fumigation with *e*CO_2 influences the response of the soil microbial communities to N inputs via fertilization and compared to *a*CO_2 distinct parts of the soil community were transcriptionally activated. However, the input of N by fertilization seems to exert short term effects on the expression of functional marker genes with consequences for N-transformations but which does not translate into the development of distinct communities under *e*CO_2 in the long-term.

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4.8. Supplementary Material

Table S4.1. Primer pairs and PCR conditions used for amplification of the functional marker genes *nirK*, *nirS*, *nosZ*, *nifH*, archaeal and bacterial *amoA*, *nrfA*, archaeal and bacterial 16S rRNA by qPCR.

Gene	Primer sets	Forward primer	Reverse primer	PCR conditions	PCR product length (bp)	References
nifH	PolF/ PolR	TGCGA(C/T)CC(G/C)A ARGC(C/G/T)GACTC	AT(G/C)GCCATCAT(C/T) TC(A/G)CCGGA	95 °C/15min, 6 cycles of (95°C/15sec, 60°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 55°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	360	Poly *et al*., 2001
nirK	qnirK876/ qnirK1040	AT(C/T)GGCGG(A/C/G) A(C/T)GGCGA	GCCTCGATCAG(A/G)TT (A/G)TGGTT	95 °C/15min, 6 cycles of (95°C/15sec, 63°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 58°C/30sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	165	Henry *et al*., 2004
nirS	qCd3af/ qR3cd	AACG(C/T)(G/C)AAGG A(A/G)AC(G/C)GG	GA(G/C)TTCGG(A/G)TG (G/C)GTCTT(G/C)A(C/T)G AA	95 °C/15min, 6 cycles of (95°C/15sec, 63°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 58°C/30sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	425	Kandeler *et al*., 2006
nosZ	nosZ2F/ nosZ2R	CGC(A/G)ACGGCAA (G/C)AAGGT(G/C) (A/C)(G/C)(G/C)GT	CA(G/T)(A/G)TGCA(G/T) (G/C)GC(A/G)TGGCAGA A	95 °C/15min, 6 cycles of (95°C/15sec, 65°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 60°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	267	Henry *et al*., 2006
nrfA	nrfA2aw/ nrfAR1	CA(A/G)TG(C/T)CA (C/T)GT(C/G/T)GA (A/G)TA	T(A/T)(A/C/G/T)GGCAT (A/G)TG(A/G)CA(A/G)TC	95 °C/15min, 6 cycles of (95°C/15sec, 58°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 53°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	269	Welsh *et al*., 2014; Mohan *et al*., 2004
Archaeal *amoA*	Arch-amoAF/ Arch-amoAR	(G/C)TAATGGTCTGGC TTAGACG	GCGGCCATCCATCTGTA TGT	95 °C/15min, 6 cycles of (95°C/15sec, 58°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 53°C/20sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	635	Francis *et al*., 2005
Bacterial *amoA*	amoA-1F/ amoA2R	GGGGTTTCTACTGGT GGT	CCCCTC(G/T)G(G/C)AAA GCCTTCTTC	95 °C/15min, 6 cycles of (95°C/15sec, 65°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 60°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	491	Rotthauwe *et al*., 1997
Archaeal 16S rRNA gene	Ar364f/ Ar934br	CGGGG(C/T)GCA(G/C) CAGGCGCGAA	GTGCTCCCCCGCCAATT CCT	95 °C/15min, 6 cycles of (95°C/15sec, 56°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 52°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	570	Burggraf *et al*., 1997; Großkopf *et al*., 1998
Bacterial 16S rRNA gene	Ba519f/ Ba907r	CAGC(A/C)GCCGCGG TAA(A/C/G/T)(A/T)C	CCGTCAATTC(A/C)TTT (A/G)AGTT	95 °C/15min, 6 cycles of (95°C/15sec, 54°C/30sec (-1°C every cycle), 72°C/30sec, 80°C/15sec), 40 cycles (95°C/15sec, 49°C/15sec, 72°C/30sec, 80°C/15sec), 60 to 95°C (+0.2°C/sec) for denaturation curve.	388	Lane, 1991

Table S4.2. Primer pairs and PCR conditions used for amplification of the functional marker genes *nirK*, *nirS*, *nosZ*, *nifH*, *amoA,* archaeal and bacterial *amoA*, *nrfA*, archaeal and bacterial 16S rRNA gene for T-RFLP.

Gene	Primer sets	Forward primer	Reverse primer	PCR conditions	PCR product length (bp)	References
nifH	PolF-FAM/ PolR	TGCGA(C/T)CC(G/C) AARGC(C/G/T)GAC TC	AT(G/C)GCCATCAT (C/T)TC(A/G)CCGGA	95 °C 5min, 10 cycles of (95°C/30sec, 60°C/40sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 55°C/40sec, 72°C/2min) 72°C/10min.	360	Poly *et al*., 2001
nirK	nirK1F/ nirK5R-FAM	GG(A/C)ATGGT (G/T)CC(C/G)TGGC A	GCCTCGATCAG(A/G) TT(A/G)TGG	95 °C/5min, 10 cycles of (95°C/30sec, 56°C/40sec (-0.5°C every cycle), 72°C/40sec), 25 cycles (95°C/30sec, 54°C/40sec, 72°C/40sec), 72°C/7min.	514	Braker *et al*., 1998
nirS	cd3aF-FAM/ R3cd	GT(C/G)AACGT (C/G)AAGGA(A/G)A C(C/G)GG	GA(C/G)TTCGG(A/G) TG(C/G)GTCTTG	95 °C/5min, 10 cycles of (95°C/30sec, 56°C/40sec (-0.5°C every cycle), 72°C/40sec), 25 cycles (95°C/30sec, 54°C/40sec, 72°C/40sec) 72°C/7min.	425	Throbäck *et al*., 2004
nosZ	NosF-FAM/ NosR	CG(C/T)TGTTC(A/C) TCGACAGCCAG	CATGTGCAG (A/C/G/T)GC(A/G)TG GCAGAA	95 °C 5min, 10 cycles of (95°C/30sec, 59°C/90sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 56°C/40sec, 72°C/2min) 72°C/10min.	700	Kloos *et al*., 2001
nrfA	nrfA2aw-FAM/ nrfAR1	CA(A/G)TG(C/T)CA (C/T)GT(C/G/T)GA (A/G)TA	T(A/T)(A/C/G/T)GGC AT(A/G)TG(A/G)CA (A/G)TC	95 °C 5min, 10 cycles of (95°C/30sec, 57°C/40sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 53°C/30sec, 72°C/2min) 72°C/10min.	269	Welsh *et al*., 2014; Mohan *et al*., 2004
Archaeal *amoA*	Arch-amoAF-FAM/ Arch-amoAR	(G/C)TAATGGTCTG GCTTAGACG	GCGGCCATCCATCT GTATGT	95 °C 5min, 10 cycles of (95°C/30sec, 57°C/90sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 53°C/50sec, 72°C/2min) 72°C/10min.	635	Francis *et al*., 2005
Bacterial *amoA*	amoA-1F-FAM/ amoA2R	GGGGTTTCTACTG GTGGT	CCCCTC(G/T)G(G/C) AAAGCCTTCTTC	95 °C 5min, 10 cycles of (95°C/30sec, 65°C/40sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 60°C/40sec, 72°C/2min) 72°C/10min.	491	Rotthauwe *et al*., 1997
Archaeal 16S rRNA gene	Ar109f/ Ar912r-FAM	AC(G/T)GCTCAGTA ACACGT	GTGCTCCCCCGCCA ATTCCT	95 °C 5min, 10 cycles of (95°C/30sec, 58°C/60sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 53°C/60sec, 72°C/2min) 72°C/10min.	803	Großkopf *et al*., 1998; Lueders and Friedrich, 2000
Bacterial 16S rRNA gene	Ba27f-FAM/ Ba907r	GAGTTTG((A/C)TCC TGGCTCAG	CCGTCAATTC(A/C)T TT(A/G)AGTT	95 °C 5min, 10 cycles of (95°C/30sec, 49°C/60sec (-0.5°C every cycle), 72°C/2min), 25 cycles (95°C/30sec, 44°C/30sec, 72°C/2min) 72°C/10min.	898	Weisburg *et al*., 1991; Lane, 1991

Table S4.3. Abundance of functional marker genes for denitrification for *a*CO_2 and *e*CO_2, respectively. Analysis of variance (ANOVA) was performed to test for differences in copy numbers at different sampling times.

Time [h]	Denitrification						Nitrification				DNRA		N-fixation	
	nirK		*nirS*		*nosZ*		*AamoA*		*BamoA*		*nrfA*		*nifH*	
	eCO_2	*a*CO_2	*e*CO_2	*a*CO_2	*e*CO_2	*a*CO_2	*e*CO_2	*a*CO_2	*e*CO_2	*a*CO_2	*e*CO_2	*a*CO_2	*e*CO_2	*a*CO_2
-11	4.95E+08^{A} ± 2.35E+08	4.76E+08^{A} ± 2.39E+08	2.02E+08^{A} ± 1.47E+08	2.20E+08^{A} ± 1.53E+08	1.76E+08^{A} ± 6.98E+07	3.58E+08^{A} ± 2.73E+08	1.58E+09^{A} ± 6.58E+08	8.14E+08^{A} ± 1.04E+09	3.84E+06^{A} ± 2.42E+06	4.93E+06^{A} ± 1.68E+06	3.93E+09^{A} ± 1.97E+09	3.38E+09^{A} ± 1.73E+09	5.13E+08^{AC} ± 6.38E+07	5.47E+08^{A} ± 5.69E+07
0	3.70E+08^{A} ± 1.53E+08	4.21E+08^{A} ± 2.00E+08	1.59E+08^{A} ± 1.05E+08	1.92E+08^{A} ± 1.41E+08	1.67E+08^{A} ± 5.01E+07	2.51E+08^{A} ± 1.79E+08	9.23E+08^{A} ± 3.68E+08	7.83E+08^{A} ± 5.00E+08	3.86E+06^{A} ± 1.00E+06	4.03E+06^{AB} ± 2.29E+06	2.83E+09^{A} ± 2.04E+09	3.77E+09^{A} ± 2.29E+09	4.29E+08^{ABC} ± 4.67E+07	4.88E+08^{AB} ± 8.45E+07
3	5.37E+08^{A} ± 2.29E+08	3.79E+08^{A} ± 2.37E+08	2.20E+08^{A} ± 1.39E+08	1.93E+08^{A} ± 1.53E+08	1.91E+08^{A} ± 5.12E+07	2.78E+08^{A} ± 2.29E+08	1.24E+09^{A} ± 5.81E+08	1.06E+09^{A} ± 7.36E+08	8.81E+06^{A} ± 6.60E+06	3.18E+06^{B} ± 2.05E+06	3.24E+09^{AB} ± 2.08E+09	2.01E+09^{AB} ± 1.66E+09	5.66E+08^{A} ± 8.21E+07	3.89E+08^{AB} ± 1.91E+08
6	4.34E+08^{A} ± 2.27E+08	3.54E+08^{A} ± 1.59E+08	2.24E+08^{A} ± 1.56E+08	1.73E+08^{A} ± 1.38E+08	1.69E+08^{A} ± 8.81E+07	2.58E+08^{A} ± 1.24E+08	1.46E+09^{A} ± 6.79E+08	7.50E+08^{A} ± 6.66E+08	4.23E+06^{A} ± 1.01E+05	4.27E+06^{AB} ± 3.59E+06	3.30E+09^{ABC} ± 3.25E+09	1.80E+09^{AB} ± 1.05E+09	4.43E+08^{ABC} ± 1.83E+08	3.42E+08^{B} ± 8.74E+07
11	4.23E+08^{A} ± 1.36E+08	4.42E+08^{A} ± 4.36E+07	1.84E+08^{A} ± 1.38E+08	1.57E+08^{A} ± 1.60E+08	1.42E+08^{A} ± 7.64E+07	2.54E+08^{A} ± 1.47E+08	7.91E+08^{A} ± 4.70E+08	8.70E+08^{A} ± 9.25E+08	2.83E+06^{A} ± 5.12E+05	2.57E+06^{B} ± 1.01E+06	9.77E+08^{C} ± 4.29E+08	1.02E+09^{B} ± 4.49E+08	3.05E+08^{BC} ± 3.43E+07	3.57E+08^{B} ± 1.09E+08
23	4.43E+08^{A} ± 5.33E+07	4.13E+08^{A} ± 3.97E+07	2.14E+08^{A} ± 1.65E+08	2.19E+08^{A} ± 1.80E+08	1.87E+08^{A} ± 4.57E+07	2.67E+08^{A} ± 1.68E+08	6.19E+08^{A} ± 4.94E+08	7.78E+08^{A} ± 4.71E+08	5.63E+06^{A} ± 5.31E+06	4.17E+06^{AB} ± 2.23E+06	1.28E+09^{BC} ± 6.74E+08	1.30E+09^{AB} ± 5.25E+08	3.31E+08^{BC} ± 4.54E+07	3.57E+08^{B} ± 4.50E+07
46	4.81E+08^{A} ± 1.23E+08	3.72E+08^{A} ± 8.59E+07	2.01E+08^{A} ± 1.41E+08	2.10E+08^{A} ± 1.57E+08	1.99E+08^{A} ± 1.73E+07	2.62E+08^{A} ± 1.73E+08	9.98E+08^{A} ± 3.74E+08	1.00E+09^{A} ± 7.14E+08	6.15E+06^{A} ± 3.27E+06	3.13E+06^{AB} ± 1.54E+06	1.16E+09^{BC} ± 7.59E+08	1.37E+09^{AB} ± 6.18E+08	3.69E+08^{C} ± 3.24E+07	3.56E+08^{B} ± 7.55E+07

^{A,B} Identical letters behind the numbers indicate no significance differences ($P > 0.05$). n.d. = not detectable

Table S4.4. Abundance of reverse transcribed mRNA (cDNA) of functional marker genes for denitrification for aCO_2 and eCO_2, respectively. Analysis of variance (ANOVA) was performed to test for differences in copy numbers at different sampling times.

Time [h]	Denitrification						Nitrification				DNRA		N-fixation	
	nirK		*nirS*		*nosZ*		A*amoA*		B*amoA*		*nrfA*		*nifH*	
	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2	eCO_2	aCO_2
-11	4.80E+05^{A} ± 3.71E+05	1.56E+05^{A} ± 1.65E+05	1.29E+05^{A} ± 8.16E+04	3.57E+04^{AB} ± 3.73E+04	2.65E+05^{A} ± 2.33E+05	4.75E+04^{A} ± 1.20E+04	1.00E+06^{AB} ± 6.27E+05	1.09E+06^{A} ± 1.68E+06	n.d.	n.d.	6.46E+06^{A} ± 4.43E+06	3.92E+06^{A} ± 1.93E+06	1.66E+06^{A} ± 1.83E+06	2.47E+06^{A} ± 2.06E+06
0	7.92E+05^{A} ± 6.30E+05	1.36E+05^{A} ± 8.09E+04	1.30E+05^{A} ± 8.53E+04	4.09E+04^{AB} ± 2.98E+04	9.87E+04^{A} ± 7.53E+04	1.54E+05^{AB} ± 6.66E+04	8.12E+05^{AB} ± 7.41E+05	1.07E+06^{AB} ± 1.16E+06	n.d.	n.d.	1.76E+06^{A} ± 3.50E+05	2.90E+06^{A} ± 1.09E+06	1.41E+06^{A} ± 1.27E+06	1.01E+06^{A} ± 1.03E+06
3	3.81E+05^{A} ± 3.08E+05	1.12E+05^{A} ± 6.89E+04	2.67E+05^{A} ± 1.53E+05	1.44E+04^{AB} ± 8.47E+03	1.28E+05^{A} ± 5.93E+04	1.98E+05^{B} ± 1.39E+05	1.85E+06^{AB} ± 1.19E+06	1.87E+06^{AB} ± 1.44E+06	n.d.	n.d.	5.18E+06^{A} ± 4.22E+06	3.19E+06^{A} ± 2.86E+06	2.69E+06^{A} ± 2.23E+06	2.09E+06^{A} ± 1.83E+06
6	1.93E+05^{A} ± 1.07E+05	1.53E+05^{A} ± 1.93E+05	1.71E+05^{A} ± 1.42E+05	3.12E+04^{AB} ± 2.15E+04	1.83E+05^{A} ± 1.82E+05	3.24E+05^{B} ± 2.80E+05	1.36E+06^{AB} ± 2.89E+05	2.37E+06^{B} ± 2.30E+06	n.d.	n.d.	2.35E+06^{A} ± 2.02E+06	2.45E+06^{A} ± 1.82E+06	2.03E+06^{A} ± 2.54E+06	3.70E+06^{A} ± 3.86E+06
11	1.15E+05^{A} ± 5.42E+04	1.01E+05^{A} ± 3.91E+04	1.55E+05^{A} ± 1.21E+05	1.29E+04^{A} ± 9.97E+03	8.15E+04^{A} ± 2.34E+04	1.31E+05^{AB} ± 6.51E+04	1.59E+06^{AB} ± 1.10E+06	1.77E+06^{AB} ± 1.51E+06	n.d.	n.d.	1.70E+06^{A} ± 5.39E+05	3.46E+06^{A} ± 1.27E+06	4.19E+06^{A} ± 3.79E+06	4.30E+06^{A} ± 3.69E+06
23	1.30E+05^{A} ± 1.22E+05	8.20E+04^{A} ± 4.79E+04	5.74E+04^{A} ± 3.09E+04	4.15E+04^{AB} ± 2.24E+04	8.95E+04^{A} ± 5.13E+04	1.82E+05^{AB} ± 1.20E+05	5.34E+05^{B} ± 4.48E+05	2.18E+06^{B} ± 2.32E+06	n.d.	n.d.	2.68E+06^{A} ± 1.42E+06	4.32E+06^{A} ± 1.95E+06	1.48E+06^{A} ± 1.30E+06	2.84E+06^{A} ± 2.93E+06
46	3.52E+05^{A} ± 1.48E+05	1.87E+05^{A} ± 5.21E+04	2.48E+05^{A} ± 8.97E+04	5.12E+04^{B} ± 2.96E+04	6.25E+05^{B} ± 1.07E+05	4.63E+05^{B} ± 2.92E+05	2.28E+06^{A} ± 9.11E+05	2.90E+06^{B} ± 3.44E+06	n.d.	n.d.	3.66E+06^{A} ± 1.43E+06	2.97E+06^{A} ± 1.66E+06	3.03E+06^{A} ± 2.66E+06	3.08E+06^{A} ± 4.31E+06

^{A,B} Identical letters behind the numbers indicate no significance differences ($P > 0.05$). n.d. = not detectable

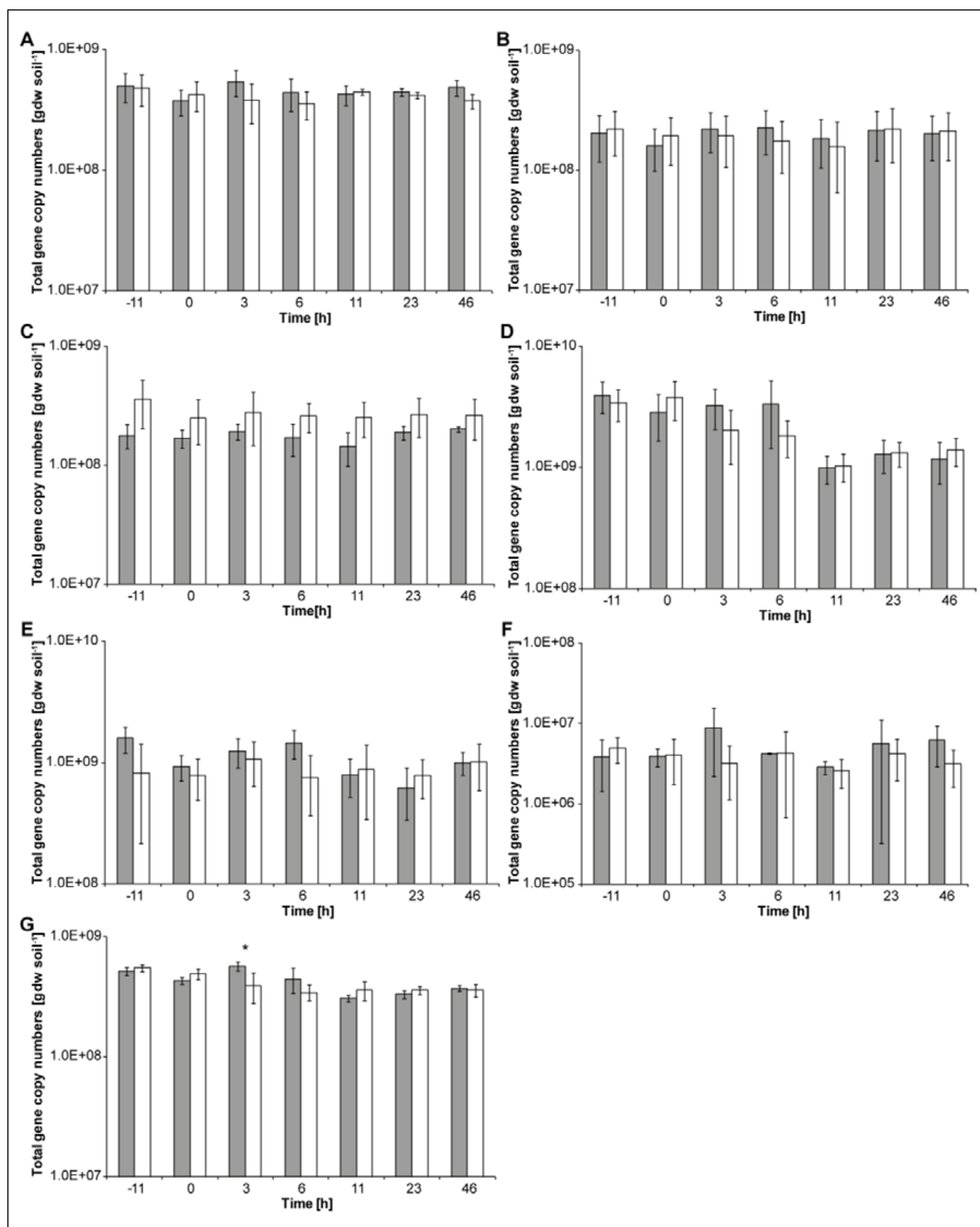

Figure S4.1. Gene copy numbers of functional marker genes involved in N-cycling. Bars indicate the total gene copy numbers. Asterisks indicate significant differences between eCO_2 and aCO_2 (ANOVA: $P < 0.05$). (A) *nirK*; (B) *nirS*; (C) *nosZ*; (D) *nrfA*; (E) archaeal amoA; (F) bacterial *amoA*; (G) *nifH*. Grey bars = eCO_2 plots; white bars = aCO_2 plots. (Mean ± SD, n=3).

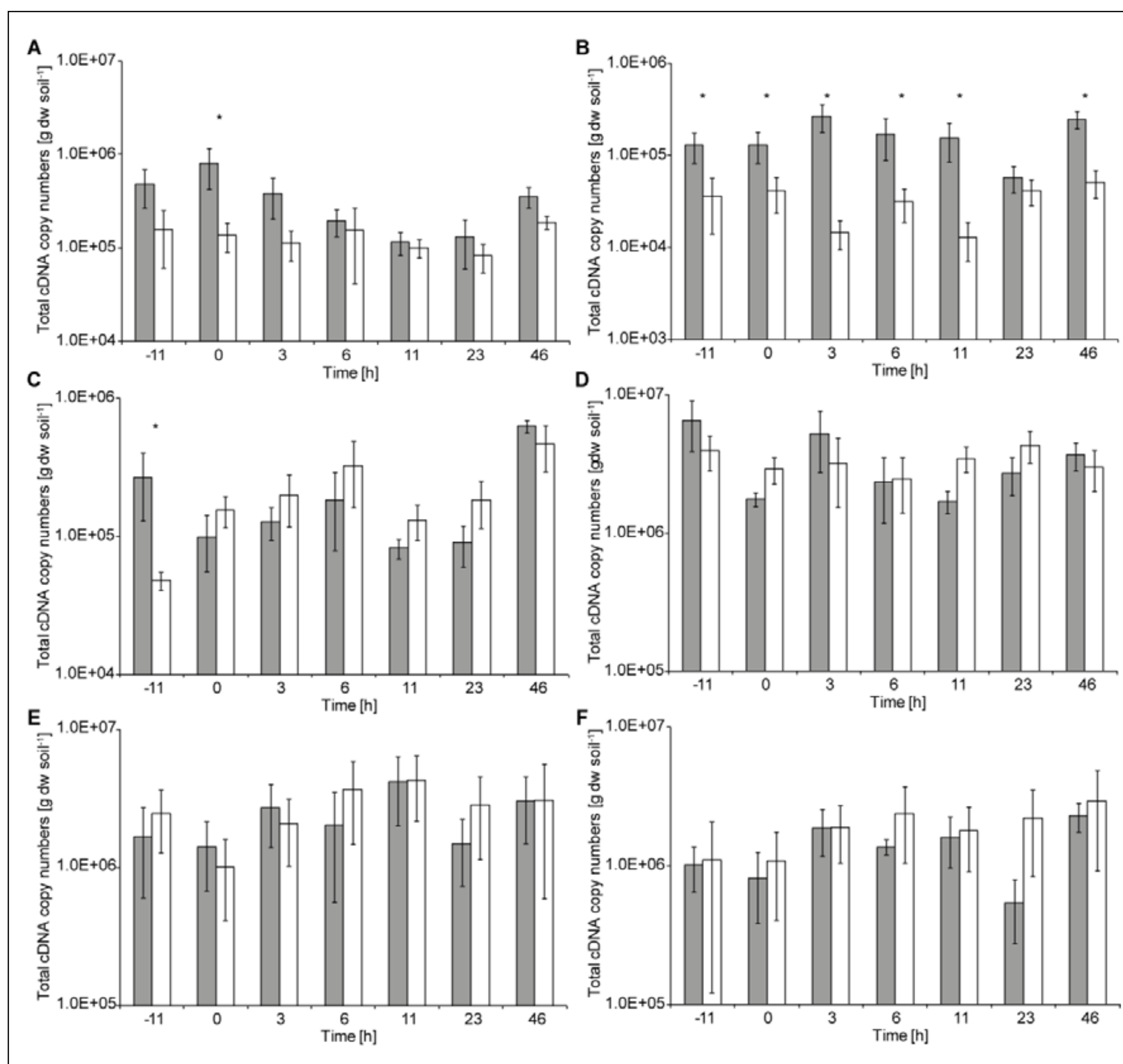

Figure S4.2. Transcript (cDNA) copy numbers of functional marker genes involved in N-cycling. Bars indicate the total cDNA copy numbers. Asterisks indicate significant differences between eCO_2 and aCO_2 (ANOVA: $P < 0.05$). (A) *nirK*; (B) *nirS*; (C) *nosZ*; (D) *nrfA;* (E) *nifH*; (F) archaeal *amoA*. Grey bars = eCO_2 plots; white bars = aCO_2 plots. (Mean ± SD, n=3).

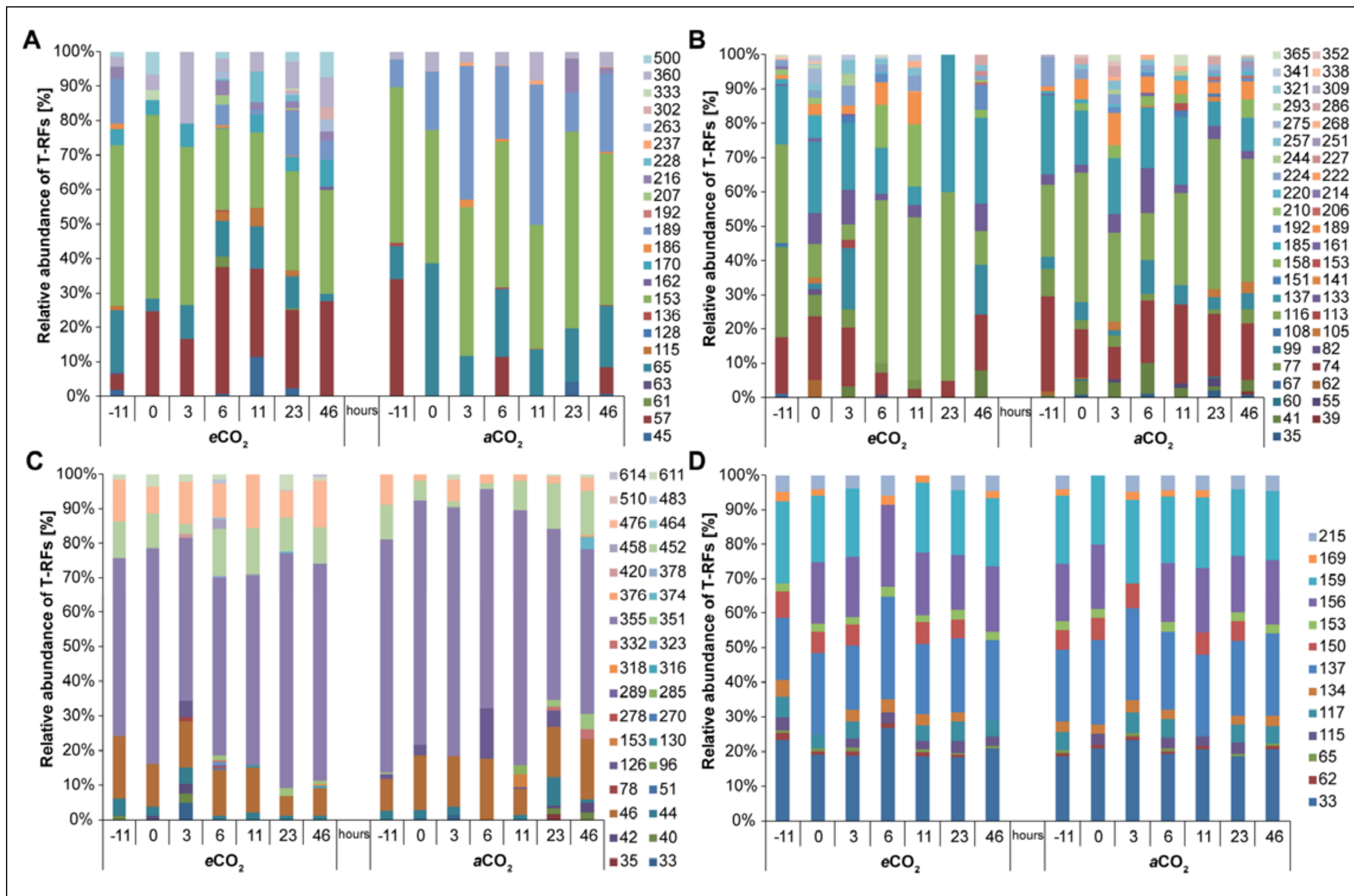

Figure S4.3. T-RFLP profiles of functional marker genes involved in N-cycling at different sampling times. Left, average of three replicate samples from eCO_2, right, aCO_2. Different colors of the bars indicate relative abundance of single T-RFs. T-RFs with minimum 1% relative abundance in at least one sample are plotted (n=3). Numbers in the figure legend indicate the size of the T-RFs in base pairs. (A) *nirK*; (B) *nirS*; (C) *nosZ*; (D) *nrfA*; (E) *nifH*; (F) archaeal *amoA*; (G) bacterial 16S rRNA; (H) archaeal 16S rRNA.

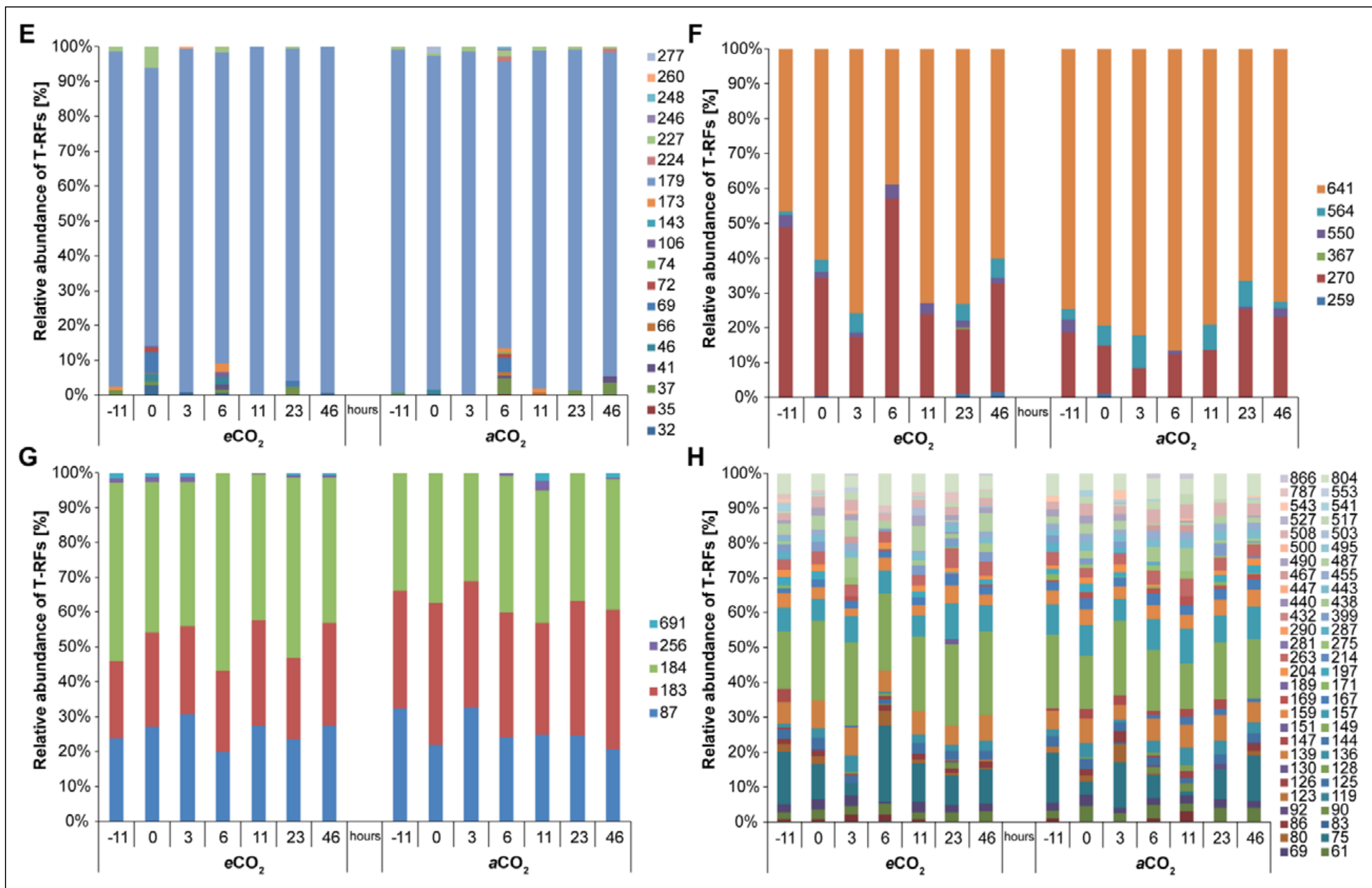

Figure S4.3. continued.

Chapter V

5. Discussion

The nitrogen cycle and the corresponding pathways are very complex and hard to predict in their response to climate changes. However, a shift in N-cycling has far reaching consequences regarding emissions of trace gas emissions such as N_2O and also management consequences to adapt to changing environmental conditions. Thus, it is of great interest to explore shifts in the N-transformations, N-balance and reactions associated with the impact of climate changes, to offer more accurate predictions. Of particular interest are the microbial mediated transformations causing emission of gaseous N-components in particular N_2O, which has a global warming potential of 298 times that of CO_2 (IPCC, 2013). Increasing CO_2 concentrations in the atmosphere or changes in the pH of agricultural fields due to anthropogenic influences often leads to changes in the N-transformation rates, along with an increase of N_2O emission (Kammann *et al*., 2008; Müller *et al*., 2009). The contribution of the underlying microorganisms, which are responsible for the production of N_2O, is at least controversial discussed. Unfortunately, many of the interactions between microorganisms and their contribution to N-transformation rates and N_2O emission are not well understood and plenty of important interactions remain unclear. Therefore, the main objective of this thesis was to shed light on the interaction of the overall and active microbial communities with pH shifts elevated atmospheric CO_2 in soils.

In the first study, the impact of pH on denitrification kinetics, end product ratios and the underlying denitrifier community was explored. pH is one of the major factors influencing the emission of N_2O produced by denitrification in agricultural soils (Liu *et al*., 2010; Raut *et al*., 2012; Šimek and Cooper, 2002). In those studies, an acidification of soils led to an increased N_2O flux. However, little is known about direct influence and changes on the

composition and abundance of the overall and in particular on the transcriptionally active microbial community. Therefore, we approached these questions by a model community obtained by extracting microbial cells from a soil with an initial neutral pH, which was then incubated at acidic pH (**Chapter II**). Microbial community composition and abundance was assessed using molecular techniques (T-RFLP and qPCR) targeting marker genes of denitrification (*nirK*, *nirS* and *nosZ*). Further, the products of the denitrification pathway (NO_2^-, NO, N_2O and N_2) were monitored using an analytical approach allowing the study of the synergy between denitrification rates and microbial dynamics.

The second aim of this thesis was to shed light on the impact of increasing atmospheric CO_2 concentrations on microbial communities involved in N dynamics under field conditions at GiFACE. One goal was to study the abundance and composition of the overall microbial community under *e*CO_2 in comparison to *a*CO_2 (**Chapter III**). In a more in depth approach the impact of *e*CO_2 upon simultaneous addition of N-fertilizer on the transcriptionally active microbial communities involved in N-transformations in soil was studied (**Chapter IV**). Increasing N_2O emissions under *e*CO_2 were reported previously (Arnone III *et al*., 1998; Gorenflo *et al*., in preparation; Ineson *et al*., 1998; Kammann *et al*., 2008; Mosier *et al*., 2002; Van Groenigen *et al*., 2011) suggesting feedback effects of *e*CO_2 on N-cycling in general and on denitrification in particular. However detailed understanding of the contribution by microorganisms promoting N_2O emission under *e*CO_2 is still marginal. To study the response of microbial communities adapted to *e*CO_2 to fertilization community composition and abundance was assessed using molecular techniques (T-RFLP, 454 pyrosequencing, qPCR) targeting several functional marker genes for pathways involved in N-transformations (B*amoA*, A*amoA*, *nirK*, *nirS*, *nosZ*, *nifH* and *nrfA*,) as well as bacterial and archaeal ribosomal 16S rRNA genes and transcripts. This project to monitor soil parameters, N-transformation/gaseous products in conjunction with molecular approaches was realized

through a close cooperation with the plant ecology department at the Justus-Liebig University Giessen.

5.1. N_2O emission controlled by microorganisms in soil

Soils are the most important contributors of emitted N_2O to the atmosphere (Thompson *et al*., 2012). Many chemical and physiological factors exist that control the fluxes of N_2O from soils (Table 5.1). It is known that proximal regulators of denitrification, i.e. temperature, soil moisture, N-status, composition and abundance of microbial communities influence N_2O production (Bateman and Baggs, 2005; Braker *et al*., 2010; Benoit *et al*., 2015; Dambreville *et al.*, 2006; Enwall *et al.*, 2005; Gödde and Conrad, 1999; Wertz *et al*., 2013). Distal regulators like soil type, microbial functional diversity and geography were suggested to determine the ability of different ecosystems to emit N_2O as a response to changes in proximal regulators (Bonnett *et al*., 2013; Braker *et al*., 2012; Cosentino *et al*., 2013; Dörsch *et al*., 2012; Philippot *et al*., 2013). Morales *et al*. (2015) showed that distal factors like latitude or soil type are best suited to predict N_2O emissions from varying environments, even though certain conditions (e.g. high NO_3^- , high soil water content) have additional impact on emitted N_2O (Table 5.1).

Table 5.1. Impact of environmental parameters on N_2O emission caused by denitrification, DNRA and nitrification.

Environmental parameters	Increase/ Decrease	Denitrification	References	DNRA	References	Nitrification	References
C:N ratio	↑	n.e.		⊕	Tiedje, 1982	n.e.	
Addition Fertilizer	↑	⊕	Dambreville *et al.*, 2006;	n.e.		⊕	Hallin *et al.*, 2009
NO_3^-	↑ ↓	⊕ ⊖	Firestone, 1982	⊖ ⊕	Tiedje, 1982; 1988	n.e.	
CO_2	↑	⊕	Kammann *et al.*, 2008; **Chapter III and IV**	⊕	Müller *et al.*, 2009; **Chapter III and IV**	n.e.	
NH4+	↑ ↓	Indirect effect by increase NO_3^- from nitrification		Indirect effect by increase NO_3^- from nitrification		⊕ ⊖	Roberstson, 1982a; 1982b
Microbial community composition	Differences in composition	⊕/⊖	Rich *et al.*, 2003; Enwall *et al.*, 2005; Hallin *et al.*, 2009; Braker *et al.*, 2012; **Chapter II, III and IV**	⊕	**Chapter III**	⊕/⊖	Smith *et al.*, 2010
Microbial community abundance	↑ ↓	⊕/⊖ ⊕/⊖	Dandie *et al.*, 2008; Hallin *et al.*, 2009; Morales *et al.*, 2010; **Chapter II, III and IV**	No interaction	**Chapter III**	⊕ ⊖	Hallin *et al.*, 2009
O_2 partial pressure	↑ ↓	⊖ ⊕	Bergaust *et al.*, 2008; 2011; van Spanning *et al.*, 2007	⊖ ⊕	Tiedje, 1982; 1988	⊕ ⊖	Conrad, 1996; van Spanning *et al.*, 2007
pH	↑ ↓	⊖ ⊕	Čuhel *et al.*, 2010; Bakken *et al.*, 2012; **Chapter II**	⊕ ⊖	Stevens *et al.*, 1998	⊖ ⊕	Cheng *et al.*, 2013
Soil moisture	↑ ↓	⊕ ⊖	Bateman and Baggs, 2005	n.e.		⊖ ⊕	Bateman and Baggs, 2005
Temperature	↑	⊕	Braker *et al.*, 2010; Wertz *et al.*, 2013	n.e.		⊕	Benoit *et al.*, 2015; Wertz *et al.*, 2013

n.e.: not evaluated; ↑↓**:** indicates increase/decrease of environmental parameter; ⊕: increase N_2O emission; ⊖: decrease N_2O emission.

Impact of acidic pH on N_2O emission by denitrification

pH is one of the key factors influencing denitrification control in soils as it affects denitrification rates, denitrification end product ratios, denitrifier community composition and abundance (Šimek and Cooper, 2002). In agricultural soil, intensive cropping systems (Raut *et al*., 2012) and repeated addition of N-fertilizer (Cheng *et al*., 2015) led to decreasing soil pH. This acidification then resulted in an accumulation of N_2O produced by denitrification processes (Cai *et al*., 2012; Liu *et al*., 2010; 2014; Raut *et al*., 2012; Šimek and Cooper, 2002; Zhang *et al*., 2015). It was speculated that pH < 6.1 induces post-translational inhibition of the N_2O reductase and therefore controls N_2O emissions (Bergaust *et al*., 2010; Liu *et al*., 2014). However, this is not fully explained yet. Further, acidic pH in soil also negatively effects diversity of the denitrification gene pool compared to neutral soils (Čuhel *et al*., 2010; 2011; Fierer and Jackson, 2006; Braker *et al*., 2012). Expression of denitrification genes of a microbial community extracted from a soil with neutral pH was impaired during the incubation at acidic pH (**Chapter II**). A sequential and slightly enhanced transient accumulation of denitrification intermediates (NO, N_2O) occurred under acidic pH. However, growth of nitrite- (*nirK*-type) and N_2O-reducers (*nosZ*) was observed and only < 1% of available N accumulated as N_2O and NO at low pH. Denitrifiers of the *nirK*-type present in the native community of the soil seemed to tolerate a broad range of pH levels as the composition of the growing community remained was unaltered during the incubation at low pH (**Chapter II**). We concluded that acid-tolerant or acidophilic denitrifier species maintained their functionality and thus fully converted NO_3^- to N_2 under extended incubation periods. Recent studies showed this for *nosZ*-type denitrifiers in acidic peatland soils (Palmer and Horn, 2012). At low pH, acid tolerant nosZ-containing denitrifiers seemed to functionally substitute N_2O-reducers that were more prevalent in the initial community. Hence, in communities and ecosystems at different environmental conditions distinct species perform similar roles and may therefore be substitutable with little impact on ecosystem processes

(Rosenfeld, 2002). It is noteworthy that the reduction of nitrogen oxides which led to an increased pH was necessary for the reduction of nitrite by *nirS*-type denitrifiers. Moreover, the occurrence of e.g. N_2O reduction in acidic soils can be explained by denitrification activity in neutral microsites as proposed by Liu *et al*. (2014). Consequently, soil denitrifier communities might be comprised of taxa differing in pH sensitivity, which jointly emulate the kinetic response of soils to pH changes.

Impact of anthropogenic climate change on N_2O emission from soil

N_2O emissions drastically increased through addition of N-fertilizer to soils. Smith *et al*. (2012) suggested that ~ 3-5% of annually introduced N into the soils is converted to N_2O. Furthermore, anthropogenic induced climate change may cause feedback effects of N_2O emissions because the biochemical processes responsible for N_2O emissions are influenced by water content and temperature. For example, as soils temperature increases, microbial decomposition and CO_2 emissions increase (Bond-Lamberty and Thomson, 2010), which can further led to higher N_2O emission rates (Kammann *et al*., 2008). Studies on FACE facilities world-wide showed that elevation of atmospheric CO_2 led to an increase of N_2O fluxes from soils (Arnone III *et al*., 1998; Ineson *et al*., 1998; Kammann *et al*., 2008; Mosier *et al*., 2002; Van Groenigen *et al*., 2011). Moreover, enhanced CO_2 concentration led to higher plant biomass production (Lenhart, 2008) and resulted in enhanced consumption of CO_2 via photosynthesis (Keidel *et al*., 2015). Consequently, plants transfer higher amounts of carbon compounds into the soil via root exudates and rhizodeposition, leading to an additional input of C-compounds which can be used by microorganisms for their metabolism. Furthermore, higher CO_2 availability altered N-transformations in soil, which resulted in higher N_2O emission under *e*CO_2 (Kammann *et al*., 2008; Müller *et al*., 2009). However, the underlying mechanism is not resolved yet. Recent studies were carried out to identify the contribution of

microorganisms to increased N_2O fluxes under *e*CO_2. It was speculated that differences in either altered N_2O:N_2 ratios during denitrification (Regan *et al*., 2011) or enhanced fungal activities were responsible for the enhanced N_2O emissions (Denef *et al*., 2007). In our study, samples collected at GiFACE in spring showed only marginal effects of *e*CO_2 on the abundance and composition of microbial communities involved in N-transformations (**Chapter III**). Our results indicate that the GiFACE grassland site harbors a relatively stable microbial community. Main differences occurred rather between soils from different sampling sites within the GiFACE than between soils under *e*CO_2 and *a*CO_2 (**Chapter III**). Just recently, a study suggested that geographic position or distal factors are the most important drivers for N_2O emission (Morales *et al*., 2015). Only the composition of nitrate-reducers to ammonia (*nrfA*) was effected by *e*CO_2 based on T-RFLP fingerprinting analyses which may explain the observed 141% higher DNRA rates under *e*CO_2 at GiFACE (Müller *et al*., 2009). N_2O is also a byproduct of DNRA in the reduction from NO_3^- to NH_4^+ (Tiedje, 1982; 1988). Higher DNRA rates were not surprising, given that the GiFACE is an N-limited grassland site, which is also promoted by a C:N ratio of 12, when DNRA seems to be prevail (Müller *et al*., 2009) . N limitation as well as high amounts of labile C provided via enhanced rhizodeposition following *e*CO_2 promotes DNRA processes (Bonin, 1996; Fazzolari *et al*., 1998; Nijburg *et al*., 1997; Tiedje, 1982; Yin *et al*., 2002). However, the higher resolution of 454 pyrosequencing revealed more specific differences between the communities of the sampling sites than between *e*CO_2 and *a*CO_2 sites. Although N_2O emission rates are relatively low during the year, N_2O emitted from soils under *e*CO_2 is twice as high as under *a*CO_2 at almost every sampling time point. Our results suggest that this is mainly due to differences in the community structure of DNRA performing microorganisms, because the prevailing conditions in *e*CO_2 plots (N-limited and more labile C input) may favor DNRA at GiFACE (**Chapter III**).

A peak in N_2O emission from the GiFACE soil occurred upon N-input, provided by fertilization, rain events or snow melting (Kammann *et al*., 2008; Regan *et al*., 2011; **Chapter III)**. At our experimental site N_2O emissions under *e*CO_2 were more enhanced than under *a*CO_2 directly after the application of N-fertilizer (Gorenflo *et al*., in preparation; Kammann *et al*., 2008; Regan *et al*., 2011). Changes in the composition and abundance of microbial communities involved in N-transformations seem responsible for increased N_2O fluxes (**Chapter IV**). For example, the abundance of active *nirS*-type denitrifier was stimulated by *e*CO_2 and simultaneous input of N (**Chapter IV**). Differences in the overall abundance of *nirS*-type denitrifiers were already observed in recent studies, but under a much higher *e*CO_2 level and on N-rich FACE site (He *et al*., 2010; 2012; Xu *et al*., 2013). Increase in the activity of *nirS*-type denitrifiers led to a change in the ratio of transcriptionally activated N_2O reducers (*nosZ*) and N_2O producers (*nirK*+*nirS*). Ratios were lower under *e*CO_2 and thus the relative abundance of N_2O producers was higher under *e*CO_2. Even without N-addition due to increasing photosynthesis at day light, the ratio of transcriptionally active *nosZ*/(*nirK*+*nirS*) indicated a higher relative abundance of transcriptionally active N_2O producers under *e*CO_2. During night time, however, the ratio between transcriptionally active N_2O reducers and NO_2^- reducers was almost equal for soil under *e*CO_2 and *a*CO_2. Also the composition of the transcriptionally active microbial communities was significantly different between *e*CO_2 and *a*CO_2, even though most were only minor changes (ANOSIM: R values between 0.2-0.5) (**Chapter IV**). Nevertheless, this differences in composition of denitrifiers (*nirK*, *nirS* and *nosZ*) and DRNA (*nrfA*) performers may have a direct impact on N_2O emission (e.g. Cole, 1988; Conrad, 1996), since both pathways can produce N_2O. Even though, also composition of archaeal ammonia oxidizers (A*amoA*) was significantly different, we could not detect a great impact of nitrification to the N_2O production under *e*CO_2 and the N_2O mainly originated from denitrification or DNRA (Gorenflo *et al*., in preparation; Moser *et al*., in preparation). Additionally, previous studies suggested that archaeal ammonia oxidizers generally only

contribute a smaller amount to the N_2O emission from soil, since they are not capable to perform nitrifier-denitrification under oxygen limiting conditions in contrast to bacterial ammonia oxidizers (Stieglmeier *et al*., 2014). Taking all results into account, we hypothesize that based on differences in the size and composition of the transcriptionally active part of the community higher N_2O fluxes at *e*CO_2 and *a*CO_2 were mainly the result of differences in activity of *nirS*-type denitrifiers. These reactions are possibly caused by an increase of labile C into the rhizosphere by root exudates, increase of neutral/alkaline pH zones in the rhizosphere through higher NO_3^- uptake by plants (Nye, 1981) or more anoxic zones under *e*CO_2 through higher consumption of O_2 by microorganisms, which benefit from higher C content in the rhizosphere. It is probably a combination of these three factors that in the end is responsible for an increase in N_2O fluxes under *e*CO_2.

5.2 Sensitivity of *nirS*-type denitrifiers to changes of environmental conditions in soil

Denitrifiers of the *nirS*-type repeatedly appeared to be particularly vulnerable to different stress factors or changes in environmental conditions and that *nirK*-type denitrifiers are more abundant than *nirS* ones in soil (Chen *et al*. 2010; Graf *et al*., 2014; Maeda *et al*. 2010b; Yoshida *et al*. 2009; Zhang *et al*., 2015; Zhou *et al*. 2011) whereas *nirS* had an advantage over *nirK* in marine environments or sea sediments (Graf *et al*., 2014; Lindemann *et al*., 2015; Smith *et al*., 2014). Nevertheless, controversial results for soil and other environments between *nirS* and *nirK* competition were reported (Kleineidam *et al*., 2010; Vilar-Sanz *et al*., 2013). In microbial fuel cells, inoculated with wastewater treatment, *nirS* outnumbered *nirK* by two orders of magnitude at the cathode (Vilar-Sanz *et al*., 2013). Vilar-Sanz *et al*. (2013) argued that a former selective enrichment of cytochrome *c* family mediators

was the reason for higher *nirS* abundance, since NirS is a cytochrome c dependent enzyme. Additionally, in two arable soils *nirS*-harboring denitrifiers were more abundant than *nirK*-harboring, however an explanation for this occurrence was missing (Kleineidam *et al*., 2010). They hypothesize that *nirK*-type denitrifiers might be more related to sites with high substrate conditions, like the rhizosphere, while *nirS*-containing bacteria might be more related to primary colonizers of ecological niches (Sharma *et al*., 2005). Additionally, though no clear taxonomic differentiation between *nirK* and *nirS* denitrifiers exists (Sharma *et al*., 2005), there are indications that *nirS*- and *nirK*-harboring communities colonize different microhabitats in soil and are stimulated by different carbon sources (Philippot *et al*., 2007). All this indicates that the complexity of a soil and the underlying chemical and physical factors are most likely the main drivers to differentiate between the two types of nitrite reducers.

In the first part of this thesis *nirS*-type denitrifiers were most sensitive to pH manipulation (**Chapter II**). We observed persistently reduced relative *nirS* transcription at acidic pH compared to neutral pH and inhibited growth of *nirS*-type denitrifiers at low pH. Activity and growth were restored only after pH values shifted to more neutral (**Chapter II**). Further, it was reported that a pure culture of the *nirS*-type *P. denitrificans* was unable to build up a functional denitrification pathway at a slightly acidic pH of < 6.8 (Baumann *et al*., 1997). Although the nitrite reductase gene was properly induced, sufficient amounts of NirS-enzyme was not detected in the culture. This indicates that either translation was inhibited, or once synthesized, nitrite reductase was inactivated, possibly by high concentrations of nitrous acid (**Chapter II**). The higher susceptibility of *nirS*-type denitrifiers to low pH has been repeatedly reported in other soil studies (Čuhel *et al*., 2010; Bárta *et al*., 2010). Additionally, samples from four acidified soils showed always higher abundance for *nirK*-harboring denitrifiers over *nirS*-types (Chen *et al*., 2010). Nevertheless, a recent study showed the opposite, that lower *nirK*-transcript numbers than *nirS* during incubations at an acidic pH (Liu

et al., 2014). However, in this study, starting conditions were different; the community had a native pH of 6.1 and the extracted community was preincubated under oxic conditions for several hours. In our study the initial abundance of *nirK*- and *nirS*-type denitrifiers in the soil and in the inoculum was equal whereas in the incubation of Liu *et al*. (2014), *nirS*-type denitrifiers were nearly 100 times more abundant than *nirK*-harboring bacteria. Therefore our provided results indicate greater robustness of *nirK*-type versus *nirS*-type denitrifier communities to acidity. Generally, long-term exposure to low pH in the natural environment will shape soil microbial community composition and predetermine a dominance of either *nirK* or *nirS* (Chen *et al*., 2014), which leads in most cases to dominance of *nirK*-harboring nitrite reducers (Bárta *et al*., 2010; Chen *et al*., 2010; Čuhel *et al*., 2010) (**Chapter III**).

The increase of atmospheric CO_2 by +20% stimulated the transcript abundance of *nirS*-type denitrifiers in comparison to *a*CO_2 concentration (**Chapter IV**). Under ambient conditions *nirS*-harboring denitrifiers seem to be outcompeted by *nirK*-type nitrite reducers, since they compete for the same substrates (**Chapter IV**). We hypotheses that this is caused by plant-, root-biomass production and higher photosynthesis rates (Lenhart, 2008) which, (i) lead to increase of labile C into the rhizosphere by root exudates (ii) an increase of neutral/alkaline pH zones in the rhizosphere through higher NO_3^- uptake by greater plant biomass (Nye, 1981) or (iii) through higher labile C content in the rhizosphere leads to enhanced respiratory activity causing enhanced O_2 consumption and the development of increased anaerobicity. Conclusively, a dramatic increase of the greenhouse gas N_2O is induced, by the differences in *nirS*-harboring denitrifier activity.

Furthermore, several other soil physical and chemical factors have a higher impact on *nirS*-type denitrifiers, while *nirK*-harboring denitrifiers seem more robust to environmental variances (**Chapter II**, Zhang *et al*., 2015). For instance, at low temperatures (≤ 4°C) *nirK* communities were still detectable, whereas *nirS* communities could only be observed for

higher temperatures (Braker *et al*., 2010). Clark *et al*. (2012) observed that the abundance of *nirS* was repressed by long-term fertilization (addition of NO_3^-/NH_4^+), which provides fresh electron acceptors, while *nirK*-type denitrifiers undergo stimulation by the addition of NO_3^-. The Increase of growth by *nirK*-type denitrifiers can be associated with increasing denitrification rates, while *nirS* showed no correlation. In conclusion our results indicate that *nirS*-type denitrifiers are on the hand more vulnerable to environmental stress factors, e.g. pH changes (**Chapter II, III and IV**) and on the other hand would benefit the most from increasing anthropogenic CO_2 concentrations (**Chapter IV**).

5.3 Outlook

This thesis provides evidence that changes in the abundance and composition of microbial communities involved in N-transformations in soil influence N_2O fluxes. Acidic pH seems to have a high impact on N_2O emission (**Chapter II**). However, pH and other factors do not strongly affect microbial communities that were shaped by constant soil chemical and physical parameters for extended time periods. Therefore, overall microbial communities involved in N-cycling seems remarkably stable to changes in environmental conditions (**Chapter III**) and only changes in transcriptional activity may explain changes in trace gas production (**Chapter IV**). Nevertheless, even if a community is relatively stable under environmental changes, *nirS*-type denitrifiers seem to be most responsive to e.g. eCO_2 and contribute significantly to different N_2O emissions between eCO_2 and aCO_2 in soils (**Chapter IV**).

We suggest that low pH episodes alter transcriptionally active populations which shape denitrifier communities and determine their gas kinetics (**Chapter II**). Still, further analyses are necessary to gain better understanding of how posttranscriptional regulation determining N_2O production under acidic conditions. For instance, a metaproteomic analyses might help to identify denitrifiers whose mRNA is not translated into proteins. However, the existing protein database is limited to the most abundant denitrifier enzymes and the protein pool in cells is dominated by ribosomes and all of their regulation factors. Additionally, direct purification of proteins from soil is not trivial. Therefore, experiments with extracted cells would minimize inhibitory effects of soil particles. Moreover, since extracted cells are most responsive to acidification they should be used to further study the effects of changes from neutral to acidic pH and back to neutral. It would also be of great interest to study if microbial communities which underwent a structural shift during a short-term pH manipulation have the potential to shift back, if the pH value were readjusted to the initial soil pH. These

experiments have the potential to reveal a deeper understanding of the interaction between acid pH, N_2O emission and the underlying microbiota. Additionally, since acidification in agricultural soil increases through repeated addition of N-fertilizer which results in higher N_2O emission (**see 5.1.1.**), it would be of interest to identify if more N_2O reducers exist capable of performing N_2O reduction even under acidic conditions. Palmer and Horn (2012) identified acidophilic N_2O reducers in peatland soils. However, it is unclear if these denitrifiers are also able to reduce N_2O in other habitats. Maybe through this approach new fertilization strategies can be established to mitigate the emission of N_2O from acidified soils.

The second and third part of this thesis provide evidence that the increase of anthropogenic caused CO_2 concentrations in the coming two to three decades have a stimulation effect on the N_2O emission which seem to be associated with enhanced activity of *nirS*-type denitrifiers (**Chapter IV**). Additionally, during periods of N-limitation, higher N_2O emissions under *e*CO_2 are mainly caused by differences in the community composition of *nrfA* (DNRA). This warrants a closer look, especially into feedback mechanism occurring at the rhizosphere and in particular at the rhizoplane. Studies combining detailed analyses on the nature of rhizodeposits and the response to nitrite reducers would be most promising to reveal processes responsible for the observed feedback effects on gaseous N emissions. There are indications that the additional carbon available via *e*CO_2 is directly consumed at the root-soil interface, since no additional labile C was measured in the bulk soil (Lenhart, 2008), also the plants consumed the additional provided CO_2 (Keidel *et al*., 2015). Furthermore, a $^{13}CO_2$ stable isotope probing (SIP) experiment under laboratory conditions could reveal a direct link between C- and N-cycle and monitor microorganisms that benefitting from higher CO_2 concentrations. Using SIP the ^{13}C could be detected in the organisms that used the C-substrates provided by the plants. So far, a $^{13}CO_2$ SIP experiment could not be established for functional marker genes under field conditions, because of difficult weather conditions and the high amount of different microorganisms that compete for the labeled CO_2. Overall, this

thesis showed that increasing anthropogenic CO_2 emissions promote feedback effects on the emission of other GHG such as N_2O that are even more potent and enhance the anthropogenic greenhouse effect further. To understand these feedback loops, it is important to understand in detail the dynamics of the microbial communities responsible for N-cycling to be able to avoid adverse effects on our environment due to global climate change.

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Appendices

Wissenschaftliche Publikationen

***Brenzinger*, *K.*,** Brandt, F.B., Breidenbach, B. and Conrad, R. (2014). Impact of short-term storage temperature on determination of microbial community composition and abundance in aerated forest soil and anoxic pond sediment samples. *Systematic and applied microbiology*, **37**:570-577.

Brenzinger, K., Dörsch, P. and Braker, G. (2015). pH-driven shifts in overall and transcriptionally active denitrifiers control gaseous product stoichiometry in growth experiments with extracted bacteria from soil. *Frontiers in Microbiol*. **6**:961.

Braker, G., Matthies, D., Hannig, M., Brandt, F. B., ***Brenzinger, K.*** and Gröngröft, A. (2015). Impact of Land Use Management and Soil Properties on Denitrifier Communities of Namibian Savannas. *Microbial ecology*, 1-12.

Beiträge zu wissenschaftlichen Tagungen

Annual Conference of the Association for General and Applied Microbiology (VAAM), Karlsruhe, Deutschland, 2011

Brenzinger, K., Braker, G., Dörsch, P. and Bakken, L. *Intrinsic differences in denitrifier community structure and abundance determine functional responses of denitrification in three organic soils* (Poster Präsentation)

Life in Microhabitats of Soils – Microbial Ecology of Biogeochemical Interfaces, Jena, Deutschland, 2012

Brenzinger, K., Braker, G., Dörsch, P. and Bakken, L. *Intrinsic differences in denitrifier community structure and abundance determine functional responses of denitrification in three organic soils* (Poster Präsentation)

17th European Nitrogen Cycle Meeting, Oslo, Norwegen, 2012

Brenzinger, K., Dörsch, P. and Braker, G., *Dynamics of the active and overall denitrifier communities from three soils in response to pH* (Oral Präsentation)

European Geosciences Union General Assembly 2014, Wien, Österreich, 2014

Moser, G., Gorenflo, A., Keidel, L., ***Brenzinger, K.,*** Elias, D., McNamara, N., Maček, I., Vodnik, D., Braker, G., Schimmelpfennig, S., Gerstner, J. and Müller C. *The effect of elevated atmospheric CO_2 concentration on gross nitrogen and carbon dynamics in a permanent grassland: A field pulse-labeling study* (Oral Präsentation)

European Geosciences Union General Assembly 2014, Wien, Österreich, 2014

Suleiman, M., ***Brenzinger, K.,*** Brandt, B., Martinson, G. and Braker, G. *Denitrifier communities in tank bromeliads and prospected N_2O emissions from tank substrate upon increasing N-deposition* (Poster Präsentation)

19th European Nitrogen Cycle Meeting, Ghent, Belgium, 2014

Brenzinger, K., Moser, G., Gorenflo, A., Keidel, L., Müller, C. and Braker, G. *Effect of eCO_2 on microbial communities involved in N cycling in soils* (Oral Präsentation)

Annual Conference of the Association for General and Applied Microbiology (VAAM), Marburg, Deutschland, 2015

Brenzinger, K., Moser, G., Gorenflo, A., Keidel, L., Müller, C. and Braker, G. *Effect of* eCO_2 *on microbial communities involved in N cycling in soils* (Oral Präsentation)

Annual Conference of the Association for General and Applied Microbiology (VAAM), Marburg, Deutschland, 2015

Suleiman, M., ***Brenzinger, K.,*** Brandt, B., Martinson, G. and Braker, G. *Denitrifier communities in tank bromeliads and prospected* N_2O *emissions from tank substrate upon increasing N-deposition* (Poster Präsentation)

European Geosciences Union General Assembly 2015, Wien, Österreich, 2015

Moser, G., Gorenflo, A., Keidel, L., ***Brenzinger, K.,*** Elias, D., McNamara, N., Clough, T., Maček, I., Vodnik, D., Braker, G., Schimmelpfennig, S., Gerstner, J. and Müller, C. *Tracing changes in N transformations in a permanent grassland under elevated atmospheric* CO_2 (Oral Präsentation)

European Geosciences Union General Assembly 2015, Wien, Österreich, 2015

Gorenflo, A., Moser, G., Keidel, L., ***Brenzinger, K.,*** Elias, D., McNamara, N., Clough, T., Maček, I., Vodnik, D., Braker, G., Schimmelpfennig, S., Gerstner, J. and Müller, C. *Tracing changes of* N_2O *emission pathways in a permanent grassland under elevated atmospheric* CO_2 *concentrations* (Poster Präsentation)

20th European Nitrogen Cycle Meeting, Aberdeen, Scotland, 2015

Brenzinger, K., Moser, G., Gorenflo, A., Keidel, L., Müller, C. and Braker, G. *Response of active microbial communities involved in N-cycling in soils to* eCO_2 *during fertilization* (Oral Präsentation)

Abgrenzung der Eigenleistung

Diese Arbeit wurde von der Deutschen Forschungsgemeinschaft (DFG) und durch die Max-Planck-Gesellschaft finanziert. Die Haupthemen dieser Arbeit wurde von meiner Betreuerin, PD. Dr. Gesche Braker, konzipiert. Soweit nicht anders erwähnt, wurden alle Experimente von mir selbst geplant und durchgeführt, sowie anschließend in Form eines Manuskriptes ausgewertet. Das Verfassen des Manuskriptes erfolgte in Zusammenarbeit mit meiner Betreuerin, PD. Dr. Gesche Braker.

Erklärung

Ich versichere, dass ich meine Dissertation

„Impact of changes in environmental parameters (pH and elevated CO_2) on soil microbial communities involved in N-cycling“

selbständig und ohne unerlaubte Hilfe angefertigt habe und mich keiner als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Diese Dissertation wurde in der jetzigen oder ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

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