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Formation of hybrid N_2O and hybrid N_2 due to codenitrification and its analysis by a ^{15}N tracer technique

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Formation of hybrid N_2O and hybrid N_2 due to codenitrification and its analysis by a ^{15}N tracer technique

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„Trägheit ist eine Tugend, wenn sie einen daran verhindert, im Topf herumzurühren, nur damit gerührt wird. Die Bessermacher haben so viel schlechter gemacht, daß von dieser Art von Besserem wegzubleiben eine Tugend geworden ist"

(Das Feuer des Heraklit, E. Chargaff 1979)

II. Zusammenfassung

Der Stickstoffkreislauf ist einer der wichtigsten Nährstoffkreisläufe innerhalb der Biosphäre. Er beinhaltet die vier wesentlichen mikrobiellen Prozesse der Stickstoff-Fixierung, der Mineralisation, der Nitrifikation und der Denitrifikation (Hayatsu et al., 2008). Die Bildung der gasförmigen Stickstoffverbindungen N_2O und N_2, welche im Zuge des mikrobiellen Stickstoffkreislaufs gebildet werden, wurde bisher überwiegend auf die Prozesse der Nitrifikation und der Denitrifikation zurückgeführt (Jetten, 2008). Innerhalb der letzten Jahrzehnte haben zahlreiche Untersuchungen jedoch gezeigt, dass die mikrobielle N_2O und N_2 Bildung in der Biosphäre weitaus komplexer ist als bisher angenommen. Dies zeigt sich vor allem in einer wesentlich größeren Vielfalt an beteiligten mikrobiellen Spezies, möglichen Reaktionswegen und involvierten Stickstoffverbindungen (Hayatsu et al., 2008).

Der Prozess der ko-metabolischen Denitrifikation (i.e. Codenitrifikation; Shoun et al. (1992) and Tanimoto et al. (1992)) bildet N_2O und N_2 in einem erstaunlichen Reaktionsweg, welcher es einer Reihe von organischen sowie anorganischen Stickstoffverbindungen erlaubt im Zuge der Denitrifikation ko-metabolisiert zu werden. Da die Gasbildung durch Codenitrifikation auf einer N-Nitrosierung basiert, bei der sich ein Stickstoffatom eines elektrophilen Denitrifikationsintermediärs direkt mit einem Stickstoffatom einer nucleophilen Spezies (bspw. Amine) verbindet, stellen die freigesetzten Gase N_2O und N_2 hybride Stickstoffverbindungen dar. Trotz der Einzigartigkeit des aufgedeckten Reaktionsweges wurde dem Prozess der Codenitrifikation, auch fast 20 Jahre nach seiner ersten Beschreibung durch Tanimoto et al. (1992), nur wenig wissenschaftliches Interesse entgegengebracht. Demnach besteht auch noch heute ein enormes Wissensdefizit über die Bedeutung der Codenitrifikation für den Stickstoffkreislauf der Biosphäre.

Basierend auf einer intensiven und tiefgründigen Literaturauswertung zeigt die vorliegende Dissertation, dass der Prozess der Codenitrifikation als prinzipiell ubiquitär innerhalb des mikrobiellen Stickstoffkreislaufs angenommen werden muss. Demnach sind eine Vielzahl bereits bekannter denitrifizierender Spezies aller drei Domänen (*Archaea*, *Bacteria* und *Eukarya*) in der Lage hybrides N_2O und/oder hybrides N_2 im Zuge der Codenitrifikation freizusetzen. Nahezu alle dieser durchgeführten Untersuchungen basieren auf Experimenten mit Zellsuspensionen oder denitrifizierenden Enzymen (bspw. Shoun et al., 1992; Tanimoto et al., 1992; Kumon et al., 2002; Immoos et al., 2004; Shoun, 2004; Su et al., 2004; Okada et al., 2005). Nur eine Studie wurde bisher auf der Basis von Bodenproben durchgeführt (Laughlin und Stevens, 2002). Die ^{15}N-Isotopen-Technik bildete dabei die zentrale Untersuchungsmethode.

Der deutliche Mangel an Forschungsarbeiten zur Codenitrifikation, insbesondere im Hinblick auf Untersuchungen jenseits der mikrobiellen Skala, ist im Wesentlichen verursacht durch (i) die extremen methodischen Anforderung bei der Messung mikrobieller N_2 Freisetzung (siehe Davidson und Seitzinger, 2006) sowie (ii) durch die Tatsache, dass die Freisetzung hybriden

N_2O/N_2 im Zuge der Codenitrifikation, welche zeitgleich mit der Freisetzung nicht-hybriden N_2O/N_2 aus der Denitrifikation erfolgt, in den bisherigen ^{15}N-Isotopen-Modellen nur unzureichend berücksichtigt wird. Zur Überwindung dieser methodologischen Einschränkungen wurden im Rahmen der vorliegenden Dissertation ein neuartiges Boden-Inkubationssystem sowie zwei neue ^{15}N-Isotopen-Modelle entwickelt. Das entwickelt Labormesssystem ermöglicht eine ^{15}N-gestütze Online-Messung der simultanen N_2O und N_2 Freisetzung aus inkubierten Bodenproben. Das erste ^{15}N-Isotopen-Modell erlaubt eine exakte Quantifizierung der hybriden N_2O/N_2 Freisetzung (Codenitrifikation) einerseits, sowie zeitlichgleich die exakte Quantifizierung der nicht-hybriden N_2O/N_2 Freisetzung (Denitrifikation) anderseits. Das zweite ^{15}N-Isotopen-Modell wurde entwickelt, um eine prinzipiell einfache und schnelle Identifizierung einer hybriden N-Gasbildung zu ermöglichen. Die Anwendbarkeit der beschriebenen methodischen Entwicklungen wurde in individuellen Experimenten verifiziert. Daraus kann zusammenfassend geschlussfolgert werden, dass die im Rahmen der vorliegenden Dissertation entwickelten neuartigen methodischen Ansätze eine zuverlässige und umfassende Untersuchung mikrobieller N_2O/N_2 Freisetzung im Zuge der Denitrifikation sowie der Codenitrifikation ermöglichen.

III. Summary

The nitrogen cycle is one of the most important nutrient cycles within the biosphere. It includes four main microbiological processes: nitrogen fixation, mineralization, nitrification, and denitrification (Hayatsu et al., 2008). A production of the gaseous nitrogen species N_2O and N_2 in the course of microbial nitrogen cycling was so far considered to be largely related to denitrification and nitrification (Jetten, 2008). However, during the recent decades plenty of studies could reveal that microbial N_2O and N_2 formation in the biosphere might be significantly more complex inasmuch as it includes a much broader variety of microbial species, reaction pathways, and nitrogen compounds (Hayatsu et al., 2008).

The process of co-metabolic denitrification (i.e. codenitrification; Shoun et al. (1992) and Tanimoto et al., (1992)) was shown to produce N_2O and N_2 in an intriguing reaction pathway, which permits a variety of organic and inorganic nitrogen species to be co-metabolised during denitrification. Since nitrogen gas production by codenitrification is based on an N-nitrosation, where a nitrogen atom of an electrophilic denitrification intermediate binds directly to a nitrogen atom of a nucleophilic species (e.g. amines), the liberated N_2O and N_2 represent hybrid nitrogen gas species. In spite of the uniqueness of the reaction pathway of codenitrification it remained almost unconsidered even two decades after its first description by Tanimoto et al. (1992). Thus, still today a tremendous gap of knowledge exists concerning its impact on nitrogen cycling in the biosphere.

Based on a profound literature review the present doctoral thesis reveals that codenitrification has to be considered as a ubiquitous process in microbial nitrogen cycling. It was found that a

variety of denitrifying species of all three domains (*Archaea*, *Bacteria*, and *Eukarya*) were already reported to be capable of hybrid N_2O and/or hybrid N_2 formation in the course of codenitrification. In general, the ^{15}N tracer technique was the method of choice to study the phenomenon of codenitrification. Almost all of these studies were based on cell suspension experiments or were carried out with denitrifying enzymes (e.g. Shoun et al., 1992; Tanimoto et al., 1992; Kumon et al., 2002; Immoos et al., 2004; Shoun, 2004; Su et al., 2004; Okada et al., 2005). Until now only one study was conducted with soil samples (Laughlin and Stevens, 2002). The lack of research, in particular with respect to studies beyond the microbial species level, is mainly due to the fact that (i) a study of N_2 formation due to microbial nitrogen transformation is still today a tremendous methodological challenge (see Davidson and Seitzinger, 2006) and (ii) a formation of codenitrified N_2O/N_2 simultaneously with denitrified N_2O/N_2 is inadequately considered in contemporary ^{15}N tracer models. To overcome these methodological constraints a novel soil incubation system and two novel ^{15}N tracer models have been developed. The laboratory system was designed to permit a ^{15}N-aided online determination of N_2O and N_2 gas simultaneously released by an incubated soil sample. The first ^{15}N tracer model was designed to permit an individual quantification of non-hybrid and simultaneous hybrid N_2O/N_2, while the second ^{15}N tracer model was developed to allow an easy and rapid identification of hybrid N_2O/N_2 gas production. The applicability of all methodological developments has been individually verified by experimental studies. Recapitulatory, it can be concluded that the novel approaches presented in this doctoral thesis represent reliable tools to study most comprehensively non-hybrid and hybrid N_2O/N_2 formation due to denitrification and codenitrification, respectively.

IV. List of Tables

V. List of Figures

VI. List of abbreviations and units

anammox	-	anaerobic ammonium oxidation
at%	-	atom percent
bspw.	-	beispielsweise
C	-	carbon
C_2H_2	-	acetylene
C_6H_5-NH_2	-	aniline
CH_3O-C_6H_5-NH_2	-	p-methoxyaniline
C_2H_6N-C_6H_4-NH_2	-	dimethyl-p-phenylene diamine
$C_7H_7NO_3$	-	salicylhydroxamic acid
CO_2	-	carbon dioxide
ConFlow IRMS	-	continuous-flow isotope-ratio mass spectrometer
Cu	-	copper
d	-	day
DNRA	-	dissimilatory nitrate reduction to ammonium
e.g.	-	exempli gratia
et al.	-	et alii
etc.	-	et cetera
E	-	enzyme
F_3CCH_2-HN_2	-	trifluoroethylamine
Fe	-	iron
g	-	gram
GC	-	gas chromatography
h	-	hour
H^-	-	hydride ion
H_2N-NH_2	-	hydrazine
H_2N-CH_2CH_2-NH_2	-	ethylenediamine
H_2N-C_6H_4-NH_2	-	p-phenylene diamine
He	-	helium
HNO_2	-	nitrous acid
HON=NOH	-	hyponitrous acid
i.e.	-	id est
IPT	-	isotope pairing technique
KCl	-	potassium chloride
KNO_3	-	potassium nitrate
L	-	liter
n	-	number
N	-	nitrogen
N-N	-	here refers to N_2O and N_2

^{13}N	-	radioactive nitrogen isotope with the molecular mass 13 u
^{14}N	-	stable nitrogen isotope with the molecular mass 14 u
^{15}N	-	stable nitrogen isotope with the molecular mass 15 u
N_2	-	molecular nitrogen or dinitrogen
$^{28}N_2$	-	molecular nitrogen consisting of two ^{14}N atoms (^{14}N-^{14}N)
$^{29}N_2$	-	molecular nitrogen consisting of a ^{14}N and a ^{15}N atom (^{14}N-^{15}N)
$^{30}N_2$	-	molecular nitrogen consisting of two ^{15}N atoms (^{15}N-^{15}N)
N_2H_4	-	hydrazine
N_3^-	-	azide
NH_2	-	amino group
NH_2OH	-	hydroxylamine
NH_3	-	ammonia
NH_4^+	-	ammonium
NH_4NO_3	-	ammonium nitrate
N_2O	-	nitrous oxide
$^{44}N_2O$	-	nitrous oxide consisting of two ^{14}N atoms (^{14}N-^{14}N-^{16}O)
$^{45}N_2O$	-	nitrous oxide consisting of a ^{14}N and a ^{15}N atom (^{14}N-^{15}N-^{16}O)
$^{46}N_2O$	-	nitrous oxide consisting of two ^{15}N atoms (^{15}N-^{15}N-^{16}O)
$N_2O_2^{2-}$	-	hyponitrite anion
N_2O_3	-	dinitrogen trioxide
NO^+	-	nitrosyl cation or nitrosonium ion
NO	-	nitric oxide
NO^-	-	nitroxyl anion
NO_3^-	-	nitrate
NO_2^-	-	nitrite
NO_x	-	mono-nitrogen oxides
m	-	meter
M	-	molarity
min	-	minute
Mn	-	manganese
mol	-	amount of substance
m/z	-	mass-to-charge ratio
^{16}O	-	stable oxygen isotope with the molecular mass 16 u
^{17}O	-	stable oxygen isotope with the molecular mass 17 u
^{18}O	-	stable oxygen isotope with the molecular mass 18 u
O_2	-	molecular oxygen or dioxygen
OH	-	hydroxyl
$^-ON{=}NO^-$	-	hyponitrite
Pa	-	Pascal

pH	-	potentia Hydrogenii
ppm	-	parts per million
QPMS	-	quadrupole mass spectrometry
R-NH_2	-	primary amine
RSD	-	relative standard deviation
sp.	-	species
SPINMAS	-	sample preparation of inorganic nitrogen mass spectrometry
TMPD	-	N,N,N',N'-tetramethyl-p-phenylendiamine
u	-	atomic mass unit
w/w %	-	weight-per-weight in percent
°C	-	degree Celsius
V	-	volt
vol.%	-	volume percent

VII. Appendix

The present doctoral thesis is based upon the following four papers, hereafter given by the respective chapters they are related to:

Chapter 1, 2, and 6 — Spott O., Russow R., Stange C.F., 2011. Formation of hybrid N_2O and hybrid N_2 due to codenitrification: First review of a barely considered process of microbially mediated N-nitrosation. *Soil Biology and Biochemistry.* published online 2nd of July 2011.

Chapter 3 — Spott O., Russow R., Apelt B., Stange C.F., 2006. A ^{15}N-aided artificial atmosphere gas flow technique for online determination of soil N_2 release using the zeolite Köstrolith SX6®. *Rapid Communications in Mass Spectrometry*. 20 (22), p. 3267 – 3274.

Chapter 4 — Spott O., Stange C.F., 2007. A new mathematical approach for calculating the contribution of anammox, denitrification and atmosphere to an N_2 mixture based on a ^{15}N tracer technique. *Rapid Communications in Mass Spectrometry*. 21 (14), p. 2398-2406.

Chapter 5 — Spott O., Stange C.F., 2011. Formation of hybrid N_2O in a suspended soil due to co-denitrification of NH_2OH. *Journal of Plant Nutrition and Soil Science*. published online 26th of January 2011.

1. Introduction and objectives

The nitrogen (N) cycle is one of the most important nutrient cycles in terrestrial environments and includes four main microbiological processes: N_2 fixation, mineralisation, nitrification, and denitrification (Hayatsu et al., 2008). Microbial production of nitrous oxide (N_2O) and molecular nitrogen (N_2) in the course of N cycling was initially supposed to occur only among particular prokaryotic species either via (i) autotrophic nitrification (e.g. by *Nitrosomonas sp.*) or by (ii) heterotrophic denitrification (e.g. by *Pseudomonas sp.*). While the former process was shown to produce N_2O during the aerobic oxidation of ammonia (NH_3) under limited oxygen (O_2) availability (Bremner and Blackmer, 1978; Klemedtsson et al., 1988; Bollmann and Conrad, 1998; Khalil et al., 2004), the latter process was shown to produce both N-N gases (i.e. N_2O and N_2) during a stepwise anaerobic reduction of nitrate (NO_3^-) via nitrite (NO_2^-) and nitric oxide (NO) to N_2O and ultimately N_2 (Knowles, 1982; Zumft, 1997). However, during recent decades many studies have revealed that microbial N_2O and N_2 formation within the biosphere is significantly more complex than originally assumed, inasmuch as it exhibits a much broader diversity of microbial species, possible reaction pathways, and range of N compounds utilised (e.g. eukaryotic nitrification and denitrification, heterotrophic nitrification, denitrification by nitrifying species, or anaerobic ammonium (NH_4^+) oxidation; e.g. Hora and Iyengar, 1960; Bollag and Tung, 1972; Castignetti and Hollocher, 1981; Poth and Focht, 1985; van de Graaf et al., 1990; Shoun et al., 1992; Anderson et al., 1993; Bock et al., 1995; Wrage et al., 2001; Jetten et al., 2005; Müller et al., 2006; Op den Camp et al., 2006; Stange et al., 2009).

Given that our knowledge on microbial N transformations has evolved significantly over the recent decades, Jetten (2008) is correct in stating that even today the microbial world still hides an enormous metabolic capability of N conversion. The intriguing, but less known process of codenitrification appears to perfectly demonstrate his supposition. Codenitrification was first described by Shoun et al. (1992) and Tanimoto et al. (1992) and has been demonstrated to produce N_2O and N_2 in a different manner compared to nitrification and denitrification. According to these two reports codenitrification refers to a co-metabolic process which utilises N compounds (e.g. NH_4^+ or amines) differently to the known denitrification pathway (i.e. $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) yet converts them to N_2O or N_2. By means of a ^{15}N tracer technique they revealed that N-N gas production by codenitrification results in a hybrid N-N species, where the N-N bond originates from a combination of an N atom from NO_2^- and an N atom from a co-metabolised compound. Concomitantly, non-hybrid N-N gas is also formed via the conventional denitrification pathway.

In spite of the unique characteristics found for the codenitrification process (Shoun et al., 1992; Tanimoto et al., 1992) only a few studies have since focused on its elucidation (in particular with respect to microbial N-N gas formation in soils). Most of these were based on cell suspension experiments or were carried out with purified enzymes of denitrifying species

(Shoun et al., 1992; Tanimoto et al., 1992; Kumon et al., 2002; Su et al., 2004; Immoos et al., 2004; Shoun, 2004). Only one codenitrification study has been conducted using soil samples (Laughlin and Stevens, 2002). This is despite the fact that microbial N_2O and N_2 formation due to codenitrification, as defined in 1992 by Tanimoto et al. and Shoun et al., was obviously already known at about the end of the 19th century.

In 1899 Grimbert reported N gas formation by a denitrifying species, where N gas production significantly exceeded the amount of NO_3^--N supplied to a nutrient solution (peptonised broth). It was concluded that the observed surplus of N gas release was caused by amine compounds, which can be utilised in the course of conventional denitrification.

> *"The evolution of nitrogen is no doubt due to a secondary reaction between the nitrous acid produced by the reduction of the nitrate by the bacillus, and the amino-nitrogen contained in the broth or extract,..." Grimbert (1900)*

Half a century later, Allen and van Niel (1952) pointed out that, if an amino group ($-NH_2$) is considered as an additional source of N gas formation during denitrification, then plenty of amines could be considered as possible reactants. Only a few years later Iwasaki et al. (1956; 1958) was able to identify some amine species, which in fact caused "excess" N-N gas production when supplied during denitrification. They concluded that in the denitrifying system an N-N-linkage can occur between the amino group of an amine species and an N compound of the denitrification pathway (e.g. NO_2^-), which then results in the observed "excess" N-N gas production. By means of a ^{15}N tracer technique Garber and Hollocher (1982b) and Kim and Hollocher (1984) confirmed the expected hybrid character of N_2O and N_2 formed by this reaction. It thus appears that these early reports of hybrid N_2O and N_2 production are consistent with the definition of codenitrification later on given by Tanimoto et al. (1992) and Shoun et al. (1992). Even in the light of these more recent findings it is intriguing that this apparently significant microbial process has remained almost unconsidered for over a century. As recently pointed out by Baggs and Philippot (2010) the contribution of codenitrification to soil N_2O (and N_2) release is still today mostly unknown. This substantial gap of knowledge appears to be mainly caused by the following two methodological constraints: (i) the tremendous methodological challenge of studying N_2 formation by microbial processes (see Davidson and Seitzinger, 2006) and (ii) the lack of ^{15}N tracer models, which adequately consider the formation of codenitrified N_2O/N_2 simultaneously with denitrified N_2O/N_2. In order to overcome these deficiencies the present doctoral thesis is aimed at the following objectives:

- to review the fundamentals of hybrid N_2O and hybrid N_2 formation due to codenitrification (Chapter 2)
- to develop a laboratory method for an ^{15}N-aided online determination of N_2O and N_2 gas simultaneously released from a soil sample (Chapter 3)

- to develop a ^{15}N tracer approach for a determination of hybrid N_2O/N_2 formation by codenitrification (Chapter 4)
- to evaluate the applicability of the methodological developments by an experimental study on hybrid nitrogen gas production due to codenitrification (Chapter 5)

2. The process of codenitrification

For more than a century studies have reported co-metabolic formation of N_2O and/or N_2 during conventional denitrification, when particular N compounds (generally amines) are supplied in addition to NO_3^- (Grimbert, 1899; Renner and Becker, 1970; Kumon et al., 2002), NO_2^- (Iwasaki et al., 1956; Iwasaki and Mori, 1958; Pichinot et al., 1969; Renner and Becker, 1970; Matsubara, 1970; Garber and Hollocher, 1982b; Kim and Hollocher, 1984; Aerssens et al., 1986; Weeg-Aerssens et al., 1987/1988; Hulse et al., 1989; Goretski and Hollocher, 1991; Shoun et al., 1992; Tanimoto et al., 1992; Usuda et al., 1995; Kumon et al., 2002; Immoos et al, 2004; Sameshima-Saito et al., 2004), or NO (Aerssens et al., 1986; Ye et al., 1991; Su et al., 2004; Okada et al., 2005), respectively. Because of the similarity between hybrid N-N gas formation via codenitrification and the abiotic process of N-nitrosation (e.g. Stevenson and Swaby, 1964; Bennett et al., 1982; Zollinger, 1994), it was suggested by a number of authors (e.g. Weeg-Aerssens et al., 1988; Shoun et al., 1992; Tanimoto et al., 1992) that the codenitrification process might be based on a microbially mediated (i.e. biotic) N-nitrosation reaction.

2.1. Biochemical fundamentals

2.1.1. Formation of hybrid N_2O and hybrid N_2 by abiotic N-nitrosation reactions

The mechanism of nitrosation belongs to a particular class of electrophilic substitution reactions (Zollinger, 1994; Römpp, 1999). During this reaction a hydrogen atom of a nucleophilic substrate (negatively charged or with a free electron pair) is replaced by a nitroso group (-N=O) supplied by an electrophilic reactant (e.g. the nitrosonium cation NO^+ formed by NO_2^- under acidic conditions). The bonding of the electrophile is realized by the free electron pair of the nucleophile. The remaining positive charge from the electrophile is neutralized by deprotonation. This N-N bonding of two different N species (e.g. NO_2^- and amines) leads to a hybrid N-N linkage.

In this context, abiotic N-nitrosation reactions are already well known to result in the formation of hybrid N_2O as well as hybrid N_2 gas (e.g. Bothner-By and Friedman, 1952; Kainz and Huber, 1959; Bottomley et al., 1974; Bennett et al., 1982; Stamler et al., 1992; Zollinger, 1994/1995; Thorn and Mikita, 2000; Clough et al., 2001; Williams, 2004). The type of N-N gas formed was shown to be largely dependent on the nucleophilic substrate. Hybrid N_2 gas formation mostly occurs due to N-nitrosation reactions with primary amines or amides via a diazo ($R_2C=N_2$) and/or diazonium compound ($R_2C-N_2^+$) as the intermediate species (= diazoniation). The cleavage (= dediazoniation) of the N_2-group can proceed by various ways (e.g. heterolytic dissociation) depending on the current reaction conditions (e.g. temperature, nucleophilic substrate etc.) (Hart, 1989; Zollinger, 1994/1995; Römpp, 1999; Williams, 2004) (Figure 2.1). The formation of hybrid N_2O was demonstrated to occur largely

upon N-nitrosation reactions with oxime compounds (C=N-OH) or hydroxylamine (NH_2OH) (Freeman, 1973; Zollinger, 1995; Brand et al., 2006) (Figure 2.2).

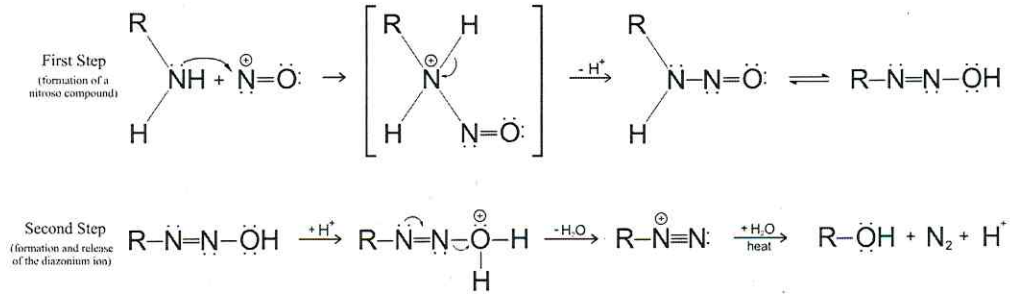

Figure 2.1 *Abiotic formation of hybrid N_2 gas due to an N-nitrosation of a nucleophilic primary amine by the electrophile NO^+ (after Hart, 1989; modified).*

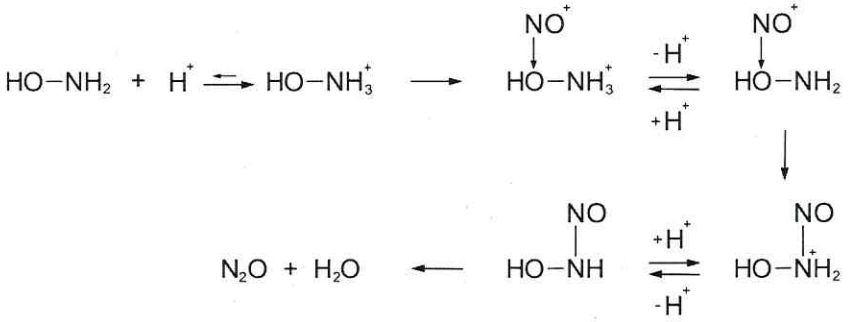

Figure 2.2 *Abiotic formation of hybrid N_2O gas due to an N-nitrosation of NH_2OH by the electrophile NO^+ (after Zollinger, 1988; modified).*

It thus follows that a formation of hybrid N-N gas due to an N-nitrosation reaction generally proceeds in the sense of an N comproportionation. The type of N-N gas which is formed by the reaction with the electrophilic N species (formal oxidation state of N is +3, e.g. NO^+) can then be deduced by the following rules:

i. N_2 is formed, if the formal oxidation state of the nucleophilic N is -3 (e.g. R-NH_2, NH_3)
ii. N_2O is formed, if the formal oxidation state of the nucleophilic N is -1 (e.g. NH_2OH)
iii. N_2O and N_2 are formed, if the formal oxidation state of the nucleophilic N is -2 (e.g. hydrazine)

2.1.2. Biotic N-nitrosation due to an enzymatic catalysis

In abiotic N-nitrosation reactions different N species (e.g. NO^+, N_2O_3, etc., largely formed by NO_2^- under acidic conditions) have been reported to act as free nitrosating agents (Freeman, 1973; Mirvish, 1975; Bosch and Kochi, 1994; Zollinger, 1995; Da Silva et al., 2007). However, concerning biotically mediated N-nitrosation it was suggested (e.g. Kim and Hollocher, 1984; Leach et al., 1987) that free reactants such as those noted above have to be ruled out for the following two reasons. Firstly, at neutral and alkaline pH-values the equilibrium concentration of possible free nitrosating agents is insufficient to permit measurable rates of nitrosation. Secondly, observed biotic nitrosation revealed reactivities rather different from those known for abiotic nitrosating agents.

Both Grimbert (1899) and Allen and van Niel (1952) assumed that during denitrification a reaction of NO_2^- with amino compounds could occur via a biotically mediated catalysis, using particular enzymes of the denitrification pathway. Later on it was concluded by others (e.g. Mills and Alexander, 1976; Kim and Hollocher, 1984; Calmels et al., 1985; Leach et al., 1987; Weeg-Aerssens et al., 1987/1988; Hulse et al., 1989; Ye et al., 1991; Kumon et al., 2002) that this type of reaction originates from a microbially mediated nitrosation process, which proceeds via an enzyme (E) bound mono-N-species the type of E-NO. The proposed enzymatic nitrosyl compound is capable of attracting nucleophilic compounds (e.g. primary amines, NH_2OH) under conditions inappropriate for abiotic nitrosation. As pointed out by Stamler et al. (1992) NO complexes as proposed above (i.e. E-NO^-, E-NO, and E-NO^+) exhibit a varying NO stretching frequency from ca. 1500 to 2000 cm^{-1} (unbound form: NO^+ = 2300 cm^{-1} / NO = 1840 cm^{-1} / NO^- = 1290 cm^{-1}), which fundamentally alters the electrophilic character of bound NO. As reported by Bottomley et al. (1973) NO complexes with a NO stretching frequency greater than ca. 1886 cm^{-1} would be susceptible to attack by nucleophiles. Hence, these species can be in principle considered as electrophilic nitrosating agents. However, as shown by Conelly (1972) NO complexes can also exhibit stretching frequencies between 1500 and 1700 cm^{-1}. In this case they behave formally as a NO^- species (i.e. as nucleophiles) and thus are unable to combine with nucleophiles (e.g. NH_3). Hence, one can conclude that an enzyme bound N species such as E-NO^+ or E-NO, formed via denitrification of NO_3^- or directly delivered by NO_2^- or NO, acts as an electrophilic denitrification intermediate (i.e. nitrosating agent) and attracts nucleophiles in the sense of N-nitrosation reactions.

In fact, both NO_2^- and NO were shown to be directly involved in biotically mediated formation of hybrid N-N gas (e.g. Garber and Hollocher, 1982b; Ye et al., 1991; Su et al., 2004). In addition, it was demonstrated (Garber and Hollocher, 1981; Averill and Tiedje, 1982; Hollocher, 1982; Garber and Hollocher, 1982a; Kim and Hollocher, 1984; Jackson et al., 1991) that under appropriate conditions the reactions between NO_2^- and NO can be assumed as reversible, and thus might explain why the observed biotic N-nitrosation reactions occurred equally in the presence of NO_2^- and NO, respectively. By contrast, the utilisation of

NO_3^- (reported by e.g. Grimbert, 1899; Renner and Becker, 1970; Kumon et al., 2002) was assumed to be only related to its function as a precursor to NO_2^- and NO in the denitrification pathway (Kumon et al., 2002; Okada et al., 2005). Considering both NO_2^- and NO as the nitroso donors responsible, then enzymes such as nitrite reductase (NIR) and nitric oxide reductase (NOR) can be expected to play a role as biotic catalysts in codenitrification. Averill (1996) notes that in many denitrifying species NIRs and NORs appear to be strongly coupled and therefore, may act as multi-enzyme complexes allowing NO_2^- and NO conversion. Until now denitrifying enzymes such as cytochrome cd_1 NIR gained from prokaryotic species (e.g. from *Pseudomonas aeruginosa*) and cytochrome P450 NOR gained from fungi species (e.g. *Fusarium oxysproum*) have been reported to (i) supply an enzyme bound nitrosating agent (e.g. $E\text{-}NO^+$), (ii) perform nitrosation reactions, or (iii) form hybrid N_2O and/or N_2. Analogous to abiotic NO transformations via metal-nitrosyl complexes (e.g. with Fe, Cu, Mo etc.), it was suggested (Garber and Hollocher, 1982b; Kim and Hollocher, 1984; Tolman, 1995; Zhao et al., 1995; Hayton et al., 2002; Wasser et al., 2002) that an enzyme bound nitrosating agent produced during denitrification could be a biotic metal-nitrosyl complex. This assumption was supported by the fact that metal-nitrosyls are known to undergo oxygen exchange reactions, which were in fact also observed for denitrified and simultaneously codenitrified N_2O (Garber and Hollocher, 1982b; Kim and Hollocher, 1984; Ye et al., 1991). It was proposed that an enzymatic iron-nitrosyl compound could act appropriately as a nitrosating agent during denitrification (Averill and Tiedje, 1982; Garber and Hollocher, 1982b; Kim and Hollocher, 1984). In addition, biotic copper-nitrosyl complexes were also reported to act as key intermediates during denitrification (Hulse et al., 1989; Suzuki et al., 1989; Goretski and Hollocher, 1991; Jackson et al., 1991; Ye et al., 1991; Ye et al., 1994; Tolman, 1995; Tocheva et al., 2004).

2.1.3. The dual character of denitrification

Many studies have reported (e.g. Garber and Hollocher, 1981; Averill and Tiedje, 1982; Ye et al., 1991; Ye et al., 1994; Averill, 1996; Wasser et al., 2002; Schmidt et al., 2004a) that during denitrification both NO_2^- and NO can be converted to N_2O by either a parallel or a sequential reaction pathway. During the parallel pathway two NO_2^- or two NO molecules bind simultaneously to one enzyme and form directly a non-hybrid N-N linkage (e.g. Ye et al., 1991; Schmidt et al. 2004a). This reaction scheme prevents in principle a nitrosation reaction with other nucleophiles and thus, excludes the possibility of a simultaneous codenitrification pathway for obvious reasons. During the sequential pathway, however, the N-N linkage is formed by a two step reaction, where one NO_2^- or one NO molecule binds first to an enzyme and a free NO_2^- or NO molecule then reacts with the enzyme bound species to form a non-hybrid N-N bond. Due to its two-step character the sequential scheme permits the occurrence of side reaction, if the first enzyme bound N species acts as an electrophilic N intermediate susceptible to attack by nucleophiles (e.g. amines). Hence, the process of denitrification, when

taking place via the sequential pathway, could in principle exhibit a dual character, where (i) either a non-hybrid N-N linkage is formed by a two step reaction of two NO_2^- or two NO molecules or (ii) a hybrid N-N linkage is formed via the first bound N species due to a side reaction in the sense of N-nitrosation.

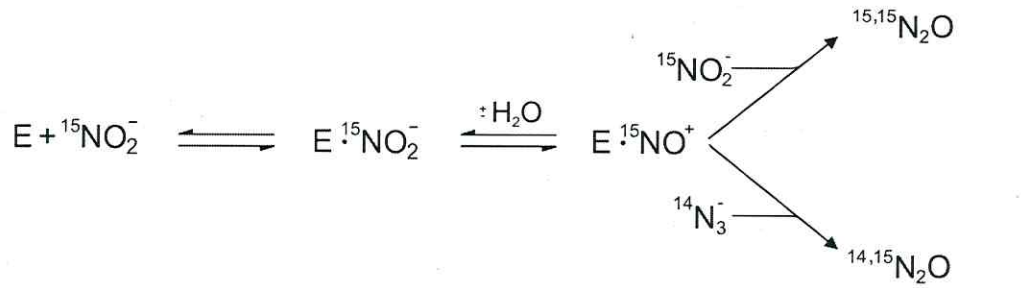

Figure 2.3. *Scheme of the dual character of denitrification, which allows simultaneous occurrence of conventional denitrification and codenitrification (E = enzyme) (after Weeg-Aerssens et al., 1988; modified).*

Concerning biotic N-nitrosation by prokaryotic denitrifying species Weeg-Aerssens et al. (1987/1988) strongly favoured a sequential reaction pathway. They suggested that different nucleophiles (e.g. NO_2^-, N_3^-, and H_2O), supplied during denitrification of NO_2^- compete for an enzyme bound nitrosyl intermediate. Accordingly, the non-hybrid N-N bond is formed via a nucleophilic attack of a second NO_2^- on a coordinated nitrosyl, while the hybrid N-N bond is formed by a competing N-nitrosation reaction with other available N nucleophiles (Figure 2.3). Also Garber and Hollocher (1982b) and Kim and Hollocher (1984) concluded that N-N gas production via prokaryotic denitrification and parallel N-nitrosation occurs largely or entirely via one common enzyme bound precursor. Later on, Goretski and Hollocher (1991) and Ye at al. (1991) demonstrated that during denitrification by prokaryotic species apparently only NO acts as the nitrosyl donor. Wang and Averill (1996) proposed a sequential mechanism for a heme cd_1-containing NIR, which includes the formation of an enzyme bound nitrosyl intermediate with either NO_2^- or NO as the substrate (Figure 2.4). In each case a ferrous heme d_1-NO^+ was assumed to be the key intermediate in the formation of the N-N linkage during denitrification (Averill, 1996). The N-O stretching frequency was found to be around 1910 cm^{-1} (Wang and Averill, 1996) and thus, indicates the electrophilic character of the heme d_1-NO^+ complex (Bottomley et al., 1973). Zumft (1997) pointed out that the redox potential of a heme Fe^{2+}-NO^0/Fe^{2+}-NO^- couple is at -0.9 V, which is too negative to be accessible by a physiological reductant. It was therefore argued that the preferred reaction of a heme Fe^{2+}-NO^0 would be the oxidation to a heme Fe^{2+}-NO^+ and thus, the formation of nitrosating agent. Recently Radoul et al. (2009) in fact proved the formation of a nitrosyl d_1 heme complex (d_1NO) during NO_2^- conversion to NO by a cd_1 NIR of *Pseudomonas aeruginosa*.

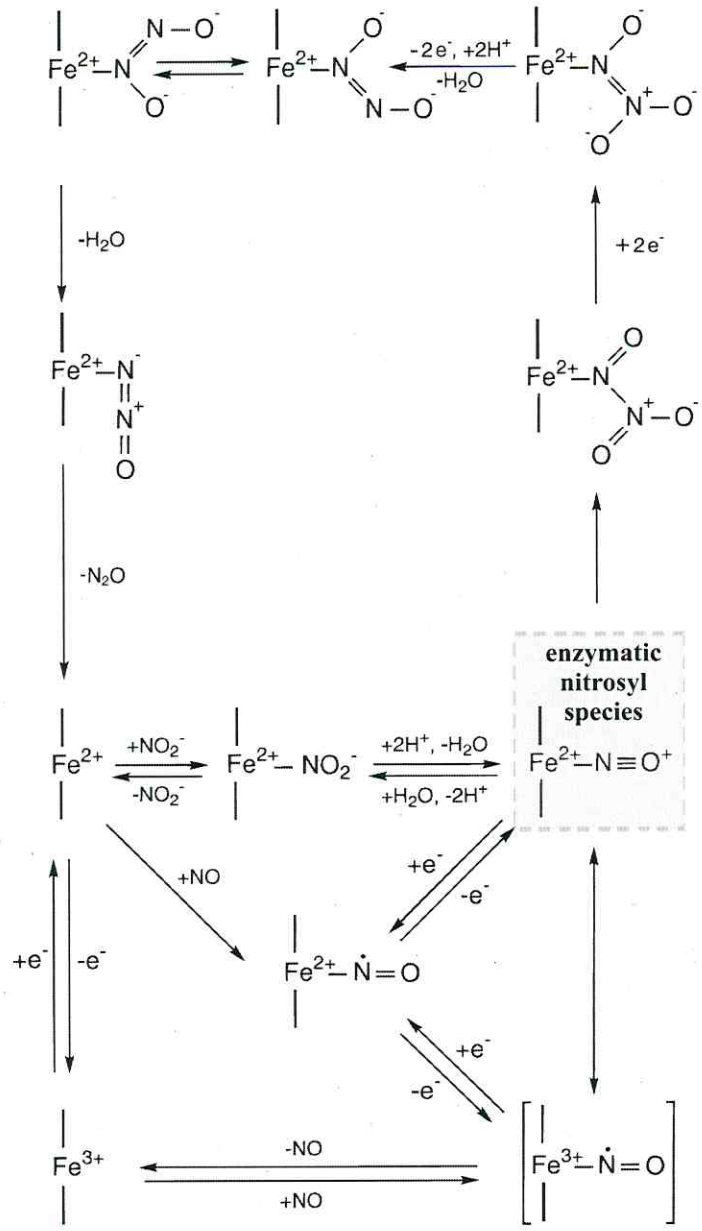

Figure 2.4 *Proposed mechanism for the reduction of NO_2^- to N_2O via an enzyme bound nitrosyl species by a heme cd_1-containing NIR (after Weeg-Aerssens et al., 1988 and Averill, 1996; modified).*

Moreover, Yoshimura et al. (1993) reported a five coordinated nitrosyl-hemoprotein (cytochrome *c'*) during denitrification of NO_3^- by the bacterium *Achromobacter xylosoxidans*. Inasmuch as Fe^{2+}-NO^+ species are highly reactive, the following reactions were proposed by

Averill (1996): (i) the NO^+ species is trapped by a nucleophile (e.g. NO_2^- or N_3^-), (ii) decomposition to NO and Fe^{3+} heme, and (iii) reduction by one electron to a stable heme Fe^{2+}-NO species.

Concerning NO_2^- reduction by Cu-NIRs a similar mechanism with a Cu^+-NO^+ intermediate was proposed by e.g. Averill (1996) (Figure 2.5) and Tocheva et al. (2004). Also Suzuki et al. (1989) found evidence for a cuprous nitrosyl complex (E-Cu^+-NO^+) as an essential intermediate during denitrification of NO by a Cu-NIR (*Achromobacter cycloclastes*). With respect to N_2O production due to denitrification by *A. cycloclastes* similar results were reported by Hulse et al. (1989) and also Goretski and Hollocher et al. (1991). Nonetheless, it was underlined by others (Shimizu et al., 2000; Shoun, 2005; Oshima et al., 2004; Xu et al., 2006; Lehnert et al., 2006; Radoul et al., 2009) that the inherent structure of involved enzymes also distinctively alters the reactivity and hence, the reaction pathways during NO_2^- and NO conversion.

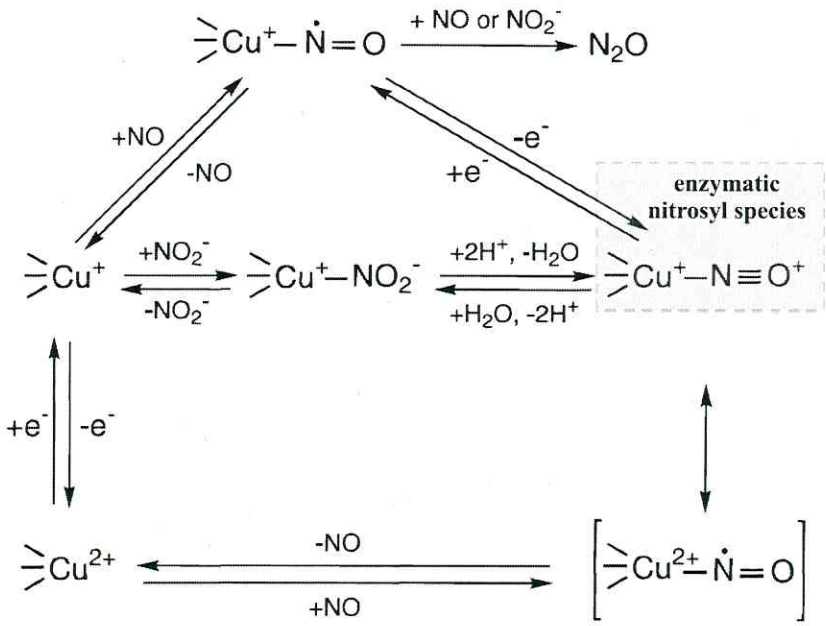

Figure 2.5 *Proposed mechanism for the reduction of NO_2^- to N_2O via an enzyme bound nitrosyl species by a Cu-containing NIR (after Averill, 1996; modified).*

In contrast to prokaryotic N-nitrosation reactions Su et al. (2004) suggested that fungal N-nitrosation might be only related to NO, which is first formed from NO_2^- by a dissimilatory NIR. Nakahara et al. (1993) reported that unlike prokaryotic NORs the responsible fungal cytochrome P450 is obviously a monomer, which contains a single protoheme. In addition,

they pointed out the unlikelihood of two NO molecules binding simultaneously to a single heme. It appears therefore not surprising that the fungal NOR P450 was later on shown to form N_2O by combining two NO molecules in a sequential two step reaction (e.g. Schmidt et al., 2004a).

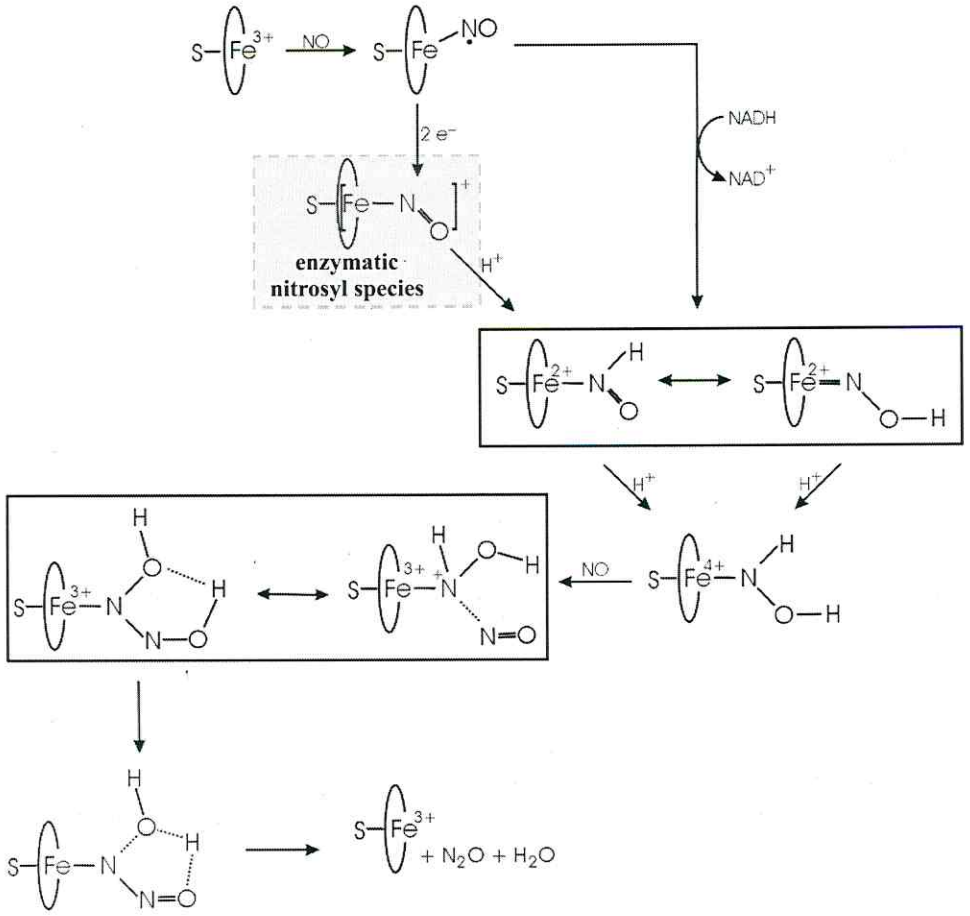

Figure 2.6 *Proposed mechanism for the reduction of NO to N_2O by fungal P450 NOR including the possibility of an enzyme bound NO^+ intermediate (after Lehnert et al., 2006; modified).*

Concerning a possible intermediate compound during NO reduction to N_2O by P450 NOR a Fe-NO complex was assumed (Xu et al., 2006; Lehnert et al., 2006). However, some studies showed (Nakahara et al., 1993; Kobayashi and Shoun, 1995; Shoun, 2005) that P450 NOR is the only heme-protein that can receive electrons directly from the physiological electron donor NADH. It was demonstrated that NO is only reduced to N_2O by P450 NOR in the presence of NADH. During this reaction two electrons are simultaneously transported from NADH to P450 NOR in form of a H^- (Shoun, 2005). Su et al. (2004) revealed that hybrid N_2O

formation takes places when NADH is replaced by a nucleophilic N co-substrate (e.g. NH_4^+). This type of multifunctional character of P450 NOR was also pointed out by Shoun (2005). According to them N_2O formation by fungal denitrification is performed by either (i) combining two NO molecules while receiving the required electrons directly from NADH or by (ii) using a nucleophile which then acts as the electron and N donor. In a similar manner to bacterial denitrification the reduction of NO by the fungal P450 NOR was assumed to include an enzyme bound nitrosyl species (Daiber et al., 2002; Lehnert et al., 2006). Lehnert et al. (2006) proposed that in the presence of NADH an enzyme bound Fe^{4+}-$NOOH^-$ species acts as the intermediate compound, which finally forms non-hybrid N_2O due to biotically mediated dimerization with a free NO molecule. By contrast, in the absence of the NADH an enzyme bound Fe-NO^+ intermediate species can be formed (see Figure 2.6). They suggested that the two-electron reduction by NADH is actually performed to avoid the ferrous nitrosyl complex. However, Daiber et al. (2002) proposed that the direct hydride transfer from NADH to an NO complex suggests that the responsible intermediate species should be predominantly present as a Fe^{2+}-$(NO)^+$ species and thus is prone to H^- acceptance.

2.2. Reports on microbial formation of N_2O and N_2 by codenitrification

Microbes belonging to *bacteria*, *archaea*, and *eukarya* (kingdom *fungi*) domains have been reported to act as codenitrifiers or can be at least assumed as codenitrifying species according to the definition of Tanimoto et al. (1992). In total one archaeal species (order *Sulfolobales*), 12 bacterial species (order *Actinomycetales*, *Burkholderiales*, *Enterobacteriales*, *Pseudomonadales*, *Rhizobiales*, and *Rhodobacterales*), and 3 fungal species (order *Hypocreales*) have been experimentally demonstrated to possess at least the first or both of the following two characteristics associated with codenitrification: (i) N compounds different to NO_3^-, NO_2^-, or NO are co-metabolically utilised during denitrification (e.g. amines) resulting in N_2O and/or N_2 formation, and (ii) the co-metabolically formed N-N gas exhibits a hybrid character. All studies presented here have been shortly summarised in Table 2.1.

2.2.1. Formation of N_2O

Co-metabolic N_2O formation in the sense of codenitrification, as described by Tanimoto et al. (1992), was shown for NH_2OH, N_3^-, NH_4^+, hydrazine (H_2N-NH_2), and salicylhydroxamic acid ($C_7H_7NO_3$). While some studies revealed an N incorporation into gaseous N species by exceeding the amount of available NO_2^--N or NO_3^--N (Iwasaki and Mori, 1958; Renner and Becker, 1970; Matsubara, 1970), others proved the hybrid character of N_2O formed via codenitrification by means of a ^{15}N tracer technique (Garber and Hollocher, 1982b; Kim and Hollocher, 1984; Aerssens et al., 1986; Weeg-Aerssens et al., 1987/1988; Ye et al., 1991; Tanimoto et al., 1992; Su et al., 2004; Immoos et al., 2004).

Table 2.1 *Summary of microbial species and N compounds reported to be involved in N_2O and/or N_2 formation by codenitrification.*

Microbial species	Denitrified compound	Co-metabolised compound	Determined N-gas	Hybrid N-N gas	Reference
Sulfolobus solfataricus	NO_2^-	ammonium	N_2O	N_2O	Immoos et al. (2004)
Corynebacterium nephridii	NO_3^-, NO_2^-	hydroxylamine	NO, N_2O	no ^{15}N [d]	Renner and Becker (1970)
Streptomyces antibioticus	NO_3^-, NO_2^-	amino compound [c]	N_2O, N_2	N_2	Kumon et al. (2002)
Achromobacter cycloclastes [e]	NO_2^-	hydroxylamine	N_2O	N_2O	Hulse et al. (1989)
	NO_2^-	azide	N_2O	N_2O	
Achromobacter cycloclastes	NO_2^-	azide	NO, N_2O	N_2O	Goretski and Hollocher (1991)
Escherichia coli	NO_3^-	amino compound [c]	total N gas	no ^{15}N [d]	Grimbert (1899)
Salmonella typhi	NO_3^-	amino compound [c]	total N gas	no ^{15}N [d]	Grimbert (1899)
Pseudomonas aeruginosa [e]	NO_2^-	p-phenylene diamine	N_2	no ^{15}N	Pichinot et al. (1969)
	NO_2^-	dimethyl-p-phenylene diamine	N_2	no ^{15}N	
Pseudomonas aeruginosa [b]	NO_2^-	azide	NO, N_2O, N_2	N_2O	Kim and Hollocher (1984)
	NO_2^-	hydroxylamine	NO, N_2O, N_2	N_2O	
	NO_2^-	hydrazine	NO, N_2O, N_2	N_2O, N_2	
	NO_2^-	p-methoxyaniline	NO, N_2O, N_2	N_2	
	NO_2^-	trifluoroethylamine	NO, N_2O, N_2	N_2	
	NO_2^-	ethylendiamine	NO, N_2O, N_2	N_2	

Microbial species	Denitrified compound	Co-metabolised compound	Determined N-gas	Hybrid N-N gas	Reference
Pseudomonas aeruginosa [b]	NO_2^-	aniline	NO, N_2O, N_2	N_2	Kim and Hollocher (1984)
	NO_2^-	ammonia	NO, N_2O, N_2	N_2	
Pseudomonas chlororaphis	NO_2^-	hydroxylamine	NO, N_2O [a]	N_2O [a]	Garber and Hollocher (1982b)
	NO_2^-	azide	NO, N_2O [a]	N_2O [a]	
Pseudomonas denitrificans	NO_2^-	p-phenylene diamine	NO, N_2	no ^{15}N [d]	Iwasaki et al. (1956)
	NO_2^-	dimethyl-p-phenylene diamine	NO, N_2	no ^{15}N [d]	
	NO_2^-	hydroxylamine	N_2O, N_2	no ^{15}N [d]	Iwasaki and Mori (1958)
	NO_2^-	hydroxylamine	NO, N_2O, N_2	no ^{15}N [d]	Matsubara (1970)
	NO_2^-	dimethyl-p-phenylene diamine	N_2	no ^{15}N [d]	
	NO_2^-	hydroxylamine	NO, N_2O [a]	N_2O [a]	Garber and Hollocher (1982b)
	NO_2^-	azide	NO, N_2O [a]	N_2O [a]	
Pseudomonas stutzeri	NO_2^-	hydroxylamine	NO, N_2O [a]	N_2O [a]	Garber and Hollocher (1982b)
	NO_2^-	azide	NO, N_2O [a]	N_2O [a]	
	NO_2^-/NO	hydroxylamine	NO, N_2O	N_2O	Aerssens et al. (1986)
	NO_2^-	azide	NO, N_2O	N_2O	Goretski and Hollocher (1991)
Pseudomonas stutzeri [e]	NO_2^-	azide	N_2O	N_2O	Weeg-Aerssens et al. (1987/1988)

Microbial species	Denitrified compound	Co-metabolised compound	Determined N-gas	Hybrid N-N gas	Reference
Mesorhizobium sp.	NO	amino compound [c]	N_2O, N_2	N_2	Okada et al. (2005)
Paracoccus denitrificans	NO_2^-	p-phenylene diamine	N_2	no ^{15}N	Pichinot et al. (1969)
	NO_2^-	dimethyl-p-phenylene diamine	N_2	no ^{15}N	
	NO_2^-	hydroxylamine	NO, N_2O, N_2	N_2O	Garber and Hollocher (1982b)
	NO_2^-	azide	NO, N_2O [a]	N_2O [a]	
Rhodobacter sphaeroides [e]	NO	azide	N_2O	N_2O	Ye et al. (1991)
Cylindrocarbon tonkinense	NO_2^-	amino compound [c]	N_2O, N_2	N_2	Shoun et al. (1992)
	NO_2^-	azide	N_2O	no ^{15}N	Usuda et al. (1995)
Fusarium oxysporum	NO_2^-	azide	NO, N_2O, N_2	N_2O	Tanimoto et al. (1992)
	NO_2^-	salicylhydroxamic acid	N_2O	N_2O	
	NO_2^-	ammonium	N_2O	N_2O	
	NO	azide	N_2O	N_2O	Su et al. (2004) [f]
	NO	ammonium	N_2O	N_2O	
Fusarium solani	NO_2^-	amino compound [c]	N_2O, N_2	N_2	Shoun et al. (1992)
	NO_2^-	amino compound [c]	N_2	N_2	Sameshima-Saito et al. (2004)

a – N_2 formation was inhibited by acetylene; b – experiments with purified dissimilatory nitrite reductase (cytochrome *cd*$_1$) of *P. aeruginosa* (Kim and Hollocher, 1984); c – unspecified amino compound contained in the peptone solution; d – N gas production exceeded the amount of supplied N substrate; e – experiments were conducted with crude or cell free extracts; f – experiments with recombinant P450 NOR produced in *Escherichia coli* and then purified

Hydroxylamine (NH_2OH) – It was first shown by Iwasaki and Mori (1958) that the denitrifier *Pseudomonas denitrificans* can co-metabolically utilise NH_2OH during denitrification. An "excess" N-N gas formation was observed when *P. denitrificans* was anaerobically incubated with NO_2^-, NH_2OH, and lactate (as electron donor). The volume of evolved N gas was 1.7 times as much as that produced using only NO_2^- and lactate. When lactate was omitted N gas evolution was even higher and corresponded to 2.1 - 2.2 times that of a nitrite-lactate-system. In the presence of NO_2^- and NH_2OH (without lactate) N_2O was almost exclusively produced with only little N_2. Matsubara (1970) subsequently reported similar results with *P. denitrificans*. Without lactate only N_2O was produced, which accounted for the N contained in NH_2OH plus NO_2^-. In the presence of lactate, however, N_2 and NO were produced simultaneously. Furthermore, N gas production increased with an increasing NH_2OH concentration. At the highest concentration (25 mM) the total N gas produced increased by a factor of 1.7 compared to the N contained in available NO_2^-. Under these conditions mainly N_2O and NO was produced (almost no N_2 was formed). The author concluded that NH_2OH tends to favour N_2O formation and furthermore, inhibits the total reduction of NO and N_2O. Renner and Becker (1970) reported a co-metabolic utilisation of NH_2OH by *Corynebacterium nephridii.* Under anaerobic conditions total N gas production by resting cells of *C. nephridii* clearly exceeded the total amount of N available in form of NO_2^-. Moreover, they proved that N gas evolution did not occur when *C. nephridii* was incubated with only NH_2OH. When NO_3^- was used instead of NO_2^- the same co-metabolic utilisation of NH_2OH has been observed. It has also been demonstrated that increasing the amount of NH_2OH (10 to 5000 µmol) affects N-N gas production. First, total N gas evolution increased and second, N gas production was simultaneously shifted from a NO/N_2O ratio of around 1:1 to solely N_2O. By means of a ^{15}N tracer technique it was first demonstrated by Garber and Hollocher (1982b) that substantial amounts of a hybrid $^{14,15}N_2O$ are formed, when a denitrifying bacterial species (see Table 2.1) is anaerobically incubated with $^{15}NO_2^-$ (99 at%) and NH_2OH (please note that in all experiments acetylene was used to inhibit N_2O reduction). Moreover, hybrid N_2O production increased with increasing NH_2OH concentration. However, as indicated by a decrease in total N_2O production, NH_2OH application caused a general inhibition of the denitrification process. Moreover, all incubated bacterial species revealed substantial ^{15}NO production in the presence of NH_2OH, which was interpreted as an evidence for incomplete denitrification. This was additionally supported by the fact that denitrification inhibitors (antimycin A and retenone) distinctively diminished NO evolution, while simultaneously produced hybrid N_2O remained unaffected. Hence, NO was apparently formed as an intermediate or abortive product of denitrification of NO_2^- and not as a side product of the nitrosation reaction. An additional application of $H_2{}^{18}O$ revealed an incorporation of H_2O-O into denitrified non-hybrid N_2O and codenitrified hybrid N_2O (Figure 2.7). It was therefore concluded that the catalyzed N-nitrosation reaction with NH_2OH proceeds via a symmetrical

dinitrogen intermediate with the molecular formula $N_2H_2O_2$ (e.g. hyponitrous acid) analogue to chemical reactions under mildly acidic conditions as reported elsewhere (see Figure 2.2).

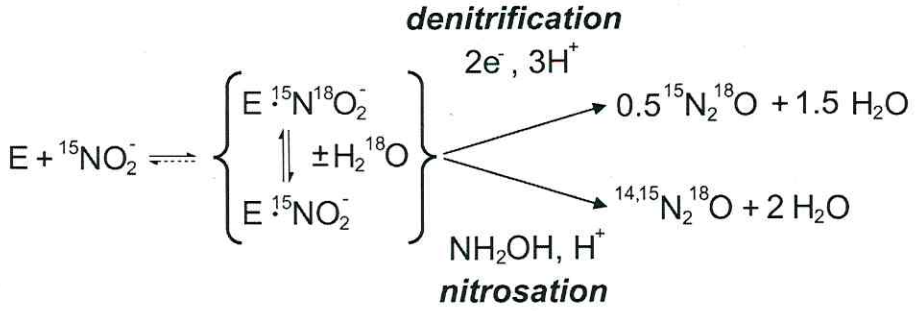

Figure 2.7 *Scheme for a co-metabolic utilisation of NH_2OH during denitrification of NO_2^- by a denitrifying bacterial species (E = enzyme) (after Garber and Hollocher, 1982b).*

^{15}N tracer experiments with NH_2OH were also reported by Kim and Hollocher (1984), Aerssens et al. (1986) and Hulse et al. (1989). The first study revealed hybrid $^{14,15}N_2O$ formation, when a dissimilatory nitrite reductase (cytochrome cd_1 purified from *Pseudomonas aeruginosa*) was incubated with $^{15}NO_2^-$ (99 at%) and NH_2OH. Later on Aerssens et al. (1986) demonstrated hybrid $^{14,15}N_2O$ formation by the denitrifying species *Pseudomonas stutzeri*, when it was anaerobically incubated either with ^{15}NO (>99.9 at%) and NH_2OH or with $^{15}NO_2^-$ (>99.9 at%) and NH_2OH. They showed that NO reduction to non-hybrid denitrified N_2O was completely inhibited by NH_2OH concentrations ≥10 mM and hence, only hybrid $^{14,15}N_2O$ production occurred. By contrast, NO_2^- reduction to non-hybrid denitrified N_2O was not significantly affected up to a maximum NH_2OH concentration of 320 mM, while hybrid $^{14,15}N_2O$ production remained constantly low. It was therefore concluded that with only NO as the source of the enzymatic nitrosyl intermediate considerable competition with NH_2OH takes place. Hulse et al. (1989) reported hybrid $^{14,15}N_2O$ production by a Cu-NIR of *Achromobacter cycloclastes* in the presence of $^{15}NO_2^-$ and NH_2OH under anaerobic conditions. Increasing amounts of NH_2OH completely stopped denitrification, while the remaining N_2O production by only N-nitrosation was significantly enhanced.

Azide (N_3^-) – It was first reported by Garber and Hollocher (1982b) that hybrid $^{14,15}N_2O$ can be produced by denitrifying bacterial species (see Table 2.1), when $^{15}NO_2^-$ (99 at%) and N_3^- are simultaneously supplied as the N substrate (please note that in all experiments acetylene was used to inhibit N_2O reduction). The N_3^- compound delivers one N atom to the enzyme-bound nitrosyl species resulting in hybrid N_2O formation, while N_2 gas is simultaneously released as the residual of this reaction. All tested microbial species revealed an inhibitory

effect of N_3^- on denitrification rates as indicated by a decrease of total N_2O production. A simultaneous increase of ^{15}N enriched NO was also detected and interpreted as an abortive denitrification product. Use of $H_2{}^{18}O$ treatments revealed the incorporation of H_2O-O into denitrified non-hybrid N_2O and codenitrified hybrid N_2O (see Figure 2.7). Kim and Hollocher (1984) reported hybrid $^{14,15}N_2O$ formation by a dissimilatory nitrite reductase (cytochrome cd_1 type purified from *Pseudomonas aeruginosa*) when it was anaerobically incubated with $^{15}NO_2^-$ (99 at%) and N_3^-. The overall reaction was dependent on a reducing system (ascorbate and TMPD), which delivers electrons to the enzyme. A high N_3^- concentration (maximally 50 mM) had only little effect on $^{15}NO_2^-$ reduction, but caused an increase of $^{14,15}N_2O$ production. Likewise Garber and Hollocher (1982b) they found that *Pseudomonas aeruginosa* produces ^{15}NO, $^{15,15}N_2O$, and $^{14,15}N_2O$ species which are similarly enriched in ^{18}O (gained from $H_2{}^{18}O$). Weeg-Aerssens et al. (1987/1988) reported hybrid $^{14,15}N_2O$ formation in a cell-free crude extract of *Pseudomonas stutzeri* when $^{15}NO_2^-$ and N_3^- were delivered under anaerobic conditions. Use of $H_2{}^{18}O$ revealed a pathway dependent ^{18}O equilibration within gaseous N products. While N_2O from denitrification of NO_2^- was 52.7 ±2 % equilibrated with ^{18}O, hybrid N_2O gained from N-nitrosation of N_3^- was 80.5 ±2.4 % equilibrated. This was interpreted as a sequential reaction pathway in which free NO_2^- without ^{18}O dilutes the ^{18}O content of the enzyme-bound nitrosyl, but does not affect the N-nitrosation with N_3^-. Hulse et al. (1989) demonstrated hybrid $^{14,15}N_2O$ formation by a Cu-NIR of *Achromobacter cycloclastes* when $^{15}NO_2^-$ and N_3^- are supplied. An increasing N_3^- supply, however, steadily decreased total N_2O production by denitrification and N-nitrosation. They suggested that N_3^- inhibits the formation of the nitrosyl intermediate. Similar to the latter study Goretski and Hollocher (1991) reported hybrid $^{14,15}N_2O$ formation when *Achromobacter cycloclastes* or *Pseudomonas stutzeri* has been anaerobically incubated with $^{15}NO_2^-$ and N_3^-. The addition of N_3^- decreased N-N gas release rate and in addition, caused a considerable release of ^{15}NO as a result of NOR inhibition by N_3^-. When ^{15}NO accumulation was inhibited (using a chemical NO trap) formation of hybrid N_2O by nitrosation of N_3^- did not occur at all, while N_2O formation by denitrification took place without any observable interference. Ye et al. (1991) revealed a hybrid $^{14,15}N_2O$ formation by a crude extract of *Rhodobacter sphaeroides* when anaerobically incubated with ^{15}NO (99 at%) and N_3^-. Application of $H_2{}^{18}O$ revealed that denitrified non-hybrid and codenitrified hybrid N_2O exhibited comparable ^{18}O incorporation.
Besides bacterial species Tanimoto et al. (1992) revealed a codenitrification of N_3^- by the denitrifying fungus *Fusarium oxysporum* with NO_2^- as the denitrification substrate. The presence of N_3^- caused a short lag phase in the N_2O production, but ultimately resulted in a rapid N_2O and N_2 production. Nitrogen gas formation significantly exceeded the amount of added NO_2^--N, but was found to be equal to the total amount of available N (i.e. $NO_2^- + N_3^-$). Su et al. (2004) reported a hybrid $^{14,15}N_2O$ formation by a P450 NOR of *F. oxysporum* when it was anaerobically incubated with ^{15}NO and N_3^-. Usuda et al. (1995) reported that the fungus *Cylindrocarbon tonkinense* shows suppressed cell growth when N_3^- is applied during the

denitrification of NO_2^-. However, since no concomitant inhibition of N_2O formation was observed, it was concluded that a large fraction of the N_2O might have been evolved due to an energy intensive codenitrification.

Ammonium (NH_4^+) – Tanimoto et al. (1992) showed that hybrid $^{14,15}N_2O$ was produced by the fungus *Fusarium oxysporum* when it was anaerobically incubated with NH_4^+ and $^{15}NO_2^-$. Although their results were not as conclusively as with other N compounds, it did prove the hybrid N_2O species evolved from a combination of a NO_2^--N and NH_4^+-N. Su et al. (2004) reported hybrid $^{14,15}N_2O$ formation by the P450 NOR of *F. oxysporum* when anaerobically incubated with ^{15}NO and NH_4^+. When NO was omitted no N_2O formation occurred. Immoos et al. (2004) revealed the formation of hybrid $^{14,15}N_2O$ during NO_2^- reduction by a NOR of *Sulfolobus solfataricus* (type of cytochrome P450) via the co-metabolic utilisation of $^{15}NH_4^+$. At an initial state only NH_4^+ was produced upon NO_2^- reduction, while in the course of ongoing incubation the efficiency significantly decreased. It was proven that NH_4^+ (if solution-based) formed by the reduction of NO_2^- can be reutilised for a simultaneous hybrid N_2O production. It was furthermore assumed that a hyponitrite anion ($N_2O_2^{2-}$) may act as an intermediate species during codenitrification of NH_4^+ to hybrid N_2O.

Hydrazine (N_2H_4) – A co-metabolic utilisation of N_2H_4 during denitrification has only been reported by Kim and Hollocher (1984). They showed that a dissimilatory NIR (cytochrome cd_1 purified from *Pseudomonas aeruginosa*) formed hybrid N_2O (and hybrid N_2) when it was anaerobically incubated with $^{15}NO_2^-$ and N_2H_4. Based on the chemical nitrosation analogue under acidic conditions (see Biddle and Miles, 1968; Perrott et al., 1976) it was suggested that biotic nitrosation of hydrazine during denitrification of NO_2^- should actually undergo a two step reaction (Figure 2.8). First, a nitrosation of N_2H_4 with $^{15}NO_2^-$ yields ^{15}N labelled N_3^- ($^{15,14,14}N_3^-$), hybrid N_2O ($^{14,15}N_2O$) and $^{14}NH_4^+$. Second, the ^{15}N labelled N_3^- again undergoes a nitrosation reaction with $^{15}NO_2^-$ resulting in either non-hybrid $^{15,15}N_2O$ and hybrid $^{14,15}N_2$ or hybrid $^{14,15}N_2O$ and non-hybrid $^{14,14}N_2$. The remaining NH_4^+ was assumed to be relatively stable under neutral pH.

Salicylhydroxamic acid ($C_7H_7NO_3$) – A co-metabolic utilisation of $C_7H_7NO_3$ during denitrification was only reported by Tanimoto et al. (1992). The fungus *Fusarium oxysporum* produced hybrid $^{14,15}N_2O$ under anaerobic conditions when $C_7H_7NO_3$ and $^{15}NO_2^-$ were simultaneously supplied. The presence of $C_7H_7NO_3$ caused a short lag phase followed by a rapid N_2O formation. The total amount of N_2O evolved during incubation was equal to the amount of N contained in NO_2^- plus $C_7H_7NO_3$. By contrast, absolutely no gas evolution occurred when only $C_7H_7NO_3$ was supplied to the fungus.

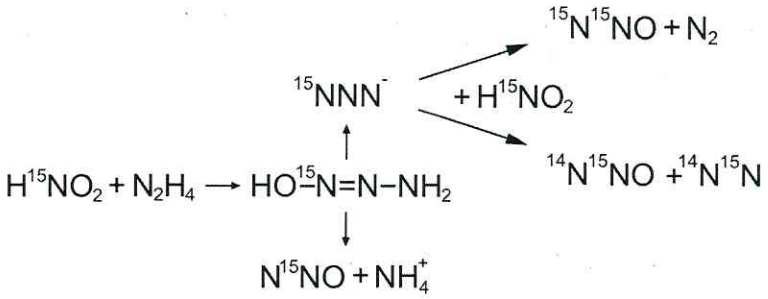

Figure 2.8 *Proposed reaction scheme for a co-metabolic utilisation of N_2H_4 during denitrification of NO_2^- (after Kim and Hollocher, 1984).*

2.2.2. Formation of N_2

Dinitrogen formation via codenitrification, as described by Tanimoto et al. (1992), was shown by means of ^{15}N tracer experiments for the N species NH_3, hydrazine (H_2N-NH_2), p-methoxyaniline (CH_3O-C_6H_5-NH_2), trifluoroethylamine (F_3CCH_2-HN_2), ethylenediamine (H_2N-CH_2CH_2-NH_2), and aniline (C_6H_5-NH_2) (Kim and Hollocher, 1984). A few authors also reported hybrid N_2 formation related to unspecified amino compounds contained in microbial nutrient solutions (Shoun et al., 1992; Kumon et al., 2002; Sameshima-Saito et al., 2004; Okada et al., 2005). In addition, hybrid N_2 formation has also been suggested to occur with respect to p-phenylene diamine (H_2N-C_6H_4-NH_2) and dimethyl-p-phenylene diamine (C_2H_6N-C_6H_4-NH_2) (Iwasaki et al., 1956; Matsubara, 1970).

Ammonia (NH_3) – Only Kim and Hollocher (1984) reported the formation of hybrid N_2 by a co-metabolic utilisation of NH_3. Hybrid $^{14,15}N_2$ evolved when a dissimilatory NIR (cytochrome cd_1 purified from *Pseudomonas aeruginosa*) was anaerobically incubated with $^{15}NO_2^-$ and NH_3.

Hydrazine (N_2H_4) – As already discussed in the previous section hybrid N_2 can be formed simultaneously with hybrid N_2O due to a two step N-nitrosation of N_2H_4 (Kim and Hollocher, 1984) (see Figure 2.8).

Amino compounds – It was proven in some studies (Iwasaki et al., 1956; Pichinot et al., 1969; Matsubara, 1970; Kim and Hollocher, 1984) that hybrid N_2 formation occurred with aliphatic or aromatic amine or diamine compounds. With respect to aliphatic species, Kim and Hollocher (1984) reported that under anaerobic conditions a dissimilatory NIR (cytochrome cd_1 purified from *Pseudomonas aeruginosa*) produced hybrid $^{14,15}N_2$ when $^{15}NO_2^-$ (99 at%) was applied simultaneously with ethylenediamine (H_2N-CH_2CH_2-NH_2) or trifluoroethylamine (F_3CCH_2-NH_2). Analogous to abiotic N-nitrosation reactions (see chapter 2.1.1) it was assumed that a diazonium intermediate was formed, where the alkyl diazonium precursor is expected to hydrolyse rapidly to form N_2. They also reported the formation of hybrid $^{14,15}N_2$

in the presence of $^{15}NO_2^-$ (99 at%) and aromatic amine species such as aniline (C_6H_5-NH_2) or p-methoxyaniline (CH_3O-C_6H_5-NH_2). The reaction was assumed to be similar to the observed N-nitrosation of aliphatic amines. Considering aromatic diamine compounds Iwasaki et al. (1956) and Matsubara (1970) suggested hybrid N_2 formation under denitrifying conditions, also by *Pseudomonas denitrificans*, due to a reaction of NO_2^- with p-phenylene diamine (H_2N-C_6H_4-NH_2) or dimethyl-p-phenylene diamine (C_2H_6N-C_6H_4-NH_2), respectively. Iwasaki et al. (1956) showed that in the presence of p-phenylene diamine (H_2N-C_6H_4-NH_2) reduction of NO_2^- by *P. denitrificans* resulted in "excess" N gas production (mostly N_2 and a little NO) corresponding to 1.8 times that of the available N in the form of NO_2^-. Concerning dimethyl-p-phenylene diamine they observed "excess" N gas production (mostly N_2 and a little NO) corresponding to 1.6 times that of the available N in the form of NO_2^-. Matsubara (1970) incubated *P. denitrificans* anaerobically with NO_2^- and dimethyl-p-phenylene diamine in the presence or absence of lactate. In each case only N_2 was produced, but N_2 formation was increased when lactate was additionally applied. They concluded that at first NO is produced from NO_2^- by the denitrifying enzyme with a cytochrome c-552 and dimethyl-p-phenylene diamine as the hydrogen donor. Afterwards NO is converted to N_2 by reacting with dimethyl-p-phenylene via diazoniation and dediazoniation (see chapter 2.1.1). Similar results with p-phenylene diamine and dimethyl-p-phenylene diamine have been reported by Pichinot et al. (1969) concerning the biotic N-nitrosation by the bacterial species *Paracoccus denitrificans* and *Pseudomonas aeruginosa*.

Shoun et al. (1992) observed that the fungi *Fusarium solani* and *Cylindrocarpon tonkinense* produce hybrid $^{14,15}N_2$, when they are anaerobically incubated with $^{15}NO_2^-$ (99 at%). When peptone was omitted from the culture medium N_2 was formed not at all. By contrast, NO and N_2O were produced with NO_2^- as the only N source. Sameshima-Saito et al. (2004) observed hybrid $^{14,15}N_2$ formation also by *F. solani*, when cultured in the presence of $^{15}NO_2^-$ (99 at%) according to the method of Shoun et al. (1992) and Tanimoto et al. (1992). Kumon et al. (2002) demonstrated hybrid N_2 formation by the *Streptomyces antibioticus* when anaerobically incubated with $^{15}NO_3^-$ (99 at%). However, since N-N gas formation continued long after NO_3^- was completely consumed, it was assumed that hybrid N_2 formation was apparently directly related to NO_2^- or NO (evolved from NO_3^- and NO_2^- reduction, respectively). Besides hybrid $^{14,15}N_2$ small amounts of denitrified non-hybrid $^{15,15}N_2$ were also simultaneously released. They concluded that the unlabelled N of hybrid $^{14,15}N_2$ originated from N compounds contained in peptone. Moreover, simultaneously produced N_2O only occurred due to conventional denitrification. In contrast to all former studies, it was demonstrated by Okada et al. (2005) that even in the presence of O_2 hybrid N_2 production by codenitrification occurs. They showed that an aerobic denitrification of $^{15}NO_2^-$ (99 at%) by a *Mesorhizobium sp.* resulted in a formation of codenitrified hybrid $^{14,15}N_2$ and denitrified non-hybrid $^{15,15}N_2O$. It was suggested that the unlabelled N of the hybrid $^{14,15}N_2$ originated from

peptone and yeast extract (i.e. amino compounds). By contrast, under anaerobic conditions only non-hybrid $^{15,15}N_2O$ was formed, while N_2 was not produced at all.

2.3. Controlling factors of codenitrification

In principle it can be assumed that the process of codenitrification is controlled by environmental constraints already recognized as controllers of denitrification (see e.g. Knowles, 1982; Zumft, 1997). Hence, an occurrence of codenitrification within the biosphere appears to be mainly controlled by (i) O_2 availability, (ii) pH, and (iii) availability of respirable organic carbon substrates. In addition, it can be assumed that codenitrification will be dependent on (iv) the type of nucleophiles and their reaction kinetics as well as (v) the capability of denitrifying species to perform codenitrification.

2.3.1. O_2 availability

Since codenitrification is dependent on the microbial reduction of NO_3^-, NO_2^-, and/or NO in the course of denitrification it can be expected to be largely restricted to anaerobic conditions. Nevertheless, as pointed out by e.g. Zumft (1997) some microbial species appear to denitrify even in the presence of O_2. A few studies (Zhou et al., 2001; Zhou et al., 2002; Takaya et al., 2003b) revealed that for fungal species (e.g. *Fusarium oxysporum*) a low O_2 level appears to be even required for denitrification. Takaya (2002) supposed that fungal denitrification was apparently a hypoxic metabolism inasmuch as it still proceeds at low O_2 concentrations. However, the phenomenon of aerobic denitrification has also been reported for bacterial species (e.g. *Pseudomonas stutzeri*) (Takaya et al., 2003a). Concerning codenitrification one study (Kumon et al., 2002) in fact proved that under low O_2 conditions (2 Vol.%) hybrid N-N gas production could take place (based on the *actinomycete Streptomyces antibioticus*). Moreover, it was demonstrated by Okada et al. (2005) that a *Mesorhizobium sp.* produced hybrid N_2 during denitrification even under nearly full atmospheric O_2 tension. Furthermore, Ralt et al. (1988) found that e.g. *E. coli* performed nitrosation of 2,3-diaminonaphtalene with NO_2^- equally efficiently under aerobic and anaerobic conditions. Nonetheless, one can finally assume that within environments characterised by variable O_2 supply and a heterogeneous O_2 distribution (e.g. soils) codenitrification will be significantly triggered by the impact of O_2, i.e. will tend to diminish with increasing O_2 availability and tend to rise with decreasing O_2 concentration.

2.3.2. pH value

It was shown in some studies that biotically mediated nitrosation occurs in principle at pH values around neutral conditions (Calmels et al., 1985; Leach et al., 1987; Smith and Smith, 1992). By contrast, abiotic acid catalysed nitrosation reaction via e.g. HNO_2 occurs on its optimum between 2 to 3 pH (Mirvish, 1975). Kim and Hollocher (1984) reported that at pH 7.5 an abiotic N-nitrosation of N_3^- by HNO_2 was not observable, while it was readily

detectable in the presence of a NIR of *Pseudomonas aeruginosa*. A similar result was reported by Calmels et al. (1985) concerning nitrosation of morpholine to N-nitrosomorpholine. Nonetheless, they also pointed out that biotic and abiotic nitrosation can occur simultaneously depending on the current pH conditions (e.g. at weakly acidic pH). Leach et al. (1987) reported that biotic nitrosation of morpholine by *P. aeruginosa* occurred at an optimum between pH 8 to 8.5. Ralt et al. (1988) demonstrated that biotic nitrosation of 2,3-diaminonaphtalene with NO_2^- by *E. coli* was indistinguishable from chemical nitrosation below pH 6, but 10 to 100 times faster than the chemical nitrosation at pH 7.3. Smith and Smith (1992) assumed that a bacterial catalysed N-nitrosation occurs over a pH-range of about 6 to 9. This is in good agreement with the pH values of all studies summarised in Table 2.1 which ranged between 6.0 and 7.5. Thus, one can conclude that biotic nitrosation (i.e. codenitrification) in the biosphere will be restricted to environments which exhibit pH values from weakly acidic to weakly alkaline conditions.

2.3.3. Respiratory substrates

Most denitrifiers represent aerobic heterotrophic organisms that under anaerobic conditions transfer redox equivalents from organic carbon sources to the respective N oxides of the denitrification chain (Zumft, 1997). Thus, denitrification is depending on the availability of respirable organic carbon substrates. For example, denitrification activity in soil has been already proven to be highly correlated with the amount of water-soluble organic carbon or mineralisable carbon (e.g. Burford and Bremner, 1975; deCatanzaro and Beauchamp, 1985). It can be therefore assumed that codenitrification is in principle also dependent on organic carbon respiration. Nonetheless, it has also been reported elsewhere (Iwasaki and Mori, 1958; Pichinot et al., 1969; Matsubara, 1970; Garber and Hollocher, 1982b; Su et al., 2004; Shoun, 2005) that some co-metabolisable nucleophiles do apparently act as both electron and nitrogen donor. Pichinot et al. (1969) demonstrated that extracts of *Paracoccus denitrificans* and *Pseudomonas aeruginosa* can catalyze the reduction of NO_2^- to hybrid N_2 even in the absence of strong organic electron donator (such as glucose or lactate) when only a nucleophilic amine species like p-phenylene diamine is delivered. Similar results have also been reported with respect to the formation of hybrid N_2O by codenitrification of NH_2OH (Iwasaki and Mori, 1958; Matsubara, 1970; Garber and Hollocher, 1982b). Su et al. (2004) demonstrated that the fungal P450 NOR catalyzes the N-nitrosation of N_3^- or NH_4^+ (with NO as the nitrosyl donor) in the absence of the commonly utilised electron donor NADH. Weeg-Aerssens at al. (1988) reported that the codenitrification/denitrification ratio increases, when the electron supply by the reductant (e.g. succinate) becomes rate limiting for denitrification of NO_2^-, i.e. with decreasing availability of electron donors the contribution of denitrification on total N-N gas formation decreases. Nonetheless, inasmuch as codenitrification is at least dependent on NO_2^- production (by e.g. NO_3^- reduction during denitrification) the question remains to what extent codenitrification can occur even in the absence of respirable organic

substrates. Besides organic reductants, however, autotrophic denitrifiers have been shown to utilise reduced sulphur compounds, H_2, or Fe^{2+} for the reduction of NO_3^- and NO_2^- to N_2O and/or N_2. As recently reported by Shao et al. (2010) autotrophic denitrifiers are apparently widely distributed within different environments (e.g. wet soils, lake sediments etc.). Until now, however, no clear evidence has been delivered that autotrophic denitrifiers perform codenitrification.

2.3.4. Type and kinetic of nucleophiles

Gutierrez et al. (2008) recently pointed out that a variety of nitrogenated species, e.g. NH_3, amines and amino acids, NH_2OH, NO_2^-, and thiolates (cysteine, glutathione, etc.) have to be considered as biorelevant nucleophiles. However, a release of hybrid N_2O or hybrid N_2 following a nitrosation is fundamentally related to the type of nucleophile which undergoes codenitrification. E.g. Iwasaki et al. (1956) demonstrated that the bacterial species *Pseudomonas denitrificans* was able to enhance N-N gas production due to N-nitrosation of p-phenylene diamine, while a similar stimulating effect on N-N gas formation could not be observed in the presence of o- and m-phenylene diamine, respectively. Similar effects concerning microbial nitrosation of amines have been reported by Pichinot et al. (1969). In principle it can be deduced that all nucleophilic N compounds known to be converted to N_2O and/or N_2 by abiotic N-nitrosation (mainly primary amines and amides as well as oxime compounds, see chapter 2.1.1) will be also co-metabolised to hybrid N_2O and/or N_2 in the course of codenitrification. In fact, all N compounds shown to be converted to hybrid N-N gas by microbial N-nitrosation fit into this scheme (see Table 2.1).

While the type of a nucleophile determines its suitability for N-N gas formation upon nitrosation (i.e. probability of dediazoniation, see chapter 2.1.1), its affinity (e.g. K_m value) to a codenitrifying enzyme (i.e. NIR/NOR) determines the nitrosation rate at a particular availability (i.e. concentration). Until now, however, only a few studies have reported K_m values of nucleophilic species which undergo hybrid N-N gas production via codenitrification (Iwasaki and Mori, 1958; Kim and Hollocher, 1984; Su et al., 2004). Among tested nucleophiles only slight differences have been observed. All determined K_m values are within a millimolar range and extent between 2 and 60 mM. Iwasaki and Mori (1958) reported that K_m values of N_2 formation via dimethyl-p-phenylene diamine (K_m value of 30 mM) are rather similar to K_m values of N_2O formation via NH_2OH (K_m value of 60 mM). Kim and Hollocher (1984) could show that at concentrations of ≥50 mM most enzymatically nitrosated nucleophiles (using prokaryotic cd_1 NIR from *Pseudomonas aeruginosa*) reveal a zero order reaction and hence, indicate a saturation kinetic (e.g. K_m value of N_3^- 11 mM, K_m value of aniline 4 mM). Similarly, Su et al. (2004) found saturation kinetics (K_m value of 3 mM) during nitrosation of N_3^- by NO, using a purified P450 NOR of the fungi *Fusarium oxysporum*. For nucleophiles which undergo microbial nitrosamine formation (e.g. pyrrolidine, dimethylamine) Calmels et al. (1985) reported K_m values between 4.5 and 58.5

mM. In addition, they revealed that K_m values of NO_2^- reduction (i.e. denitrification) and amine nitrosation, respectively, (by *Escherichia coli*) have been very similar. Furthermore, it was found that biotic nitrosation of tested amines clearly followed a Michaelis-Menten kinetic. They also showed that the degree of nitrosation was clearly dependent on the amine compound which was nitrosated to a nitrosamine. Also Leach et al. (1987) revealed that bacterial catalysed nitrosation shows a dependence on the nucleophile and can be best described by classical Michaelis-Menten kinetic. Calmels et al. (1985) reported that nitrosamine formation by microbial nitrosation appears to be dependent on the pK_a value ($-\log_{10}$ of the acid dissociation constant K_a) of a nitrosated amine species. It was shown that the logarithm of the specific nitrosation rate was inversely linear related to their p*Ka* value. Ralt et al. (1988) reported microbial nitrosamine formation by *Escherichia coli* where nitrosation of morpholine, despite of a 75-fold-higher concentration, was slower than the nitrosation of 2,3-diaminonaphtalene. Also here it was assumed that the differences in nitrosation rates were directly related to their acid dissociation constant. Similar dependencies on the pK_a vlaue have been reported by Kim and Hollocher (1984) concerning hybrid N_2O formation by enzymatic nitrosation of N_3^-, NH_2OH, and $H_2N\text{-}NH_2$, respectively. Furthermore, Kim and Hollocher (1984) demonstrated that when two nucleophiles are present at equal concentrations, the one with the lower K_m will inhibit nitrosation of the one with the higher K_m value, while an obverse inhibition is insignificant. However, since K_m values known for NO_2^-/NO reduction during denitrification mainly extent between nM and µM range (Conrad, 1996; Zumft, 1997) it appears that microbial nitrosation rates are significantly affected by the availability of NO_2^- and NO, respectively. It was shown by some studies (Weeg-Aerssens et al., 1987/88; Hulse et al., 1989) that the codenitrification/denitrification ratio decreases considerably when the ratio of nitrosyl donor concentration (e.g. NO_2^-) to nucleophile concentration (e.g. N_3^-) increases. It should be noted, however, that some microbial studies revealed significant nitrosation rates even under conditions where the nucleophile has been delivered at a similar concentration as the nitrosyl donor (e.g. NO_2^-) (Garber and Hollocher, 1982b; Calmels et al., 1985; Weeg-Aerssens, et al. 1988; Tanimoto et al., 1992).

2.3.5. Physiology of denitrifying species

As shortly summarised in Table 2.1 N_2O and N_2 gas production due to codenitrification is known to occur in all three domains of microorganisms: *bacteria*, *archaea*, and *eukarya* (kingdom *fungi*). However, many studies (e.g. Mills and Alexander, 1976; Calmels et al., 1985; Leach et al., 1987) revealed significant differences in their individual capabilities to perform codenitrification. These differences seem to depend on the setup of an enzyme and hence, its individual reaction kinetic (e.g. Kim and Hollocher, 1984; Hulse et al., 1989; Suzuki et al., 1989; Ye et al., 1991) and furthermore, appear to be affected also by the location of an enzyme within a microbial species (e.g. membrane bound or cytosolic enzymes; see Aerssens et al., 1986; Weeg-Aerssens et al., 1987/1988). For example, biotic nitrosation

activity was proven to be strongly related to the metal species of the binding site for NO and NO_2^-, respectively (e.g. exhibiting a Fe-NIR or a Cu-NIR, see also chapter 2.1.2 and 2.1.3) (e.g. Hulse et al., 1989; Suzuki et al., 1989; Ye et al., 1991). Trapping experiments with $H_2{}^{18}O$ and NO_2^- revealed that with a purified Cu-NIR of *Achromobacter cycloclastes* no detectable incorporation of ^{18}O into N_2O took place (Hulse et al., 1989), while with Fe-NIR (e.g. from *Pseudomonas stutzeri*) a considerable ^{18}O incorporation into denitrified and codenitrified N_2O occurred (Garber and Hollocher, 1982b; Aerssens et al., 1986; Weeg-Aerssens et al. 1987). Ye et al. (1991) pointed out that with microbial species using Fe-NIRs a generally extensive exchange between the oxygen of H_2O and NO_2^- takes place. By contrast, microbial species using Cu-NIRs revealed oxygen exchange rates from near zero to almost complete exchange. Ultimately it was therefore assumed that the nitrosation kinetic of an enzymatic Cu^+-NO^+ intermediate is significantly different from the that of Fe^{2+}-NO^+ intermediate. In fact, a few studies (Goretski and Hollocher, 1991; Jackson et al., 1991; Ye et al., 1991) demonstrated significantly higher N-nitrosation rates by copper-containing NIRs compared to iron-containing NIRs. As supposed by Averill (1996) NO_2^- reduction by copper NIRs appears to proceed via a more labile Cu-NO species, which is observable by an increased NO accumulation during denitrification of NO_2^- as well as hybrid $^{14,15}N_2O$ production in the presence of $^{15}NO_2^-$ (99 at%) and unlabelled NO. In fact, Jackson et al. (1991) showed that a $^{14,15}N_2O$ production was performed by a Cu-NIR (*A. cycloclastes*) in the presence of $^{15}NO_2^-$ and ^{14}NO, while with a ferrous NIR (*Pseudomonas stutzeri*) the incorporation of free NO was not found at all.

Garber and Hollocher (1982b) demonstrated that hybrid N_2O formation due to codenitrification of NH_2OH with NO_2^- as the nitrosyl donor significantly alters depending on the denitrifying species. While with *Paracoccus denitrificans* and *Pseudomonas stutzeri* the mole fraction of hybrid N_2O to total N_2O production accounted for ca. 47 and 63%, respectively, the species *Pseudomonas denitrificans* and *Pseudomonas chlororaphis* achieved mole fractions of 91 and 98%, respectively (incubation time of 30 min). Similar results have been found when NH_2OH was replaced by N_3^-. With *Pa. denitrificans* and *P. stutzeri* the mole fraction of hybrid N_2O to total N_2O production was 6 and 8%, respectively, while with *P. denitrificans* and *P. chlororaphis* a mole fraction of 54% was achieved. Two studies (Calmels et al., 1985; Leach et al., 1987) demonstrated that even different isolates of the same species can show considerable variation in their ability to catalyse nitrosation reactions. As figured out by Leach et al. (1987) the most rapid catalysis is associated with those bacteria capable of reducing NO_3^- and NO_2^- via denitrification (e.g. *P. aeruginosa*, *E. coli* etc.). Ralt et al. (1988) suggested that observed differences in nitrosation activity of different *E. coli* strains might be related to genetic or phenotypic differences. Mills and Alexander (1976) could show that even the physiological state of microorganism alters its ability to perform nitrosation reactions. It was found that *E. coli* catalyses a nitrosation of dimethylamine during a resting phase, but was incapable of performing nitrosation during the growing phase. Furthermore, they observed

differences between microbial components responsible for biotic nitrosation. While boiled cells of *P. stutzeri* maintained their ability to enhance nitrosation, other species (e.g. *E. coli*, *Cryptococcus terreus*) completely lost their capability to enhance nitrosation after boiling. It was concluded that in the latter case a nitrosation was performed enzymatically, while in the former case a nitrosation was biotically enhanced by cell constituents other than enzymes. Also Collins-Thompson et al. (1972) reported nitrosation of secondary amines with NO_2^- mediated by a *Streptococcus* species via an unknown non-enzymatic reaction with one or more metabolic products. Weeg-Aerssens et al. (1988) revealed that the accessibility of a nucleophile to an enzyme (e.g. passage through the cell membrane) can significantly alter the efficiency of hybrid N-N gas formation. While only $\leq 1\%$ of N_2O was found to be formed by biotic N-nitrosation of N_3^- using whole cells of *P. stutzeri*, ca. 30% N_2O was gained from biotic N-nitrosation when cell-free extracts (using a French Press) were used.

3. ^{15}N-aided online determination of soil N_2 and N_2O release on a laboratory scale

3.1. Abstract

N_2 is one of the major gaseous nitrogen compounds released by soils due to N-transformation processes. Since it is also the major constituent of the earth's atmosphere (78.08 vol.%), the determination of soil N_2 release is still one of the main methodological challenges with respect to a complete evaluation of the gaseous N-loss of soils. Commonly used approaches are based either on a C_2H_2 inhibition technique, an artificial atmosphere or a ^{15}N tracer technique and are designed either as closed systems (non-steady state) or gas flow systems (steady state). The intention of this work has been to upgrade the current gas flow technique using an artificial atmosphere for a ^{15}N aided determination of the soil N_2 release simultaneously with N_2O. A ^{15}N aided artificial atmosphere gas flow approach has been developed, which allows a simultaneous online determination of N_2 as well as N_2O fluxes from an open soil system (steady state). Fluxes of both gases can be determined continuously over long incubation periods and with high sampling frequency. The N_2 selective molecular sieve Köstrolith SX6® was tested successfully for the first time for N_2 collection. The present chapter mainly focuses on N_2 flux determination. For validation purposes soil aggregates of a Haplic Chernozem were incubated under aerobic (21 and 6 vol.% O_2) and anaerobic conditions. Significant amounts of N_2 were released only during anaerobic incubation (0.4 to 640.2 pmol N_2 h^{-1} g^{-1} dry soil). However, some N_2 formation also occurred during aerobic incubation.

3.2. Introduction

Scientists from different disciplines have focused their studies on processes of transformation and transport of nitrogen compounds in soils due to an increased gaseous nitrogen release from agricultural fields as a result of N fertiliser application. Different studies revealed that gaseous nitrogen species (e.g. NO, N_2O or N_2) are produced in soils mainly as a consequence of microbial oxidation and reduction processes during soil N-transformation (Quastel and Scholefield, 1951; Knowles, 1982). Since NO and N_2O occur only in trace amounts within the earth's atmosphere, flux measurements are relatively easy to carry out. In contrast to this, N_2 is the major component of the earth's atmosphere (78.08 vol.%) and thus measurements of N_2 fluxes are very difficult to realise. However, for a complete evaluation of the gaseous nitrogen release from soils during the soil/atmosphere N-cycle it is essential to consider NO, N_2O and N_2, separately. In soils N_2 is mainly formed during denitrification (Knowles, 1982). At present three basic approaches for measuring N_2 fluxes from soils have been developed: (i) application of acetylene (C_2H_2) (e.g. Weier et al., 1993; Rudaz et al., 1999), (ii) use of N isotopes (stable ^{15}N or radioactive ^{13}N) (e.g. Speir et al., 1995; Wolf and Russow, 2000) and,

(iii) creation of an artificial atmosphere (e.g. Butterbach-Bahl et al., 2002; Cardenas et al., 2003).

The use of C_2H_2 as a component of the soil atmosphere (10 kPa) will block the N_2O reductase of denitrifying species and subsequently inhibit the formation of N_2 (Yoshinari and Knowles, 1976). The N_2 flux isn't directly measured but can be calculated as the difference between the N_2O amount released by soil samples incubated with and those incubated without C_2H_2. The main advantage of this approach is that it can detect very low levels as only N_2O has to be measured by gas chromatography. However, different studies revealed some uncertainties concerning the applicability of this approach. Wrage et al. (2004) published that the inhibition effect of C_2H_2 on the N_2O release of soil samples is inconsistent even at a constant C_2H_2 headspace concentration. Furthermore C_2H_2 additionally impedes the supply of NO_3^- during nitrification by inhibiting the enzyme NH_4^+-monooxygenase produced by nitrifying species (Malone et al., 1998). Thus, N_2 fluxes can be calculated only for a short period of time due to a shortage of NO_3^- supply. Jordan et al. (1998) have shown that an in situ application of C_2H_2 affects the N_2O flux only during simultaneous addition of NO_3^-. Bollmann and Conrad (1997) showed that C_2H_2 also affects the reduction of NO to N_2O resulting in an underestimation of denitrification rates. Finally, the simultaneous determination of NO, N_2O and N_2 released from one soil sample is impossible. Taken together these factors lead to the conclusion that the acetylene inhibition technique should be replaced by other methods, as has already been proposed by Butterbach-Bahl et al. (2002).

The application of N isotopes is another basic approach for measuring N_2 fluxes from soils (e.g. Speir et al., 1995; Bergsma et al., 2001). Since the utilization of the radionuclide ^{13}N allows a measurement period of only one hour (half life time approximately 10 min) the stable ^{15}N is commonly used. Depending on the individual objectives of a study, a variety of tracer substances can be introduced into the soil ($^{15}NH_4^+$, $^{15}NO_3^-$ etc.) for labelling N_2 formed due to soil N-transformation. N_2 fluxes can easily be calculated by various mathematical approaches (Hauck and Bouldin, 1961; Mulvaney and Boast, 1986; Russow et al., 1996), the choice of which depends on the ^{15}N treatment and the method by which the output data is assayed.

A third basic approach for measuring N_2 fluxes from soil samples is based on an artificial atmosphere with helium or argon as a substitute for atmospheric N_2. Based on that principle, different gas flow soil core techniques have been developed during the last 10 years (Swerts et al., 1995; Scholefield et al., 1997a; Butterbach-Bahl et al., 2002; Cardenas et al., 2003; Mander et al., 2003). Soil cores are initially purged with an artificial gas or gas mixture (up to 200 mL min^{-1}) in order to replace atmospheric N_2 within the soil. Afterwards the artificial gas or gas mixture is used as a continuously streaming gas flowing over the soil surface (5 to 80 mL min^{-1}) (Scholefield et al., 1997a) and generating a permanent artificial atmosphere. Soil N_2 formation will be measurable as long as the N_2 efflux of the soil exceeds the N_2 background concentration of the artificial atmosphere. N_2 fluxes are determined online by gas

chromatography based on either a steady state (Scholefield et al., 1997a; Cardenas et al., 2003) or a non-steady state approach (Butterbach-Bahl et al., 2002; Mander et al., 2003).
The intention of this work has been to upgrade the common soil core gas flow technique for an improved determination of the soil N_2 release and simultaneous determination of other gaseous N species (e.g. N_2O) released by soils. Based on the experience of a former ^{15}N supported flow-through incubation system (Russow et al., 1995) we developed a ^{15}N aided artificial atmosphere gas flow technique for a continuous online determination of soil N_2 release and simultaneous determination of N_2O release. The combination of the gas flow soil core technique using an artificial atmosphere with ^{15}N tracer technique leads to a number of advantages which are essential for detailed studies of the complete gaseous N-loss of soils. First of all current detection limits concerning N_2 flux determination could be markedly extended. Furthermore investigations of different gaseous nitrogen species released simultaneously by soils (e.g. N_2O, N_2), but generated by different biotic and abiotic pathways could be conducted. Because of the steady state condition (open soil system) fluxes can be determined for long incubation periods without disturbing the gas headspace. Furthermore, online determination permits a high sampling frequency. Finally, flux calculation based on ^{15}N tracer technique is performed by a single measurement, which minimizes the amount of work and additionally increases the sampling frequency. The presented approach has been validated by soil aggregates of a Haplic Chernozem and examination will be mainly focused on N_2 flux determination.

3.3. Material and Methods

3.3.1. Basic principle of flux determination

Comparable with the cryoenrichment technique for capturing NO and N_2O (e.g. Speir et al., 1995; Sich and Russow, 1998) from a carrier gas, the basic idea of this approach was to develop a technique for a controlled capturing and release of N_2. A carrier gas continuously passes over the surface of an incubated soil core at a constant flow rate (10^0 to 10^2 mL min^{-1}). Under steady state conditions it is assumed that the current headspace gas concentration of any gas (e.g. N_2) produced in the soil is controlled only by the current biotic and abiotic conditions of the soil sample itself. Consequently the headspace concentration of a certain gas under study correlates with its current efflux from the incubated soil sample. Capturing this gas completely from the downstreaming carrier gas for a short period of time and analysing the captured amount would allow the determination of its current efflux (amount/time) under given conditions. Concerning N_2 the dinitrogen selective molecular sieve Köstrolith SX6® (Chemiewerk Bad Köstritz) has been used for dinitrogen collection. SX6® is a synthetic crystalline aluminosilicate with a regular micropore structure (faujasite type, 0.9 nm). Since contamination with ambient air is inevitable a ^{15}N tracer technique is used for a distinct

differentiation between N_2 produced during soil N-transformation (e.g. denitrification) and atmospheric N_2 derived from leakages.

3.3.2. Instrumental set-up

Stainless steel assemblies designed for sealing a soil core (0.8 dm in diameter) from bottom and top were used for soil incubation. Sealing was ensured by using wing nuts to tighten the soil core against rubber rings at the top and the bottom. Additionally silicone gel was used to improve the sealing of the soil core (Figure 3.1). He 5.3 (Linde AG, Pullach, Germany) was used as the major carrier gas and mixed with O_2 5.0 (Linde AG, Pullach, Germany) by using two mass flow controllers (Series 5850; Westphal M&R GmbH, Ottobrunn, Germany), according to experimental procedures. The actual O_2 concentration of the carrier gas was monitored by a micro-optical oxygen sensor (oxy 10 micro-trace, Precision Sensing GmbH, Regensburg, Germany). The main carrier gas stream was divided into three parallel pathways providing three separate incubation entities (Figure 3.2, IE A to C). In front of the inlets of each entity a nickel capillary (I.D. 32 µm) 50 mm in length was assembled resulting in a higher pressure upstream of the capillary and guaranteeing a continuous and constant flow rate through all incubation entities even when minor back-pressure changes occur.

To achieve controlled capture and release of N_2, an open gas circuit (Figure 3.2) was designed. During no-load running, the gas circuit was flushed with He 5.3 (50 mL min^{-1}). For flux determination the outlet of an incubation entity was connected to the inlet of the gas circuit (Figure 3.2, V1). First the carrier gas was purified from NO, CO_2 and H_2O by a NO_x filter p/n79275 (ECOM America, Gainesville, USA), Ascarrite (Fluka Chemie AG, Buchs, Switzerland) and Siccapent (Merck KGaA, Darmstadt, Germany), respectively (Figure 3.2, PP/AS/SI). Afterwards the carrier gas passed a stainless steel accumulation loop (Figure 3.2, CT1) assembled on a 6-port-valve (Figure 3.2, V2) and used for N_2O capturing by a cryoenrichment technique. O_2 was subsequently removed completely from the carrier gas by a copper oxidation furnace (500 °C) (Figure 3.2, CF), since it is readily adsorbed by SX6® resulting in interference with the N_2 flux determination. In front of the furnace a second cryotrap (Figure 3.2, CT2) was inserted to assure that no N_2O entered the furnace. Otherwise N_2O would be partially reduced to N_2 resulting in an erroneous N_2 flux determination. At the outlet of the furnace a third cryotrap was assembled for NO removal (Figure 3.2, CT3), since NO is formed in very small amounts by N_2 and O_2 passing the furnace. The carrier gas subsequently passed a second stainless steel accumulation loop (Figure 3.2, Z) assembled on a 6-port-valve (Figure 3.2, V3) and filled with SX6® (0.1 g; granular 1.2 - 2 mm) for N_2 capture by cooling with liquid dinitrogen.

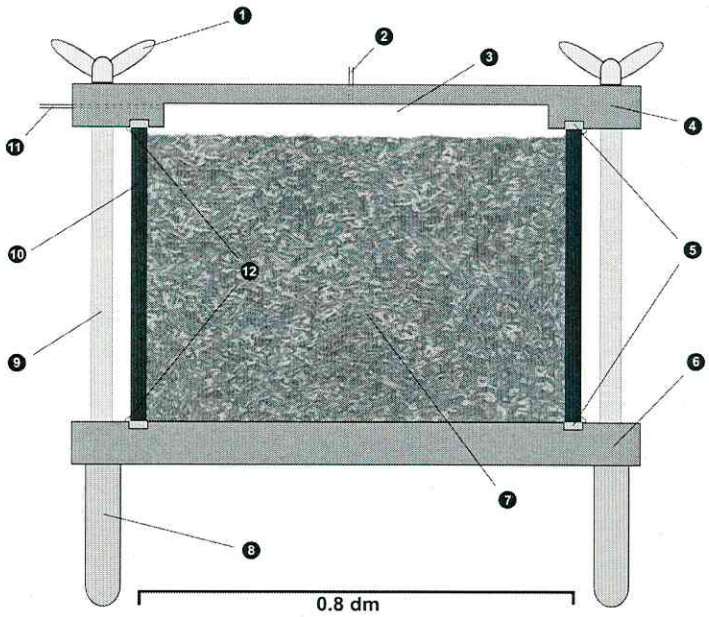

Figure 3.1 *Schematic cross-section of an incubation entity: 1 - wing nut; 2 - headspace outlet; 3 - headspace; 4 - cap; 5 - upper and lower sealing ring; 6 - base; 7 - soil core; 8 - stand; 9 - screw thread; 10 - steel cylinder; 11 - headspace inlet; 12 - silicone gel sealing.*

Finally the carrier gas was streamed through the outlet of the open gas circuit (Figure 3.2, V3). By switching valve 2 and 3 captured N_2O and N_2 were transferred into the gas circuit of a continuous-flow isotope-ratio mass spectrometer (ConFlow IRMS - delta S; ThermoFinnigan MAT, Bremen, Germany) downstreaming to a gas chromatograph (GC 5890 Series II; HP – Agilent, Palo Alto, USA). Captured N_2 was released by electrical resistance heating, which resulted in a rapid heating-up of the N_2 accumulation loop to approximately 200 °C. Activation of SX6® was carried out at 450 °C for 5 min and repeated every day prior to the flux measurements. Captured N_2O was released by removing rapidly the N_2O accumulation loop from the Dewar vessel. Total amount of gas and abundance of ^{15}N was measured by a ConFlow IRMS equipped with a triple collector for a simultaneous detection of mass-to-charge ratios (m/z) 28, 29, and 30. N_2O was reduced to N_2 by a copper furnace (730 °C) prior determination.

3.3.3. Flux measurement

The outlet of an incubation entity was connected to valve 1 (Figure 3.2, V1) by Swagelok® couplings. After 5 min the connected carrier gas stream was switched into the open gas circuit. For N_2O capturing, the N_2O accumulation loop (Figure 3.2, CT1) was switched into the open gas circuit and immersed in liquid dinitrogen prior to switching valve 1.

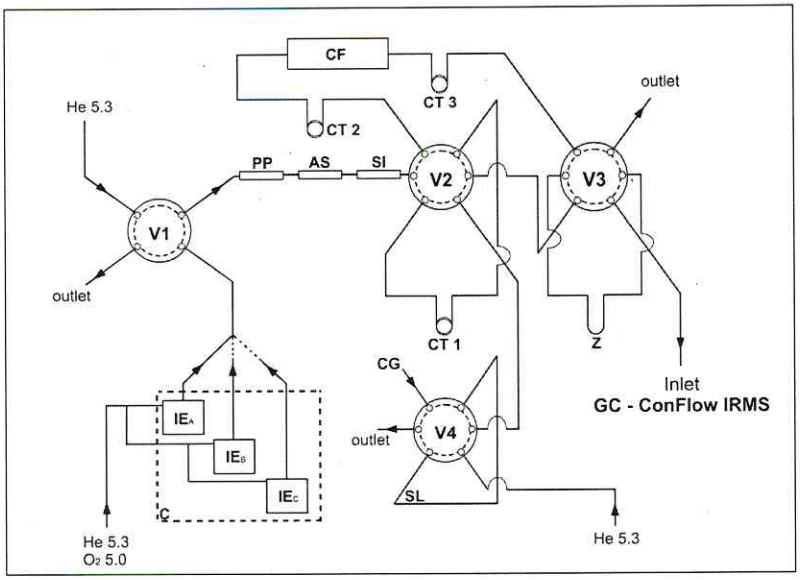

Figure 3.2 *Scheme of instrumental setup: AS - CO_2-trap (Ascarite); C - warming cupboard; CF - copper furnace; CG - Inlet for calibration gas; CT1 - cryotrap for N_2O sampling; CT2 - cryotrap for N_2O removal; CT3 - cryotrap for NO removal; IE - incubation entity A-C; PP – NO-trap (potassium permanganate); SI - H_2O-trap (Siccapent) SL - sample loop for calibration gas; V - Valve 1-4; Z - zeolite SX6® for N_2 sampling.*

The capture of N_2 was started after 15 min of continuous flushing. N_2 was captured throughout the setup for 2 min. Afterwards the N_2 accumulation loop was switched into the inner circuit of the GC-ConFlow-IRMS (Figure 3.2, V3) and N_2 was released 10 seconds later. After a total of 20 min flushing valve 1 was switched back to the starting position thereby initiating no-load running conditions. After a further 3 mins, the N_2O accumulation loop was switched into the inner circuit of the GC-ConFlow-IRMS (Figure 3.2, V2) and N_2O was released and measured. Since the three faraday cups of the delta S have a measuring range of 13 V in maximum (amplitude) the capturing time had to be shortened when soil N-fluxes increased. Thus, N_2 capture took between 1 and 2 min and N_2O capture between 3 and 20 min. When it was necessary to capture N_2O for less then 17 min, the N_2O flux was measured during a second run immediately after the N_2 flux measurement.

3.3.4. Flux calculation

Amounts of captured N_2 and N_2O were calculated via a calibration curve generated by using differently sized sample loops (Figure 3.2, SL) flushed with a standard gas mixture (2530 ppm N_2 and 2500 ppm N_2O in helium). Relative standard deviation (RSD) was ≤1.0 % (n = 5)

for N_2 and ≤3.0 % (n = 5) for N_2O. The accuracy of the delta S with regard to the determination of ^{15}N abundance beyond the natural background occurrence was confirmed by calibration standards of $^{15}N_2O$. The standards were generated by heating up (>170 °C) $^{15}NH_4{}^{15}NO_3$ salt (^{15}N abundance of 4.73, 15.45, 35.51 and 55.64 at%) contained in 12 mL vials. To remove atmospheric N_2O, vials were flushed with helium before heating them up. Measurements were conducted via an auto sampling procedure published by Schmidt et al. (1997). The RSD calculated was ≤1.5 % (n = 5).

N_2 flux calculation is based on a two-pool model using the non-random distribution approach (Hauck and Bouldin, 1961), which involves the following assumptions: (i) the soil NO_3^- pool is homogenously labelled, (ii) labelled NO_3^- is the only source of N_2 formation, and (iii) N_2 formed by denitrification is only mixed in with atmospheric N_2. The same model can be used with regard to the N_2O flux calculation. However, since no detectable amounts of atmospheric N_2O interfered with the N_2O flux determination, the two-pool model approach can be applied to two different soil N sources instead of one. Thus, the differentiation between nitrified (NH_4^+) and denitrified N_2O (NO_3^-) is possible with the following assumptions: (i) soil NO_3^- pool is homogenously labelled, (ii) soil NH_4^+ pool has natural ^{15}N abundance and, (iii) N_2O formed by denitrification is only mixed in with nitrified N_2O. The flux calculation of both gases is based on the same equations, but only presented for N_2.

Since the ^{15}N abundance of the soil N source of N_2 (NO_3^-) can not be measured directly we used a modified equation derived from equations published by Russow et al. (1996):

Equation 3.1
$$a_2 = \frac{\alpha_m - a_1 \cdot a_m}{a_m - a_1}$$

- a_2 - ^{15}N abundance of pool 2 (NO_3^-)
- a_1 - ^{15}N abundance of pool 1 (atmospheric N_2)
- a_m - measured ^{15}N abundance of captured N_2
- α_m - measured fraction of m/z 30 of captured N_2

The fraction of soil N_2 within the captured N_2 gas mixture (air/soil) was calculated subsequently by using the obtained value of the ^{15}N abundance a_2 for the following equation (Russow et al., 1996):

Equation 3.2
$$f_s = \frac{a_m - a_1}{a_2 - a_1}$$

- f_s - relative fraction of captured N_2 released by the soil (Pool a_2)

Finally the current N_2 flux was calculated as follows:

Equation 3.3 $$F = \frac{n \cdot f_s}{t \cdot m}$$

F	-	N_2 flux [mol h^{-1} g^{-1} dry soil]
n	-	amount of captured N_2 [mol]
t	-	time of N_2 capturing [h]
m	-	dry mass of the incubated soil [g]

During ionisation of N_2 small amounts of $^{14}N^{16}O$ are formed simultaneously within the ion source of the delta S resulting in an erroneous determination of m/z 30 signal when ^{15}N abundance of N_2 is near natural abundance. The application of α_m and a_m in Equation 3.1 would then give an overestimation of the ^{15}N abundance of denitrified NO_3^- (a_2) and would lead to an incorrect calculation of the soil N_2 fraction (f_s). To evaluate uncertainties of m/z 30 due to $^{14}N^{16}O$ formation, we derived an equation that calculates the soil fraction of captured N_2 (f_s) independently from m/z 30 (Equation 3.4):

Equation 3.4 $$f_s = \frac{1}{1 - \frac{R_{29}(1-a_2)^2 - 2a_2(1-a_2)}{R_{29}(1-a_1)^2 - 2a_1(1-a_1)}} \quad \text{; with} \quad R_{29} = \frac{m/z\,29}{m/z\,28}$$

R_{29} was calculated by using the isotopic distribution data of captured N_2. The required a_2 value is gained from the isotopic distribution of simultaneously determined N_2O (Equation 3.1). Since the ^{15}N abundance of captured N_2O was always > 5 at%, uncertainties on m/z 30 due to $^{14}N^{16}O$ formation are negligible concerning N_2O.

3.3.5. Soil treatment and incubation

For validation purposes soil samples of an A-Horizon of a Haplic Chernozem (Bad Lauchstädt, Germany) were used. The soil sampling area belongs to an agricultural research site unfertilized since 1905 (Körschens et al., 1981). The soil form is a well-textured loess black earth (21 % clay, 68 % silt, 11 % sand). As source material only soil aggregates were used. The aggregates were gained by sieving the collected soil (6.3 mm sieve) without prior drying. The soil NO_3^- pool was labelled with ^{15}N using a $K^{15}NO_3$ (95 at% ^{15}N) solution (120 mg N L^{-1}). The solution was added by spraying 20 mL onto a 950 g of fresh soil aggregate. Subsequently 250 g were placed within each incubation entity (three parallel entities: A, B and C). Afterwards incubation entities were sealed, placed in a warming cupboard (25.8 ± 0.3

°C) and connected to the carrier gas stream (Figure 3.2). The flushed headspace volume of the incubation entities was approximately 25 mL. The flow rate of the carrier gas was set to 6.4 mL min^{-1} and was kept constant. Soil aggregates were continuously incubated for a total of 25 days. For flux measurements only the outlets of the incubation entities were out with the warming cupboard. During the first phase of incubation (8 days) oxygen content of the carrier gas was set to 21 vol.%. After this the O_2 supply was stopped for the next 9 days and only helium was used as carrier gas (2^{nd} phase of incubation). O_2 concentration was subsequently set to 6 vol.% for the following 8 days (3^{rd} phase of incubation). First flux measurement was carried out 3 hours after incubation started.

The soil moisture (w/w %) was determined at the beginning (24 h before incubation, three samples from 950 g of ^{15}N treated soil aggregates) and the end of incubation (25^{th} day of incubation, one sample per incubation entity) by drying 10 g of the soil aggregates at 105 °C for 24 h. Additionally, initial and final ^{15}N abundance of soil NO_3^- as well as NO_3^--concentration of treated soil aggregates were determined. Soil samples (25 g of soil aggregates) were taken in the same manner as for soil moisture analysis. 100 mL 1 M KCl-solution was added to a soil sample and shaken for 2 h. Subsequently the soil slurry was filtered through a folded filter (Schleicher & Schuell MicroScience, Dassel/Relliehausen, Germany) and two 5 mL samples were used for NO_3^- analyses by the SPINMAS technique (Stange et al., 2007). Total soil N and ^{15}N abundance were determined at the end of incubation (three parallel soil samples from each incubation entity), by a CN-Analyser (Elementar Analysesysteme, Hanau, Germany) coupled to an QPMS (InProcess Instruments, Bremen, Germany) (Russow and Götz, 1998).

3.4. Results

3.4.1. Validation of N_2 capturing by Köstrolith SX6®

By using variously sized sample loops (Figure 3.2, V4) flushed with N_2 calibration gas (2530 ppm N_2 in helium) it was shown that N_2 was adsorbed completely by SX6® up to a loop volume of 5 mL (ca. 500 nmol N_2). Since the current upper detection limit of the delta S is around 100 nmol for N_2 at natural ^{15}N abundance, it is apparent that sampled N_2 was removed completely from the carrier gas during N_2 flux determination. Following release of the captured N_2, a second heating of the N_2 accumulation loop to 450 °C gave no detectable peak indicating a complete and rapid desorption of adsorbed N_2. The effect of varying duration of N_2 capture on the accuracy of N_2 flux determination was also investigated (Figure 3.3). For this purpose the incubation entity B was connected to the gas circuit on the 16^{th} day of incubation (anaerobic incubation) for 127 min and N_2 was captured for 0.5, 1, 2, 3 and 4 min (a total of three times per sample time) at time intervals of 5 to 9 min. At the end an additional single N_2 capture of 0.5 min was performed.

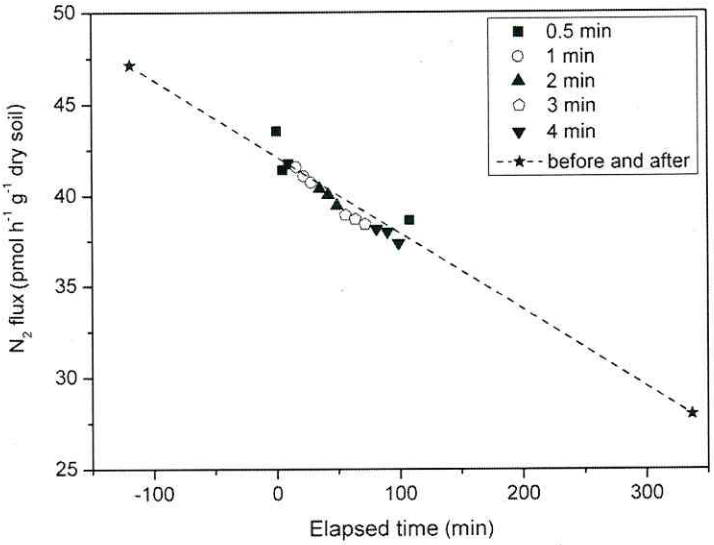

Figure 3.3 *N_2 flux determination based on different periods of N_2 capturing. (black star - regularly measured N_2 flux before and after capturing experiment on 16th day of incubation; the first determined flux value of this sub-experiment has been set at zero on the x-axis).*

Between the first and the last measurements, the N_2 fluxes decreased continuously from 43.5 pmol N_2 h^{-1} g^{-1} dry soil (0.5 min) to 38.6 pmol N_2 h^{-1} g^{-1} dry soil (0.5 min). Looking at the N_2 flux values determined before and after the experiment suggests that the observed decline occurred because the soil N_2 release decreases (Figure 3.3 and Figure 3.6), and does not occur due to changes of capture duration. With respect to 1 to 4 min capture, flux rate changes of approximately 0.5 pmol h^{-1} g^{-1} dry soil within 6 to 9 min intervals were clearly quantified, independent of the capturing period. Fluxes which were determined based on 0.5 min capture revealed slight deviations from the generally decreasing trend of soil N_2 release. Thus, the capturing period should not be set lower than 1 minute.

3.4.2. Validation of $^{14}N^{16}O$ interference on N_2 flux determination

For the evaluation of the interference of $^{14}N^{16}O$ formation on m/z 30 of captured N_2, we compared the results of the N_2 flux calculation based on Equation 3.1 to Equation 3.3 with the results based on Equation 3.4 and Equation 3.3. Figure 3.4 illustrates that there is good concordance between both approaches, which indicates a negligible influence of $^{14}N^{16}O$ formation on the N_2 flux calculation using Equation 3.1 to Equation 3.3. However, results of both approaches start to differ at the lowest end of determined fluxes (Figure 3.4b).

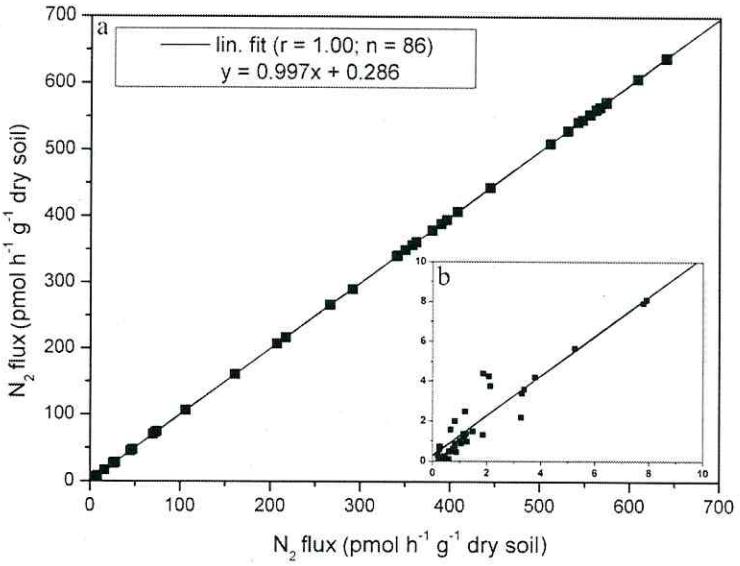

Figure 3.4 *(a) Comparison of N_2 fluxes calculated either by including m/z 30 of captured N_2 [Equation 3.1, Equation 3.2, and Equation 3.3] (x-axis) or without m/z 30 of captured N_2 [Equation 3.4 and Equation 3.3] (y-axis). (b) Magnification of lowest flux values.*

The lowest flux values are calculated from captured N_2 with the lowest ^{15}N abundance. During aerobic incubation ^{15}N abundance of captured N_2 was $\leq$ 0.42 at%, whereas during anaerobic incubation ^{15}N abundance ranged between 0.38 and 13.23 at%. A comparison of the calculated a_2 values (^{15}N abundance of denitrified NO_3^-) based upon both N_2 and N_2O (Equation 3.1) revealed that particularly during aerobic incubation the calculated ^{15}N abundance of denitrified NO_3^- based on N_2 is up to 8-times higher than values calculated for N_2O (Figure 3.5). This overestimation of a_2 concerning N_2 is caused by the exponentially increasing impact of formed $^{14}N^{16}O$ on m/z 30 against natural ^{15}N abundance. This overestimation consequently leads to an imprecise N_2 flux determination as shown in Figure 3.4b. The minimum ^{15}N abundance for an accurate flux calculation based on Equation 3.1 to Equation 3.3 was determined to be 0.5 at% (not shown) and resulted in an N_2 flux determination limit of around 4 pmol N_2 h^{-1} g^{-1} dry soil ($\approx$ 4 µg N_2-N m^{-2} h^{-1}) with respect to this study (Figure 3.4b).

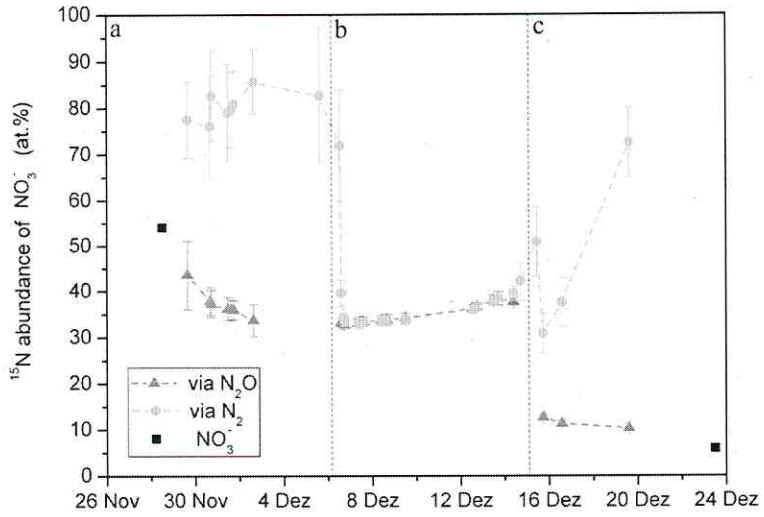

Figure 3.5 *^{15}N abundance of soil NO_3^- and denitrified NO_3^- of incubated soil aggregates: (a) aerobic (21 vol.% O_2); (b) anaerobic; (c) aerobic (6 vol.% O_2) (grey triangles: ^{15}N abundance of denitrified NO_3^- calculated via N_2O; light grey circles: ^{15}N abundance of denitrified NO_3^- calculated via N_2; black squares: ^{15}N abundance of soil NO_3^- determined by the SPINMAS technique (Stange et al., 2007); bars indicate the standard deviation).*

3.4.3. N-fluxes, soil moisture, and soil nitrate of incubated soil aggregates

Measured N_2 fluxes ranged between 0.2 and 640.2 pmol N_2 h^{-1} g^{-1} dry soil. All three soil samples, incubated in parallel, showed comparable results (Figure 3.6). Significant amounts were released only under anaerobic conditions. During the first phase of incubation (21 vol.% O_2) N_2 fluxes were very low and ranged between 0.2 and 5.0 pmol N_2 h^{-1} g^{-1} dry soil. A clearly decreasing trend with the highest fluxes at the beginning and lower fluxes towards the end was observed. On the 8th day of incubation the O_2 supply was shut off. Within 2 to 3 hours N_2 fluxes began to increase steadily and peaked after 3 days at 640.2, 608.1, and 639.6 pmol N_2 h^{-1} g^{-1} dry soil, respectively. During the following 5 days fluxes decreased to values comparable to the end of the first phase of incubation. On the 18th day of incubation O_2 concentration of the carrier gas was set to 6 vol.%. During this incubation period N_2 fluxes remained very low and ranged between 0.2 and 2.1 pmol N_2 h^{-1} g^{-1} dry soil. Two of the three incubated soil samples showed a minor and short increase of N_2 fluxes at the beginning of this incubation period. Integrated flux values of N_2 and N_2O revealed that during the anaerobic incubation 557.6 ±22.8 µg N per incubation entity was released, whereas during both aerobic incubation periods 1.5 ±0.4 µg N per incubation entity was released. Under anaerobic conditions the determined N-loss via N_2 was 489.1 ±23.4 µg N per incubation entity, whereas

N_2O accounts for 68.5 ±0.7 µg N per incubation entity. During this incubation period the N_2/N_2O ratio increased exponentially from 2.3 to 55.2 (Figure 3.7).

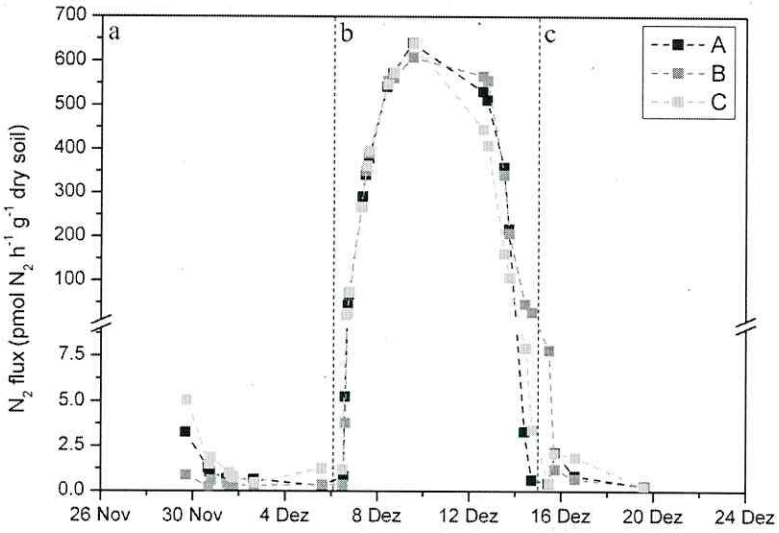

Figure 3.6 *N_2 fluxes of black earth soil aggregates during 21 days of continuous incubation: (a) aerobic (21 vol.% O_2); (b) anaerobic; (c) aerobic (6 vol.% O_2). (Note the discontinuity of the y-axis).*

Soil moisture of incubated soil aggregates decreased from 20.2 ±0.1 % to 15.7 ±0.8 % (w/w) during 25 days of incubation. The calculated water loss of an incubation entity was 0.4 ±0.1 mL d^{-1}. The initial and the final NO_3^- concentrations of the incubated soil samples were 5.3 ±0.5 mg N kg^{-1} dry soil (26.4 ±2.2 mg N L^{-1} soil solution) and 4.9 ±0.3 mg N kg^{-1} dry soil (31.5 ±2.1 mg N L^{-1} soil solution), respectively. Corresponding NO_3^- content per incubation entity was very low (initial 1.1 ±0.1 and final 1.1 ±0.1 mg N per incubation entity, respectively). The initial ^{15}N abundance of soil NO_3^- (24 h before incubation) was 54.05 ±0.83 at% (Figure 3.5). During an incubation experiment ^{15}N abundance of denitrified NO_3^- was surveyed by calculating the a_2 value via N_2O and N_2 levels (Equation 3.1), respectively. During aerobic incubation periods a_2 values calculated via the N_2 level was distinctly overestimated, as described above. ^{15}N abundance calculated via the N_2O level showed a clear decrease from 43.58 to 33.73 at% during the first phase of incubation (21 vol.% O_2). During the following anaerobic incubation ^{15}N abundance increased slightly up to 37.76 at%. The same increase was found based on calculations via the N_2 level. After O_2 concentration was set to 6 vol.%, the ^{15}N abundance of denitrified NO_3^- (based on N_2O) showed a rapid and clear decrease from 37.76 to 10.32 at% (21st day of incubation). The ^{15}N abundance of soil NO_3^- determined after the incubation experiment was 5.83 ±0.64 at% (Figure 3.5).

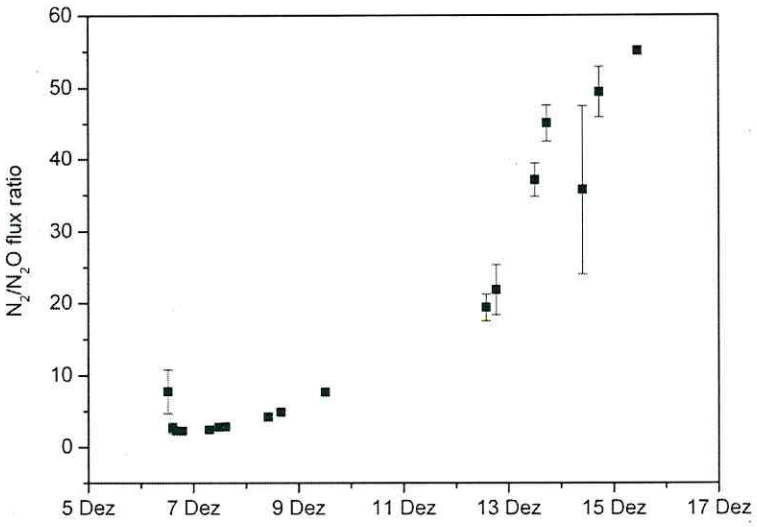

Figure 3.7 *Averaged release ratio of N_2 and N_2O during 9 days anaerobic incubation based on mol h^{-1} g^{-1} dry soil. (Bars indicate the standard deviation).*

3.5. Discussion

3.5.1. N_2 flux determination by a ^{15}N aided gas flow soil core system with an artificial atmosphere

The present ^{15}N assisted gas flow soil core system with an artificial atmosphere has significantly improved the current methodology of N_2 flux determination of soils on a laboratory scale. By combining both approaches together with the first time application of the dinitrogen selective molecular sieve Köstrolith SX6®, a number of advantages have been merged. Based on the steady state approach the present system allows a continuous determination of soil N_2 release. In contrast to non-steady state approaches (Malone et al., 1998; Bergsma et al., 2001; Stevens and Laughlin, 2001a) flux determination is not interrupted by reopening of the incubation vessel. Furthermore, soil gas release is not interfered with gas accumulation within a closed headspace. In contrast to the ^{15}N assisted offline steady state approach as e.g. published by Ruser et al. (2006), the developed online determination of soil N_2 release allows a considerably higher sampling frequency. Since N_2 fluxes can change rapidly over time (Figure 3.3 and Figure 3.6) a high resolution in short sampling times is essential for detailed studies of soil N_2 release. Using one incubation entity the present approach allows a N_2 flux determination at 5 min intervals. Based on the isotopic distribution of captured N_2 flux calculations can be performed on the basis of one single measurement, which minimises the amount of work and additionally increases the sampling frequency. In contrast to studies (Speir et al., 1995) using the radionuclide ^{13}N a lengthy

determination of soil N_2 release over several weeks or months is possible by using the stable isotope ^{15}N. During this study N_2 fluxes were studied continuously for 21 days in total.

With respect to N_2 gas sampling, the application of common sample loops allows a sampling volume of around 10 mL of carrier gas in maximum. However, in dependence on the flow rate of the carrier gas the capturing procedure of N_2 via the zeolite Köstrolith SX6® allows a preconcentration of N_2 gained from distinctively more than 10 mL of carrier gas (e.g., when the flow velocity of the carrier gas is 10 mL min^{-1} and the capture duration is 3 min then a total volume of 30 mL of carrier gas could be used for N_2 analysis). Thus, Köstrolith SX6® permits the detection of N_2 at significantly lower concentrations within the carrier gas. Additionally the preconcentration procedure allows an excellent peak performance even at high sampling volumes.

The N_2 flux determination limit is not restricted by the N_2 detection limit of the delta S, but by the ^{15}N abundance of the captured N_2 air/soil gas mixture. This is caused by the erroneous measurement of m/z 30 of N_2 due to $N^{14}O^{16}$ formation within the ion source of the delta S. In this study a ^{15}N abundance of 0.5 at% was found to be the minimum value for a precise flux calculation. However, at high ^{15}N abundance, flux determination is additionally restricted by the maximum detection range of the amplified detectors measuring m/z 29 and m/z 30. This was compensated for by shortening the time of N_2 capture when signal intensity of m/z 29 and/or m/z 30 was expected to reach the maximum (13 V). The application of a quadrupole mass spectrometer could help to overcome the upper detection limit, but would simultaneously be detrimental to the lower detection limit, which had itself been improved by using the delta S.

The combination of an artificial atmosphere with a ^{15}N tracer technique allowed a lowering of the current detection limit to 4 pmol N_2 h^{-1} g^{-1} dry soil (≈ 4 µg N_2-N m^{-2} h^{-1}). A steady state approach based on ambient air and ^{15}N tracer technique published by Ruser et al. (2006) resulted in a determination limit of ≥ 180 µg N_2-N m^{-2} h^{-1}. Compared to ambient air, however, diffusion rates of soil gases towards the headspace of an artificial helium atmosphere might be expected to differ. Scholefield et al. (1997a) and Butterbach-Bahl et al. (2002) published that at least for N_2O, no interferences concerning amounts and efflux rates could be observed.

The lengthy flushing of a soil core should affect the soil moisture. Butterbach-Bahl et al. (2002) published that no significant decrease in soil moisture during up to 48 h initial flushing (200 mL min^{-1}) through a soil core had been observed. However, during this study alone the continuously streaming carrier gas above the soil surface with 6.4 mL min^{-1} caused a significant decrease in soil moisture. After Parkin et al. (1984) this could be solved by hydrating the carrier gas immediately in front of the incubation entities. Cardenas et al. (2003) proposed that a permanent gas flow could readily be used for studies concerning the influence of different wind speeds as the flow rate of the carrier gas would alter accordingly.

In the absence of isotope tracer technique, flux determination starts with a time-lag of 36 to 48 h after incubation, since soil cores must to be flushed initially (up to 200 mL min^{-1}) in order to

displace atmospheric N_2 by an artificial substitute (e.g. helium) (Scholefield et al., 1997a; Butterbach-Bahl et al., 2002; Cardenas et al., 2003). By using the ^{15}N tracer technique a ^{15}N tracer has to be introduced, homogeneously, into the soil, but flux determination can be started quite near to the beginning of incubation.

Taking all of the above into account, the developed approach represents a comprehensive method for a continuous, long-term determination of soil N_2 release at a high sampling frequency combined with a very low determination limit.

3.5.2. Some aspects of N-transformation in soil aggregates gained by the present approach

The determined N_2 fluxes of black earth soil aggregates (0.2 to 640.2 pmol N_2 h^{-1} g^{-1} dry soil; 0.2 to 742.9 µg N_2-N m^{-2} h^{-1}) were in the same range as published elsewhere. Based on soil dry mass Malone et al. (1998) published N_2 fluxes between 235 and 5260 pmol N_2 h^{-1} g^{-1} dry soil. In relation to the horizontal soil surface, fluxes from < 0.1 to 18.75 mg N m^{-2} h^{-1} have been published (Nommik, 1956; Reddy et al., 1989; Russow et al., 1996; Scholefield et al., 1997b; Delaune et al., 1998; Butterbach-Bahl et al., 2002; Cardenas et al., 2003; Ruser et al., 2006).

Significant N_2 fluxes occurred only during anaerobic incubation (Figure 3.6). However, compared to former studies the highest fluxes seen were rather low; this is assumed to be mainly caused by the low depth of soil samples used during this study. During aerobic incubation determined N_2 fluxes were extremely low, since soil aggregates were loosely packed within the steel core, which permitted an unresisted and rapid spreading of the artificial He/O_2 atmosphere through the whole core. In combination with the small diameter of the aggregates as well as the medium water content the O_2 supply to the soil aggregates were thus sufficient to inhibit denitrification almost completely. Nevertheless N_2 was released during denitrification, even under 21 vol.% O_2 concentration and could be measured only due to the very low determination limit of the present approach. Similar results concerning denitrification within soil aggregates during aerobic incubation (20.4 kPa O_2) have been published elsewhere for N_2O (Khalil et al., 2004). This lends support to former results of anaerobic microsites within soil aggregates (Smith, 1980; Renault and Stengel, 1994) and/or to aerobic denitrification (Lloyd et al., 1987). During anaerobic incubation the ^{15}N abundance of denitrified NO_3^- increased significantly (Figure 3.5). We assume that during ongoing denitrification introduced NO_3^- is more strongly delivered to microorganisms compared to the original soil NO_3^-. This implies that soil NO_3^- as a whole doesn't need to be equally involved within the soil N-cycle. Stevens et al. (1997) found that the ^{15}N abundance of extractable soil NO_3^- was lower as the ^{15}N abundance of denitrified N_2O, an observation which they explained by an incomplete mixing of soil NO_3^- and added NO_3^-. The decreasing ^{15}N abundance of denitrified NO_3^- calculated via N_2O during aerobic incubation revealed that a direct coupling

between nitrification and denitrification occurred. Similar results have been published by Russow et al. (2000).

A comparison of the initial and the final amounts of $^{15}NO_3^-$ (597.0 ±63.4 µg ^{15}N vs. 62.2 ±10.9 µg ^{15}N per incubation entity) stocked within an incubation entity shows, that approximately 90 % of added ^{15}N atoms have left the soil NO_3^- pool after 25 days of continuous incubation. Using both the ^{15}N amount released via N_2O and N_2 during anaerobic incubation (207.6 ±13.4 µg ^{15}N in average per incubation entity) as well as the ^{15}N amount stocked in the whole soil N pool (123.4 ±19.7 µg ^{15}N in average per incubation entity) at the end of incubation gives a deficiency of around 45 % with respect to the originally added ^{15}N atoms. Since significant amounts of N_2 and N_2O have been released only during anaerobic incubation, this discrepancy can not be explained by a gaseous loss of ^{15}N (N_2O + N_2) during aerobic incubation. We assume this might be explained by a strong release of NO via denitrification during anaerobic conditions as a result of given soil structure and bulk density.

3.6. Conclusions

The developed technique represents the first approach combining a ^{15}N tracer technique with the gas flow soil core technique using an artificial atmosphere for an online determination of soil N_2 release. The application of the zeolite Köstrolith SX6® significantly improved the N_2 sampling procedure. In combination with the ^{15}N aided artificial atmosphere gas flow technique it allows an excellent online determination of the soil N_2 release simultaneously with N_2O. Fluxes of both gases can be determined continuously for long incubation periods and at a high sampling frequency. Due to the application of ^{15}N tracer technique the N_2 flux determination limit could be lowered to 4 pmol N_2 h^{-1} g^{-1} dry soil (≈ 4 µg N_2-N m^{-2} h^{-1}). Furthermore the ^{15}N tracer technique allows investigations concerning the metabolic pathways of N_2 and N_2O (denitrification/nitrification). In addition to its application in soil science, detailed studies of anaerobic ammonium oxidation (anammox) (Mulder et al., 1995) should also be possible by applying the developed approach to aquatic samples instead of to solid samples

4. A novel ^{15}N tracer model for studying hybrid N_2 formation due to anammox or codenitrification

4.1. Abstract

Denitrification and anaerobic ammonium oxidation (anammox) have been identified as biotic key processes of N_2 formation during global nitrogen cycling. Based on the principle of a ^{15}N tracer technique new analytical expressions have been derived for a calculation of the fractions of N_2 simultaneously released by anammox and denitrification. An omnipresent contamination with atmospheric N_2 is also taken into account and furthermore calculable in terms of a fraction. Two different mathematical approaches are presented which permit a precise calculation of the contribution of anammox, denitrification, and atmosphere to a combined N_2 mixture. The calculation is based on a single isotopic analysis of a sampled N_2 mixture and the determination of the ^{15}N abundance of NO_2^- and NO_3^- (simplified approach) or of NH_4^+, NO_2^-, and NO_3^- (comprehensive approach). Calculations are even processable under conditions where all basal educts of anammox and denitrification (NH_4^+, NO_2^-, and NO_3^-) are differently enriched in ^{15}N. An additional determination of concentrations of dissolved N compounds is unnecessary. Finally, the present approach is also transferable to studies focused on terrestrial environments where N_2 is formed by denitrification and simultaneously by codenitrification or chemodenitrification.

4.2. Introduction

N_2 is one of the major gaseous nitrogen compounds released by soils and aquatic environments during global nitrogen cycling (Galloway et al., 2004). Denitrification (Knowles, 1982) and anammox (van de Graaf et al., 1990) have been identified as biotic key processes of N_2 formation in the course of nitrogen cycling (Op den Camp et al., 2006; Davidson and Seitzinger, 2006). During anammox and denitrification N_2 is formed on the basis of two different metabolic pathways of microbial N transformation. Whereas N_2 gained by anammox is formed as a hybrid species due to a combination of two single N atoms of two different N species (NH_4^+-N combined with NO_2^--N) (Jetten et al., 1999), denitrified N_2 represents a combination of two single N atoms based on one single N species (e.g. NO_2^-) (Knowles, 1982). Recent studies revealed that both processes do coexist at least in aquatic environments (van de Graaf et al., 1997; Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Kuypers et al., 2003; Risgaard-Petersen et al., 2003; Rysgaard et al., 2004; Dalsgaard et al., 2005).

During the last four decades ^{15}N tracer technique (Faust, 1993) has been evolved as the most suitable tool with respect to investigations of N_2 formation by denitrification or anammox (Reddy et al., 1989; Malone et al., 1998; Dalsgaard et al., 2003; Kuypers et al., 2003; Ruser et al., 2006; Groffman et al., 2006). However, only three ^{15}N aided approaches have been developed to estimate a simultaneous N_2 formation by anammox and denitrification

(Thamdrup and Dalsgaard, 2002; Risgaard-Petersen et al., 2003; Trimmer et al., 2006). Another ^{15}N based approach (Laughlin and Stevens, 2002) facing this problem was published currently with respect to an N_2 formation in the course of denitrification and codenitrification in soils. Considering the formation of hybrid N-N gas species codenitrification is a microbiological process (Tanimoto et al., 1992) quite similar to that of anammox. The principle applicability of former approaches was shown in a few studies (Laughlin and Stevens, 2002; Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Risgaard-Petersen et al., 2003; Trimmer et al., 2003; Rysgaard et al., 2004; Risgaard-Petersen et al., 2004; Thamdrup et al., 2006). Nevertheless, basal difficulties coupled to these methods need to be discussed in detail.

The first ^{15}N based approach quantifying N_2 formation in the course of anammox and denitrification was published by Thamdrup and Dalsgaard (2002). Considering the distribution of ^{15}N and ^{14}N within process related N compounds the quantification of anammox and denitrification is based either on a time dependent production rate of the N_2 mole masses 29 and 30 ($^{14}N^{15}N$ and $^{15}N^{15}N$, respectively) in case of $^{15}NO_3^-$ treatment or on time dependent production and/or consumption rate of $^{15}NH_4^+$, NO_2^-, and NO_3^- in case of $^{15}NH_4^+$ treatment. Thus, each N species needs to be analysed leastwise at two different time steps to estimate N_2 production. Moreover, since all calculations are expressed as total N amounts (mol) quantifications based on $^{15}NH_4^+$, NO_2^-, and NO_3^- need to consider other processes (e.g. microbial assimilation of NH_4^+) interfering with the production and/or consumption of $^{15}NH_4^+$, NO_2^-, and NO_3^- during an incubation. Hence, experimental efforts increase, since separate incubation experiments are necessary to quantify these additional processes. Furthermore, the given equations derived for a quantification of N_2 production based on a $^{15}NO_3^-$ treatment neglect the fact that under the given experimental conditions ($\approx$ 95 at% $^{15}NO_3^-$ and 0.366 at% $^{15}NH_4^+$; see Thamdrup and Dalsgaard, 2002) both anammox and denitrification will produce the N_2 masses 28, 29, and 30. First, anammox is assumed to produce only mass 28 and 29 of N_2 when only NO_3^- is highly enriched in ^{15}N. And second, based on the assumption that anammox produces only mass 28 and 29, respectively, mass 30 of a sampled N_2 mixture is completely assigned to denitrification. These assumptions, however, contradicts with a possible formation of mass 30 in the course of anammox due to a combination of $^{15}NH_4^+$ and $^{15}NO_3^-$ (see Figure 4.1). The impact of these assumptions on the estimation of an N_2 production in the course of both processes may be negligible, but only as long as NH_4^+ remains at natural ^{15}N abundance (0.366 at% ^{15}N) (e.g. Trimmer et al., 2003) and NO_3^- remains highly enriched in ^{15}N (e.g. Thamdrup et al., 2006). However, it was already shown by Trimmer et al. (2003) that during an incubation experiment based on ^{15}N labelled NO_3^- a labelling of NH_4^+ could occur due to dissimilatory nitrate reduction to ammonium (DNRA). Moreover, Kartal et al. (2007) recently reported that the anammox bacteria *Kuenenia stuttgartiensis* reduced $^{15}NO_3^-$ to $^{15}NH_4^+$ via $^{15}NO_2^-$ as the intermediate which was followed by the anaerobic oxidation of the produced ^{15}N labelled NH_4^+ and NO_2^-

resulting in a significant $^{15}N^{15}N$ production. It is apparent that in consequence of DNRA N_2 production during anammox would be underestimated whereas denitrification could be significantly overestimated when the approach given by Thamdrup and Dalsgaard (2002) is applied. Finally, an interference from atmospheric N_2 (as an omnipresent contaminant) is not optimally taken into account, since it is estimated based on a single reference sample. However, an equal contamination with atmospheric N_2 among parallel samples can not be assured. Moreover, an additional contamination during sampling and measuring procedure can also not be excluded.

A second approach quantifying N_2 formation of anammox and denitrification was published by Risgaard-Peterson et al. (2003). This approach is based on the Isotope Pairing Technique (Nielsen, 1992) (IPT) and was developed in consequence of confronting the IPT (originally designed for denitrification measurements only) with a simultaneous occurring anammox process. It represents a calculation of microbial N_2 production in aquatic sediments associated with error estimation due to anammox. However, severe difficulties connected with the approach were already reported (see Risgaard-Petersen et al., 2003; Rysgaard et al., 2004; Trimmer et al., 2006). Similar to Thamdrup and Dalsgaard (2002), calculations are based on time dependent production rates of masses 29 and 30 when NO_3^- was enriched in ^{15}N. Therefore, each molecular mass needs to be analysed at least at two different time steps to estimate the current N_2 production of anammox and denitrification. Moreover, the anammox process is also mistakenly assumed to form only mass 28 and 29 of N_2 which leads to comparable erroneous calculations as already discussed above. Furthermore, a contamination by atmospheric N_2 is also not optimally taken into account, since it is also based on measurements of a single reference sample.

The most recent approach was presented by Trimmer et al. (2006). This approach is also based on the IPT (Nielsen, 1992) and represents a further development of the revised IPT (r-IPT) published by Risgaard-Peterson et al. (2003). An improved estimation of microbial N_2 production in the course of anammox and denitrification was achieved by using denitrified N_2O in addition to N_2 for a more accurate quantification of N_2 production (Trimmer et al., 2006). Nevertheless, since it is based on the same experimental principles as approaches discussed above similar difficulties have to be considered.

A fourth ^{15}N aided approach was published earlier by Laughlin and Stevens (2002) and was focused on a calculation of denitrified and codenitrified N_2 simultaneously produced in soils. The approach presented by them is based on common $^{15}NO_3^-$ treatments of soils and is associated with equations published by Siegel et al. (1982), Mulvaney (1984), Stevens and Laughlin (2001b), and Clough et al. (2001). In contrast to approaches discussed above, these mathematical expressions allow a calculation based on only one analysis of the mole fraction of mass 28, 29, and 30 within an N_2 mixture and the ^{15}N abundance of soil NO_3^-. Moreover, a contamination of a sampled N_2 mixture with atmospheric N_2 is taken into account based on the isotopic analysis of the sampled N_2 mixture. However, three principle mathematical

assumptions of this approach do also not fully correspond to the contribution of codenitrification, denitrification, and atmosphere to the final mole fraction of masses 28, 29, and 30 within a sampled N_2 mixture. First, mass 30 is completely assigned to denitrification without considering a possible contribution of codenitrification. Second, calculated N_2 production in the course of codenitrification is based on mass 29 only. Third, mass 28 is completely assigned to atmospheric N_2 without considering codenitrification or denitrification.

Taking all the above into account, former approaches do not allow a precise calculation based on analytical expressions, but serve as estimators for a simultaneous N_2 release in the course of denitrification and anammox (or codenitrification). Particularly, aspects like (i) interferences with atmospheric N_2, (ii) a low ^{15}N abundance of NO_3^-, and (iii) experimental conditions where NH_4^+ and NO_3^- are simultaneously enriched in ^{15}N could cause severe calculation errors. In order to overcome all of these difficulties and also considering the growing demand of a quantitative survey of N_2 formation by anammox and denitrification within natural environments (Galloway et al., 2004; Davidson and Seitzinger, 2006) and waste water treatment systems (Schmidt et al., 2003; Paredes et al., 2007) (e.g. fluidized batch reactor (Mulder et al., 1995; van de Graaf et al., 1997)) two mathematical approaches were developed based on a ^{15}N tracer technique. Derived analytical expressions allow a precise calculation of the contribution of denitrification and anammox to an N_2 mixture and in addition take into account a contamination with atmospheric N_2. The present chapter focuses on derivation of fundamental mathematical expressions and discusses in detail the applicability of the developed approaches.

4.3. Derivation of mathematical expressions

4.3.1. Fundamentals

A ^{15}N based characterisation of an N_2 mixture formed by anammox N_2, denitrified N_2, and atmospheric N_2 requires definite mathematical expressions which allow a precise calculation of the contribution of each individual source to the resulting mole fraction of the masses 28, 29, and 30 within the N_2 mixture. Based on probability theory this can be best solved by expressing the respective mole fractions of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ (referred here to as m_{28}, m_{29}, and m_{30}) with the isotopic abundance of involved ^{14}N and ^{15}N atoms (referred here to as a_{14} and a_{15}, respectively) which form the N_2 molecule. With regard to atmospheric N_2 the mole fractions of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ can mathematically be expressed by a binominal distribution function where (i) $m_{28} = (a_{14})^2$; (ii) $m_{29} = 2a_{14}a_{15}$; (iii) $m_{30} = (a_{15})^2$; and (iv) $(a_{14})^2 + 2a_{14}a_{15} + (a_{15})^2 = 1$ (e.g. Hauck et al., 1958). However, with respect to a ^{15}N based characterisation of anammox and denitrification, which in fact represent active N_2 forming processes, some more detailed explanations shall be given here in order to clearly demonstrate differences and similarities between the mathematical expressions applied to these two processes. With

regard to fundamental probability algebra the mole fraction of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ of each individual N_2 source considered here is expressible as the probability of an occurrence of the event $^{14}N^{14}N$, $^{14}N^{15}N$, and $^{15}N^{15}N$, respectively. By using the isotopic abundance of involved ^{14}N and ^{15}N atoms which forms a N_2 molecule of either mass 28, 29, or 30, respectively, the mole fractions of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ can mathematically be expressed as follows: (i) $m_{28} = a_{14} \cdot a'_{14}$; (ii) $m_{29} = a_{14} \cdot a'_{15} + a_{15} \cdot a'_{14}$; (iii) $m_{30} = a_{15} \cdot a'_{15}$ (where a and a' represent the isotopic abundances (^{14}N and ^{15}N, respectively) of the sources of the two N atoms which form a N_2 molecule). With respect to anammox a and a' describe two different N sources (NH_4^+ and NO_2^-) and thus the given mathematical expressions represent the fundamental equations which have to be applied considering the formation of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ during an anammox process. By contrast, N_2 formation during denitrification is based on only one N source (e.g. NO_3^-) and thus a and a' are equal. It follows that with respect to denitrification the given mathematical expressions can be simplified to the commonly used binomial distribution function: (i) $m_{28} = (a_{14})^2$; (ii) $m_{29} = 2a_{14}a_{15}$; (iii) $m_{30} = (a_{15})^2$. With regard to anammox a simplification to a binominal distribution function can only be made in the special case of an equality of the ^{15}N abundances of NH_4^+ and NO_2^-. A schematic overview of the formation of an N_2 mixture evolved from anammox, denitrification, and atmosphere concerning the fundamental mathematical expressions given above is illustrated in Figure 4.1. According to this, the contribution of each considered source to the mole fraction of mass 28, 29, and 30 of the final N_2 mixture can mathematically be expressed as follows (variables used are listed in Table 4.1):

Equation 4.1 $$\alpha_{28} = A(1-a)^2 + B(1-b)^2 + C(1-c)(1-d)$$

Equation 4.2 $$\alpha_{29} = A2a(1-a) + B2b(1-b) + C\left[c(1-d) + d(1-c)\right]$$

Equation 4.3 $$\alpha_{30} = Aa^2 + Bb^2 + Ccd$$

Where the sum of the fractions of all three N_2 sources (A, B, and C) as well as the sum of the mole fraction of mass 28, 29, and 30 (α_{28}, α_{29}, and α_{30}) within an N_2 mixture must be one:

Equation 4.4 $$1 = A + B + C$$

Equation 4.5 $$1 = \alpha_{28} + \alpha_{29} + \alpha_{30}$$

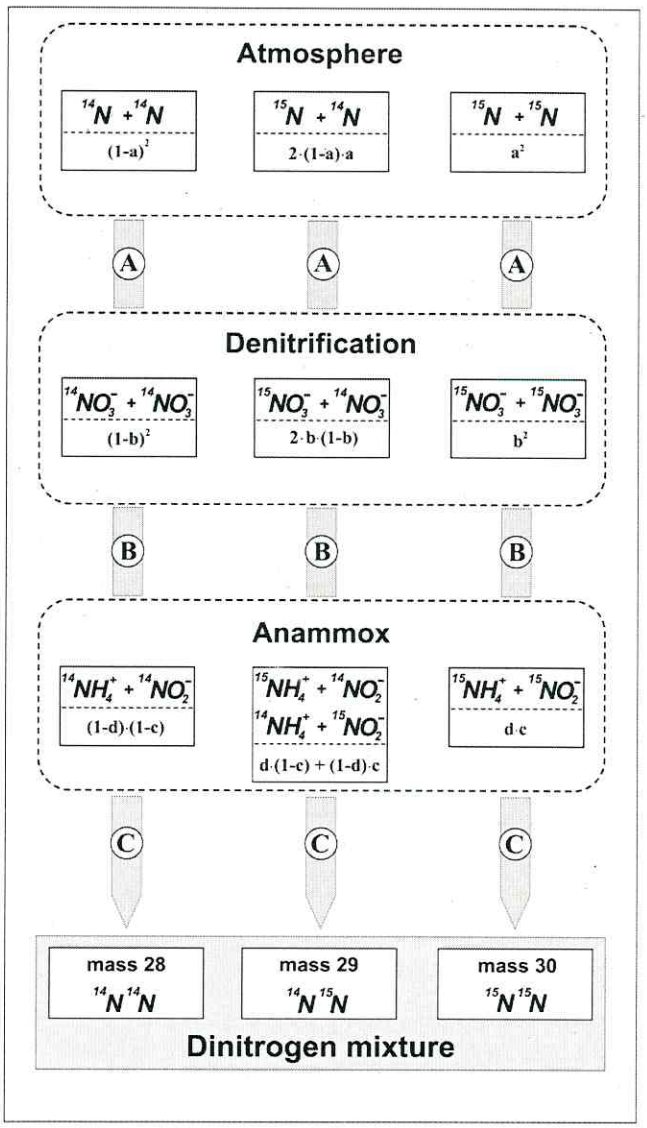

Figure 4.1 *Formation scheme of an N_2 mixture originating from atmospheric N_2, denitrified N_2, and anammox N_2 with respect to the fundamental mathematical expressions, which characterise the source of the mole fraction of masses 28, 29, and 30 (variables used are listed in Table 4.1).*

Based on Equation 4.1 to Equation 4.5 two different mathematical approaches were developed for the calculation of the contribution of denitrification and anammox to an N_2 mixture in consideration of atmospheric N_2. The first approach (comprehensive approach) was developed for experimental conditions where all of the basal nitrogen sources of denitrification (NO_3^-) and anammox (NH_4^+ and NO_2^-) are enriched in ^{15}N. However, since the anammox process is also calculable based on a ^{15}N enrichment of only one of the two

involved N species a second approach (simplified approach) was developed based only on a ^{15}N enrichment of NO_2^- and NO_3^-. Thus, the simplified approach allows less complex experimental conditions for the determination of a simultaneous N_2 production by anammox and denitrification, and still takes into account atmospheric N_2 contamination.

Table 4.1 *List of variables used for calculations related to Equation 4.1 to Equation 4.16.*

Variable	Range	Designation
$\alpha_{28...30}$	[0 ... 1]	Mole fraction of mass 28, 29, and 30 within the N_2 mixture
$a...d$	[0 ... 1]	^{15}N mole fraction of N_2 (a), NO_3^- (b), NO_2^- (c), and NH_4^+ (d)
$A...C$	[0 ... 1]	Mole fraction of atmospheric N_2 (A), denitrified N_2 (B), and anammox N_2 (C)

4.3.2. Comprehensive approach

The comprehensive approach permits a calculation of the fraction of atmosphere (A), denitrification (B), and anammox (C) as contributors to an N_2 mixture based on the ^{15}N abundance of atmospheric N_2 (a), NO_3^- (b), NO_2^- (c), and NH_4^+ (d) as well as the mole fraction of masses 28, 29, and 30 (α_{28}, α_{29}, and α_{30}) within the N_2 mixture. Therefore, the mole fraction of mass 28, 29, and 30 within the N_2 mixture and the ^{15}N abundance of NO_3^-, NO_2^-, and NH_4^+ need to be measured whereas the ^{15}N abundance of atmospheric N_2 is assumed to be at natural condition (0.366 at%). The validity of the comprehensive approach is coupled to the following assumptions: (i) atmosphere, denitrification, and anammox are the only sources contributing to the N_2 mixture; (ii) NH_4^+ and/or NO_2^- as well as NO_3^- are enriched in ^{15}N abundance; (iii) ^{15}N abundance of at least one of the inorganic N species (NH_4^+, NO_2^-, or NO_3^-) must be unequal compared to the others; (iv) anammox N_2 is based on a combination of NH_4^+ and NO_2^- at a stoichiometric ratio of 1:1; and (v) N sources of anammox and denitrification are homogeneously labelled with respect to their spatial distribution throughout the investigated environment.

Based on a Gaussian elimination approach Equation 4.1 to Equation 4.3 were solved to the variables A, B, and C and further simplified to Equation 4.6 to Equation 4.to calculate the fraction of atmosphere (A), denitrification (B), and anammox (C) which contribute to the N_2 mixture.

Equation 4.6
$$A = \frac{2b(\alpha_{30} - cd) + (c+d)(b^2 - \alpha_{30}) - (b^2 - cd)(\alpha_{29} + 2\alpha_{30})}{(a-b)\left[2(ab+cd) - (a+b)(c+d)\right]}$$

Equation 4.7
$$B = \frac{2a(cd-\alpha_{30})+(c+d)(\alpha_{30}-a^2)+(a^2-cd)(\alpha_{29}+2\alpha_{30})}{(a-b)\left[2(ab+cd)-(a+b)(c+d)\right]}$$

Equation 4.8
$$C = \frac{2ab-\alpha_{29}(a+b)+2\alpha_{30}(1-b-a)}{2(ab+cd)-(a+b)(c+d)}$$

4.3.3. Simplified approach

The simplified approach calculates the fraction of atmosphere (*A*), denitrification (*B*), and anammox (*C*) contributing to an N_2 mixture based on the same input parameters used for the comprehensive approach except for the ^{15}N abundance of NH_4^+ (*d*). In this case only the mole fraction of mass 28, 29, and 30 within the N_2 mixture (α_{28}, α_{29}, and α_{30}) and the ^{15}N abundance of NO_3^- (*b*) and NO_2^- (*c*) need to be measured whereas the ^{15}N abundance of atmospheric N_2 (*a*) and NH_4^+ (*d*) is assumed to be at natural condition (0.366 at%). The mathematical validity of this approach is associated with the following assumptions: (i) atmosphere, denitrification, and anammox are the only sources contributing to the N_2 mixture; (ii) NO_2^- and NO_3^- are enriched in ^{15}N; (iii) ^{15}N abundance of atmospheric N_2 and NH_4^+ is equal ($a = d$); (iv) anammox N_2 is based on a combination of NH_4^+ and NO_2^- at a stoichiometric ratio of 1:1; and (v) N sources of anammox and denitrification are homogeneously labelled with respect to their spatial distribution throughout the investigated environment.

Considering the assumption $a = d$, Equation 4.1 and Equation 4.2 can be rewritten as follows:

Equation 4.9
$$\alpha_{28} = (1-a)\left[A(1-a)+C(1-c)\right]+B(1-b)^2$$

Equation 4.10
$$\alpha_{29} = (1-a)\left[Aa+Cc\right]+a\left[A(1-a)+C(1-c)\right]+2Bb(1-b)^2$$

In order to remove variables *A* and *C* from both equations the term $[A(1-a)+C(1-c)]$ in Equation 4.9 and Equation 4.10 can be rewritten into $[-(Aa+Cc-1+B)]$ and both equations can be rearranged to Equation 4.11 and Equation 4.12:

Equation 4.11
$$\left[Aa+Cc\right] = 1-B-\left[\frac{\alpha_{28}-B(1-b)^2}{(1-a)}\right]$$

Equation 4.12
$$\left[Aa+Cc\right] = \left[\frac{\alpha_{29}-a+B(a-2b(1-b))}{(1-2a)}\right]$$

Equating Equation 4.11 and Equation 4.2 and rearranging to *B* gives a simple equation for the calculation of the fraction of denitrified N_2 (*B*) in dependence on the variables *a*, *b*, α_{29}, and α_{30}.

Equation 4.13
$$B = \frac{a(a-\alpha_{29}) + \alpha_{30}(1-2a)}{(a-b)^2}$$

An equation for the calculation of the fraction of anammox N_2 (*C*) can then subsequently be derived as follows. At first Equation 4.3 is rearranged to *A*, inserted it into Equation 4.4 and simplified to *C* resulting in Equation 4.14.

Equation 4.14
$$C = \frac{a^2 - \alpha_{30} + B(b^2 - a^2)}{(a^2 - ca)}$$

The variable *B* can then be substituted by inserting Equation 4.13 into Equation 4.14 giving a simple equation for the calculation of the anammox fraction (*C*) based on the variables *a*, *b*, α_{29}, and α_{30}.

Equation 4.15
$$C = \frac{(b+a)(\alpha_{29} + 2\alpha_{30}) - 2(\alpha_{30} + ab)}{(a-b)(a-c)}$$

The residual fraction of atmospheric N_2 can be easily calculated by rearranging Equation 4.4 to *A* and either inserting the calculated fractions of denitrification (*B*) and anammox (*C*) or by substituting *B* and *C* in Equation 4.4 with Equation 4.13 and Equation 4.15, respectively, and resolving it to *A*:

Equation 4.16
$$A = 1 - B - C$$

or

$$A = \frac{(a+b)(\alpha_{30} - b^2) - (ac - b^2)(\alpha_{29} + 2\alpha_{30}) - 2b(\alpha_{30} - ac)}{(a-b)^2(a-c)}$$

4.4. Results and discussion

4.4.1. General applicability of derived mathematical expressions

In contrast to all former methods the present mathematical expressions (comprehensive and simplified approach) face most comprehensively the difficulties associated with investigations of N_2 formation within environments where anammox and denitrification do coexist: (i) all equations are based on analytical expressions and thus allow a precise calculation with respect

to the given assumptions; (ii) all three sources contributing to an N_2 mixture (anammox, denitrification, and atmosphere) are adequately considered and exactly calculable as a fraction of the total N_2 mixture; and (iii) the calculation of the fraction of the three sources is based on only one single isotopic analysis of a sampled N_2 mixture and the determination of ^{15}N abundance of either NO_2^- and NO_3^- (simplified approach) or NH_4^+, NO_2^-, and NO_3^- (comprehensive approach). Furthermore, calculations are even processable under conditions where all educts (NH_4^+, NO_2^-, and NO_3^-) are enriched in ^{15}N. Thus, none of other N transforming processes, e.g. nitrification (Mosier and Schimel, 1992) or DNRA (Trimmer et al., 2003), interfere with the calculation. Moreover, a determination of concentrations of dissolved N compounds is unnecessary.

The fractions of N_2 permeated from atmosphere and released by denitrification and anammox (*A*, *B*, and *C*, respectively) can be calculated by means of derived equations as shown above. The total N_2 amount released from each source is then given by the product of the calculated N_2 source fraction and the total N_2 amount of the gas mixture, at which total N_2 of the gas mixture can be quantified by mass spectrometric analysis if a calibration function for N_2 concentration is used (see Chapter 3.3.4 or e.g. (Nielsen, 1992; Thamdrup and Dalsgaard, 2002)). An experimental set-up for the study of N_2 production by anammox and denitrification should be based on a steady state system (open system). Since microbial processes (e.g. DNRA, nitrification etc.) most likely change the ^{15}N enrichment of involved inorganic N species (i.e. NH_4^+ etc.) during incubation, a non-steady state system (closed system) would allow an accumulation of N_2 evolved from equal N compounds but with different ^{15}N enrichments. This, however, contradicts with the assumption that N_2 formed by anammox and denitrification results from homogenously labelled N species. By contrast, within a steady state system currently released N_2 is not accumulated within a headspace and thus will be always based directly on the current ^{15}N enrichment of NH_4^+, NO_2^-, and NO_3^-.

4.4.2. Major critical aspects of application

In contrast to denitrification the complete transformation pathway of nitrogen during anammox is still under investigation (Jetten et al., 1999). Thus, certain difficulties need to be considered when a ^{15}N tracer technique is applied as proposed in chapter 4. The mathematical expressions of anammox as presented here are deeply related to a 1:1 ratio of NH_4^+ and NO_2^- which form the hybrid N_2 molecule. With respect to the consumption rates of both N species in the course of anammox, recent studies (e.g. (Strous et al., 1999b)) revealed an increasing stoichiometric ratio of up to 1:4 in favour of NO_2^- with increasing NO_2^- concentration. Strous et al. (1999b) postulated an internal microbially generated electron donor differing from NH_4^+, but also reducing NO_2^-. As long as this unknown microbial reduction of NO_2^- is not coupled to an additional N_2 formation no interferences with regard to the present approach will occur. However, a redox reaction of N_2H_4 (intermediate of the anammox process (van de Graaf et al., 1997)) with NO_2^- as the electron acceptor has been reported (Schalk et al., 1998)

to form N_2 at a stoichiometric ratio of 3:4. Therefore, additional ^{15}N based studies are necessary to clearly elucidate all crucial transformation pathways coupled to anammox and possibly interfering the basal mathematics presented here. Nevertheless, at least NH_2OH, as most essential intermediate of anammox, has been reported to react with NH_4^+ always at a stoichiometric ratio of 1:1 (van de Graaf et al., 1997). However, up to now it has not been proven whether NH_2OH instead of NO_2^- could be used as a more reliable proxy value for a quantification of N_2 formation by anammox using the given mathematical expressions. A promising method with respect to a readily determination of ^{15}N abundance of NH_2OH in aqueous samples (SPINMAS technique) was published recently by Stange et al. (2007).

A second critical aspect of application arises only for the simplified approach. This approach is related to the assumption of the equality of the ^{15}N abundance of atmospheric N_2 and NH_4^+. In order to estimate the effect of a small discrepancy to that assumption simple error estimations were conducted with respect to the ratio of calculated (subscript *c*) vs real fraction (subscript *r*) of denitrification (B_c/B_r) and anammox (C_c/C_r). In general the following aspects could be deduced. When NH_4^+ is enriched in ^{15}N beyond natural condition (> 0.366 at%) the fraction of denitrification (*B*) is systematically overestimated (Figure 4.2a). By contrast, the fraction of anammox (C) will only be overestimated when ^{15}N abundance of NO_2^- is lower than 50 at% and maximally half as high as ^{15}N abundance of NO_3^- (Figure 4.2b). Otherwise the calculated anammox fraction will be systematically underestimated. The magnitude of the relative error of *B* varies depending on ^{15}N abundance of NO_3^-, NO_2^-, and NH_4^+ (*b*, *c*, and *d*) as well as the ratio of denitrification and anammox. The relative error of *C* varies in dependence on ^{15}N abundance of NO_3^-, NO_2^-, and NH_4^+ (*b*, *c*, and *d*), but is independent from the denitrification/anammox ratio. At a constant ^{15}N enrichment of 1 at% $^{15}NH_4^+$ (Figure 4.2) the relative error of *C* will generally increase with decreasing ^{15}N abundance of NO_3^- as long as the ^{15}N abundance of NO_2^- is at least 50 at%. However, the relative error of C will in general decrease with decreasing ^{15}N abundance of NO_3^- and/or increasing ^{15}N abundance of NO_2^- as long as the ^{15}N abundance of NO_2^- is lower than 50 at% and maximally half as high as ^{15}N abundance of NO_3^-.

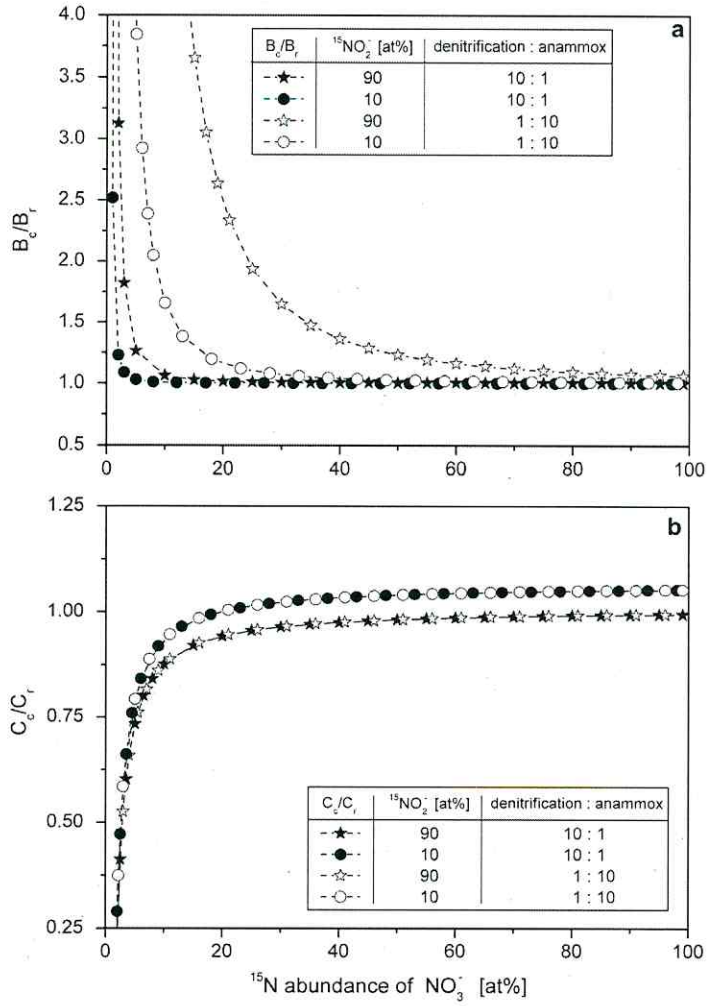

Figure 4.2 *Error estimation (calculated value (c) vs real value (r)) of the simplified approach at a ^{15}N abundance of NH_4^+ of 1 at% in dependence on ^{15}N enrichment of NO_2^- and NO_3^- and the denitrification/anammox ratio: (a) calculation of the fraction of denitrification and (b) calculation of the fraction of anammox. (atmospheric fraction (A) has been set to 0.1; note that B_c/B_r and C_c/C_r are generally independent from A).*

By contrast, the relative error of *B* will in general increase with an increasing ^{15}N abundance of NO_2^- and/or a decreasing ^{15}N abundance of NO_3^-. The fraction of atmospheric N_2 contributing to an N_2 mixture does not influence the magnitude of the B_c/B_r and C_c/C_r ratio. Moreover, under the specific condition of an equal ^{15}N abundance of NO_2^- and NO_3^- ($b = c$) the air fraction (A) would always be calculated correctly by Equation 4.16, independent from the ^{15}N enrichment of NH_4^+ (not illustrated). Under this condition the residual fraction of such an N_2 mixture is at least definable as microbial metabolised N_2 based only on the

determination of the isotopic composition of the N_2 mixture and the ^{15}N abundance of either NO_2^- or NO_3^-. However, with respect to an acceptable estimation of *C* the following conditions need to be fulfilled, when the simplified approach is used under conditions of an ^{15}N enriched NH_4^+ pool: (i) ^{15}N abundance of NH_4^+ should be 1 at% maximally; (ii) ^{15}N abundance of NO_2^- should be 10 at% at least; and (iii) ^{15}N abundance of NO_3^- does not decrease below a value of 20 at%. With regard to a calculation of *B* (denitrified fraction) the following conditions need to be fulfilled: (i) ^{15}N abundance of NH_4^+ should be 1 at% maximally; (ii) ^{15}N abundance of NO_3^- does not decrease below a value of 20 at%; (iii) the ^{15}N abundance of NO_2^- should not exceed that of NO_3^-; and (iv) at least 25 % of the microbial N_2 production results from denitrification. Within these boundary conditions the relative error of *B* and *C* will be 10 % at the most and thus appears acceptable with respect to studies of biological systems. Otherwise the comprehensive approach is recommended to be used for a calculation.

A third critical aspect is the determinability of the ^{15}N abundance of the basal N compounds needed for the calculation. Already published approaches (e.g. Kelley et al., 1991; Højberg et al., 1994; Stevens and Laughlin, 1994; Kieber et al., 1998) are in general time-consuming and mostly consist of several laborious steps. A precise determination of the N_2 production by anammox and denitrification is therefore coupled to extensive experimental efforts in consequence of the determination the ^{15}N abundance of NH_4^+, NO_2^-, and NO_3^-. However, a promising approach (SPINMAS technique) was published recently by Stange et al. (2007) allowing a readily, rapid, and automated determination of the ^{15}N abundance of NH_4^+, NO_2^-, or NO_3^- in aqueous samples.

4.4.3. Application in case of denitrification and codenitrification within soils

It was firstly reported by Shoun et al. (1992) that, additionally to denitrification, codenitrification by soil fungi also produces N_2. A number of studies (e.g. Shoun et al., 1992; Tanimoto et al., 1992; Kumon et al., 2002) demonstrated that (similar to anammox) during this process N_2 is formed by a combination of two N atoms from two different N species, e.g. NH_4^+ and NO_2^-. Until now only Laughlin and Stevens (2002) could show that codenitrification by soil fungi significantly contributes to a soil N_2 release. The present mathematical expressions appear therefore essential with respect to a reliable calculation of the N_2 production within soils where denitrification and codenitrification occur simultaneously. Otherwise severe calculation errors were generated when common ^{15}N based equations were used. As illustrated in Figure 4.3 former approaches would generally overestimate the fraction of denitrified N_2 (or even give a value exceeding 1) when ^{15}N labelled NO_3^- is simultaneously metabolised to NO_2^- and finally to N_2 in the course of codenitrification. With respect to equations presented by Mulvaney and Boast (1986), Khalil et al. (2004) and Equation 3.4 (see chapter 3.3.4) an overestimation depends on ^{15}N

enrichment of NO_3^-, the contamination with atmospheric N_2, and the ratio between denitrified N_2 and codenitrified N_2.

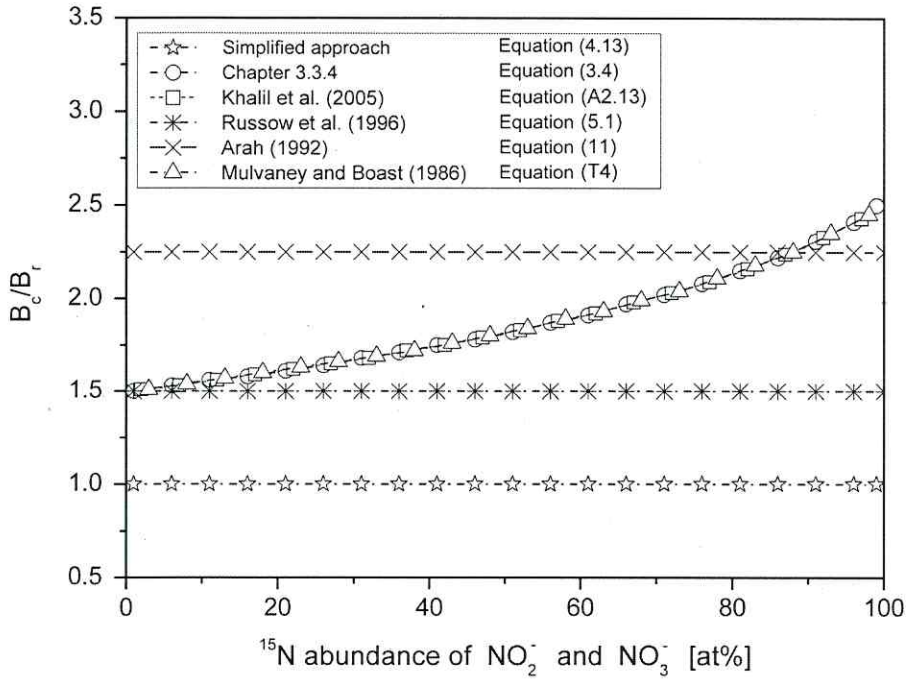

Figure 4.3 *Error estimation [calculated value (c) vs. real value (r)] of the calculated fraction of denitrified N_2 based on a theoretical N_2 mixture evolved from denitrification, codenitrification and atmosphere with respect to values gained by former approaches compared to values gained by Equation 4.13 of the simplified approach. The following conditions were set: denitrification/codenitrification ratio 1:1; atmospheric fraction (A) = 0.2; ^{15}N abundance of NH_4^+ (d) = 0.366 at%; ^{15}N abundance of NO_2^- equals that of NO_3^- (b=c). (Note that all presented approaches are based on equal input variables).*

If $b = c$ (^{15}N abundance of NO_3^- is equal to that of NO_2^-), highest calculation errors with respect to denitrified N_2 occur at high ^{15}N enrichments of NO_3^- and large fractions of atmospheric N_2 and codenitrified N_2. Under the same conditions equations published by Russow et al. (1996) and Arah (1992) do overestimate the fraction of denitrified N_2 only in dependence on the ratio between denitrified N_2 and codenitrified N_2. However, in contrast to all former approaches (Mulvaney and Boast, 1986; Arah, 1992; Russow et al., 1996; Khalil et al., 2004) Equation 4.13 of the simplified approach presented here permits an exact calculation of the fraction of denitrified N_2 based on the same input variables (α_{29}, α_{30}, a, and b), but considers a hybrid N_2 formation by codenitrification which also contributes to the N_2 mixture (Figure 4.3). Hence, if only denitrification has to be determined Equation 4.13 of the

simplified approach should be preferred, if the ^{15}N abundance of NO_3^- is known and another source forming hybrid N_2 can not be excluded. Equation 4.13 would also be correct if the ^{15}N abundance of denitrified NO_3^- differs from codenitrified NO_2^-. However, it should be noted that the calculation would be only accurate if NH_4^+ is at natural ^{15}N abundance (see Figure 4.2). A comparison of values gained by Equation 4.13 and those from former approaches could help to evaluate the impact of other N_2 sources, which may additionally contribute to an N_2 mixture.

Besides a codenitrification, abiotic denitrification (e.g. chemodenitrification) has been also reported to produce N_2 in soils based on an abiotic reaction combining one N atom of NO_2^- with one N atom of an organic N compound (Ewing and Bauer, 1966; Thorn and Mikita, 2000; Kappelmeyer et al., 2003). However, even if codenitrification and chemodenitrification would occur simultaneously with denitrification, Equation 4.13 would be still correct as long as chemodenitrification and codenitrification use N compounds with equal ^{15}N enrichments. This should be mostly the case, if only NO_3^- is labelled with ^{15}N in order to determine N_2 formation due to denitrification.

4.5. Conclusions

With respect to a ^{15}N tracer technique new analytical expressions were derived allowing a precise calculation of the fractions of three different N_2 sources simultaneously contributing to an N_2 mixture, and which are based on (i) atmospheric N_2; (ii) N_2 generated by a process combining two single N atoms gained from one N species (e.g. denitrification of NO_3^- Knowles, 1982); and (iii) N_2 evolved as a hybrid due to a combination of two single N atoms gained from two different N species (e.g. utilisation of NH_4^+ and NO_2^- by anammox Jetten et al., 1999). The calculation of the fraction of anammox, denitrification, and atmosphere is based on only one single isotopic analysis of a sampled N_2 mixture and the ^{15}N abundance of NO_2^- and NO_3^- (simplified approach) or NH_4^+, NO_2^-, and NO_3^- (comprehensive approach). In contrast to former approaches one essential advantage is an adequate consideration of atmospheric N_2 as an omnipresent contaminant. Since it is included in all calculations and furthermore precisely quantifiable as a fraction, the demands on purity during experimental studies are less extensive concerning a contamination by atmospheric N_2. Moreover, since the present comprehensive approach is also valid under conditions where all educts (NH_4^+, NO_2^-, and NO_3^-) are enriched in ^{15}N, other N transforming processes (e.g. nitrification; Mosier and Schimel, 1992) or dissimilatory nitrate reduction to ammonium (Trimmer et al., 2003) do not interfere with the calculation. Therefore, present mathematical expressions handle most comprehensively the difficulties associated with ^{15}N based investigations of N_2 formation within environments where anammox and denitrification coexist.

Instead of anammox the present approach could also be applied for soil studies focused on N_2 and N_2O formation by denitrification and simultaneous codenitrification (Shoun et al., 1992; Tanimoto et al., 1992) or by denitrification and chemodenitrification (Kappelmeyer et al.,

2003). Furthermore, based on the same input variables as required in former approaches (Mulvaney and Boast, 1986; Arah, 1992; Russow et al., 1996; Khalil et al., 2004) the presented simplified approach (Equation 4.13) permits a precise quantification of denitrified N_2 or N_2O, since it considers a simultaneous production of hybrid N_2 or N_2O (e.g. by codenitrification). Nevertheless, it needs to be proven yet how far the present mathematical equations will help to elucidate these processes with respect to future experimental studies.

5. Formation of hybrid N_2O in a suspended soil due to codenitrification of NH_2OH

5.1. Abstract

A few studies have shown that amine compounds (e.g. hydroxylamine) can be co-metabolically introduced into the reaction pathway of denitrification. During this microbial process the nitrogen atom of the amine species is bound to a nitrogen atom of nitrite. In case of hydroxylamine this concomitant reaction ultimately results in the formation of hybrid N_2O. Due to its co-metabolic character the process has been termed codenitrification. Hybrid N_2O production during codenitrification has been proven to occur in prokaryotic (e.g. *Pseudomonas sp.*) as well as eukaryotic (e.g. *Fusarium sp.*) species. Many of them are already well known as common denitrifiers. However, until now no clear evidence has been provided to show that N_2O production by codenitrification really takes place in a soil. In the present study a formation of hybrid N_2O was revealed by an adapted ^{15}N tracer model, when both hydroxylamine and [^{15}N]-nitrate were applied (mol ratio 10:1) to an anaerobically incubated soil suspension (Haplic Chernozem). The presence of hybrid N_2O was also indicated by a novel characteristic factor (R_{binom}) developed for a hybrid N-N gas detection. By contrast, no hybrid N_2O was found when either an autoclaved soil suspension, only nitrate or only hydroxylamine was used. Thus it appears that hybrid N_2O formation occurred due to codenitrification of hydroxylamine. Hence, this is the first study which demonstrates hybrid N_2O production by codenitrification beyond a microbial species level. The ^{15}N tracer model revealed that under the given experimental conditions N_2O production by codenitrification prevailed against N_2O from denitrification and abiotic hydroxylamine decomposition. In addition, a formation of hybrid N_2 was also calculated by the model. However, the experimental results lead to the conclusion that it was most likely caused by a reduction of hybrid N_2O due to conventional denitrification.

5.2. Introduction

During the last decades many studies have revealed that microbial nitrous oxide (N_2O) production in terrestrial environments appears to be more complex than commonly thought. Biotic N_2O production includes a broader variety of microbial species, reaction pathways, and nitrogen (N) substrates (Hayatsu et al., 2008; Jetten, 2008). Jetten (2008) recently pointed out that even today the microbial world still hides an enormous biodiversity and metabolic capability. The unique and less known process of co-metabolic denitrification (i.e. codenitrification) appears to perfectly demonstrate this. Codenitrification was shown to produce N_2O in an intriguing reaction pathway, which permits a variety of N compounds (atypical for denitrification) to be co-metabolized during denitrification (Tanimoto et al., 1992; Immoos et al., 2004; Su et al., 2004). Nonetheless, it has remained almost unregarded

since its first description in 1992 (Tanimoto et al., 1992) and still today belongs to those "microbial worlds" aloof from scientific perception.

According to Tanimoto et al. (1992) the phenomenon of codenitrification refers to a microbially mediated process (described for *Fusarium oxysporum* MT-811) which utilizes inorganic (e.g. ammonium – NH_4^+) and organic N compounds (e.g. salicylhydroxamic acid – $C_7H_7NO_3$) totally different to the known denitrification pathway (i.e. $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$) to form N_2O under conditions favorable for denitrification. Codenitrification is only induced by the major electron acceptors of denitrification (NO_2^- and NO_3^-), while the co-metabolically utilized N species are incapable of inducing this cooperative respiration process alone. By means of a ^{15}N tracer technique Tanimoto et al. (1992) revealed that N_2O gas production due to codenitrification forms a hybrid N gas species. The N-N bond originates from a combination of an N atom from NO_2^- and an N atom from the co-metabolized compound (e.g. NH_4^+ or organic N). Concomitantly, also non-hybrid N_2O is formed by conventional denitrification.

However, microbial N_2O production in the sense of codenitrification, as later on defined by Tanimoto et al. (1992), has been already reported early (Iwasaki and Mori, 1958; Matsubara, 1970; Renner and Becker, 1970; Garber and Hollocher, 1982; Kim and Hollocher, 1984; Aerssens et al., 1986; Weeg-Aerssens et al., 1987/1988; Hulse et al., 1989; Ye et al., 1991). Some of these earlier studies (e.g. Garber and Hollocher, 1982) postulated that an N-nitrosation reaction during denitrification might be responsible for the observed co-metabolism. In this type of reaction an N atom of an enzyme-bound electrophilic mono-nitrogen intermediate of the denitrification pathway can bond to an N atom of a nucleophilic compound (e.g. amine compound; $R\text{-}NH_2$). The resulting hybrid N-N linkage can finally be found in the N_2O gas released by this reaction.

So far, hybrid N_2O formation due to codenitrification has only been revealed by cell suspension experiments or purified enzymes of denitrifying species (e.g. Ye et al., 1991; Immoos et al., 2004; Su et al., 2004). Until now codenitrification in a soil has only been proven by one single study (Laughlin and Stevens, 2002). They reported that soil N_2 formation in a Typic Dystrochrept (acidic brown earth) was clearly dominated by fungi species which largely produced hybrid N_2 (up to 92 %) due to codenitrification. By contrast, soil N_2O release was found to only be related to denitrification. To our best knowledge N_2O formation by codenitrification has never been proven to really occur in a soil. Nonetheless, it has at least been reported for prokaryotic (e.g. *Pseudomonas sp.*) and eukaryotic (e.g. *Fusarium sp.*) species, which are well-known in soil N cycling (Kim and Hollocher, 1984; Tanimoto et al., 1992). Hence, it is reasonable to assume that N_2O formation by codenitrification also occurs in a soil.

Production of N_2O via codenitrification has generally been reported with hydroxylamine (NH_2OH) as the co-metabolized N species (Iwasaki and Mori, 1958; Renner and Becker, 1970; Matsubara, 1970; Garber and Hollocher, 1982; Kim and Hollocher, 1984; Aerssens et

al., 1986; Hulse et al., 1989). NH_2OH appears particularly interesting as it represents a well-known key compound of terrestrial N cycling. As already shown by others (Lees, 1952; Verstraete and Alexander, 1972) NH_2OH is an essential intermediate during the nitrification process. In addition, it has also been proven to be directly involved in soil N_2O production via nitrification (Khalil et al., 2004) as well as via abiotic NH_2OH decomposition (Bremner et al., 1980; Nelson, 1985).

Analogue to former microbial studies (Iwasaki and Mori, 1958; Renner and Becker, 1970; Matsubara, 1970; Garber and Hollocher, 1982; Kim and Hollocher, 1984; Aerssens et al., 1986) the present study focuses on hybrid N_2O production by codenitrification of NH_2OH in an anaerobically incubated soil suspension. An adapted ^{15}N tracer model is applied to evaluate simultaneous N_2O production by codenitrification and denitrification. In addition, the novel characteristic factor R_{binom} (see chapter 5.7.1) is introduced to improve the identification of hybrid N-N gas formation.

5.3. Materials and Methods

5.3.1. Soil treatment and incubation

Soil suspension experiments were conducted with a Haplic Chernozem (A-horizon). The soil was sampled from an agricultural research site (Bad Lauchstädt, Germany) described in detail by Körschens et al. (1981). Sampled soil was stored at a constant temperature of 4 °C, and only subsamples have been used for a series of five individual ^{15}N tracer experiments. For each ^{15}N tracer experiment (described in the next paragraph) a subsample of 150 g of stored soil has been prepared for incubation as follows. The subsample was mixed with 300 ml distilled H_2O and stirred manually with a spoon for one minute. Afterwards, gained soil suspension (pH-value 7.8 ±0.1; n = 5) was divided into two equal replicates of 150 ml (approx. 25 g of dry soil) each placed in a glass vessel (230 ml volume). In addition, a magnetic stir bar was added to both vessels. Each glass vessel was then placed in a metal core (8 cm in diameter, 6 cm height) of an incubation unit. Afterwards incubation units were sealed, placed on a magnetic stirrer and maintained at ≈25 °C (for all five ^{15}N tracer experiments 26.2 ±1.1 °C in average; n = 479; measured hourly by a digital online thermometer). Each incubation unit was then connected to a separate carrier gas stream (He 5.0, Linde AG, Pullach, Germany) which constantly flowed through the headspace above the soil suspension (flow rate of 27 ml min^{-1}). After the carrier gas had been connected for 10 minutes 5 ml of glucose solution (≈55 µM) was added to each replicate via the gas outlet of its incubation unit (using a sterile syringe and needle). Subsequently replicates were pre-incubated for 72 hours. In addition, soil dry weight was determined for each experiment by drying two subsamples (each approx. 20 g) of stored soil at 105 °C for 24 h. Prior to any experiments being conducted under autoclaved conditions the soil suspension, incubation units, glass vessels, and magnetic stir bars were autoclaved (for 15 min at 121 °C and 2.1 bar; Laboklav, SHP Steriltechnik AG, Germany).

The following five individual ^{15}N tracer experiments were conducted: (I) adding only $^{15}NO_3^-$, (II) adding only $^{15}NH_2OH$, (III) adding only $^{15}NH_2OH$ with autoclaved soil suspension, (IV) adding $^{15}NO_3^-$ + NH_2OH, and (V) adding $^{15}NO_3^-$ + NH_2OH with autoclaved soil suspension. The ^{15}N abundance of added $^{15}NO_3^-$ was 30 at% (applied as $K^{15}NO_3$). Added $^{15}NO_3^-$ solution (5 ml; 1.5 g $^{15}NO_3^-$ L^{-1}) was adjusted to gain an initial NO_3^- concentration in the soil suspension of 50 mg NO_3^- L^{-1} (≈800 µM). To prevent a complete depletion of respirable carbon during incubation glucose (≈55 µM) was also contained in NO_3^- solution. NH_2OH solution (5 ml) was adjusted to gain an initial concentration 10 times of that of NO_3^- on a molar basis (≈8 mM). For that purpose NH_2OH·HCL salt was dissolved in distilled H_2O and immediately adjusted to neutral conditions (pH 7) by adding NaOH solution (32 %). In case of isotopically enriched NH_2OH a ^{15}N abundance of 30 at% was used. In experiment IV and V NH_2OH was applied 1 to 2 h after the addition of the $^{15}NO_3^-$/glucose solution. A time lag was applied for the following two reasons: (i) since codenitrification only occurs in the presence of denitrification, we wanted to assure that $^{15}NO_3^-$ reduction to N_2O/N_2 really occurs before NH_2OH is applied; (ii) as we had to consider that sterilization might have been unsuccessful, we had to assure that after autoclaving no $^{15}NO_3^-$ was reduced to N_2O/N_2. Correspondingly, in experiment II and III a blank solution containing only glucose (≈55 µM) was added prior to $^{15}NH_2OH$ application. All added solutions were always freshly prepared for each experiment. Moreover, all solutions were flushed with helium via a submerged needle for at least 30 min before application. Solutions added to autoclaved soil suspension were applied via a sterilized 0.2 µm filter (Minisart RC 25, Sartorius Stedim Biotech GmbH, Germany).

During incubation N_2O and N_2 gas release was determined at an interval of 1 to 2 h (except the night period) as described in the following section. In addition, soil suspension samples (≈5 ml) were taken from each replicate at an interval of 2 to 5 h (except the night period) via the gas outlet of the incubation unit by a sterilized syringe and needle. Solution samples were filtered by a 0.2 µm filter with a Luer Lock coupling. Then they were immediately stored under constant cooling at 4 °C. 1 to 7 days after sampling filtered solution samples was analyzed for $^{15}NO_3^-$ and $^{15}NO_2^-$ by the SPINMAS technique (Stange et al., 2007). The former species was measured in two parallel aliquots (each 2 ml), while the latter was measured in only one aliquot (0.5 ml).

5.3.2. $^{15}N_2O$ and $^{15}N_2$ determination

N_2O/N_2 release was measured based on an artificial atmosphere gas flow incubation system, which is coupled online to a gas chromatograph (GC 5890 Series II; HP – Agilent, USA) and a continuous-flow isotope ratio mass spectrometer (ConFlow IRMS - delta S; Thermo-Finnigan MAT, Bremen, Germany) (see chapter 3). N_2O and N_2 can be sampled by a direct capturing from a carrier gas using pre-concentration traps. During this study N_2O and N_2 was pre-concentrated for 5 to 32 min and 1 min, respectively. In addition, the system was

modified to also allow N_2O/N_2 sampling via a sample loop. The carrier gas was then routed via the loop (0.5, 1, 2 or 4 ml) and switched into the GC-IRMS. This type of gas sampling was applied when the N_2O/N_2 release was too high to be determined by direct capturing. To assure comparable peak shapes for both sampling methods N_2O/N_2 sampled via the loop was pre-concentrated by using the N_2O/N_2 pre-concentration traps within the GC-IRMS circuit. Gas samples were analyzed for $^{14}N/^{15}N$ distribution within N_2O and N_2, respectively. N_2O was reduced to N_2 (copper oven; 750 °C) before measuring. Hence, for each gas species only $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ was determined. The total amount of sampled N_2O/N_2 (mole) was calculated using a calibration function gained from standard gas calibration (see chapter 3.3.4). The current N_2O and N_2 release rate can then be calculated by either (i) the amount of gas captured from the carrier gas per time (mole/time) or (ii) the flow rate of the carrier gas (volume/time) and the amount of N gas in the sample loop (mole/volume). The flow rate was determined at the outlet of the sample loop using a Flowmeter (ADM1000, Agilent, USA).

5.3.3. ^{15}N calculations

Non-hybrid N_2O/N_2 formation in the presence of one uniformly labeled N source (Experiment I, II, III, and V as well as first two measurements of N_2O determination and first three measurements of N_2 determination in Experiment IV) was determined by the approach given in chapter 3.3.4. First, the ^{15}N abundance of the labeled N source (e.g. NO_3^-) is calculated by the determined $^{14}N/^{15}N$ distribution of produced N-N gas species (Equation 3.1). Second, the calculated ^{15}N abundance is used to calculate the contribution of this source on the total gas release of the respective N-N gas species (Equation 3.2). However, it was demonstrated in chapter 4.4.3 that this former approach fails when also hybrid N gas formation occurs. Since a hybrid N_2O formation was expected in experiment IV (non-autoclaved, presence of NH_2OH + $^{15}NO_3^-$) we developed an adapted ^{15}N tracer model. The model considers two different N sources (e.g. NH_2OH and NO_2^-), where each source generates non-hybrid N_2O (e.g. by NH_2OH decomposition and denitrification of NO_2^-) and simultaneously both N sources can be combined to form hybrid N_2O (e.g. by codenitrification). The adapted ^{15}N tracer model is related to the following assumptions: (i) one of two considered N sources is constantly at natural ^{15}N abundance (0.366 at%), while the other is enriched in ^{15}N; (ii) the ^{15}N abundance of the enriched N sources is homogeneously distributed within the studied environment (i.e. unaffected by spatial heterogeneity); and (iii) no other differently labeled N source is converted to N_2O. Assuming an N_2O production based on a labeled and unlabeled N source, both simultaneously utilized by the three different pathways described above, the resulting mole fractions of the species $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$ in the N_2O mixture can be calculated as follows (Equation 5.1 to 5.3).

Equation 5.1 $$\alpha_{44} = A(1-a)^2 + B(1-b)^2 + C(1-a)(1-b)$$

Equation 5.2 $$\alpha_{45} = 2A(1-a)a + 2B(1-b)b + C[(1-a)b + (1-b)a]$$

Equation 5.3 $$\alpha_{46} = Aa^2 + Bb^2 + Cab$$

where α_{44}, α_{45}, and α_{46} ($\alpha_{44} + \alpha_{45} + \alpha_{46} = 1$) represent the mole fraction of $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$, respectively, and the variables *a* and *b* represent the ^{15}N mole fraction ($^{15}N/[^{14}N+^{15}N]$ = relative ^{15}N abundance) of the two considered N sources. The variables *A* and *B* represent the respective fractions of non-hybrid N_2O, while *C* represents the fraction of hybrid N_2O (*A* + *B* + *C* = 1). Applying a Gaussian elimination approach to Equation 5.1 to 5.3 results in Equation 5.4 to 5.6, which permit the calculation of the relative contribution of each considered N_2O forming process.

Equation 5.4 $$A = \frac{b^2 + b(-2\alpha_{46} - \alpha_{45}) + \alpha_{46}}{(a-b)^2}$$

Equation 5.5 $$B = \frac{a^2 + a(-2\alpha_{46} - \alpha_{45}) + \alpha_{46}}{(a-b)^2}$$

Equation 5.6 $$C = \frac{a(2\alpha_{46} + \alpha_{45} - 2b) + b(2\alpha_{46} + \alpha_{45}) - 2\alpha_{46}}{(a-b)^2}$$

where *A* represents the fraction of non-hybrid N_2O based on the unlabeled source (*a*), *B* represents the fraction of non-hybrid N_2O formed by the labeled source (*b*), and *C* represents the fraction of hybrid N_2O formed by a 1:1 linkage of both sources (*a* and *b*). The contribution of each pathway on the total N_2O formation can then be calculated based on the determination of the mass distribution within released N_2O (i.e. α_{44}, α_{45}, and α_{46}) and the current ^{15}N mole fraction of the labeled N source (i.e. *b*). As reported by others (e.g. Kim and Hollocher, 1984; Weeg-Aerssens et al., 1988) NO_2^- rather than NO_3^- can be assumed as the direct precursor of denitrified and codenitrified N_2O. Therefore, in experiment IV ($^{15}NO_3^-$ + NH_2OH, non-autoclaved) the ^{15}N mole fraction of NO_2^- (measured and interpolated values; see chapter 5.3.2) was used as variable *b*. Variable *a* was assumed to be represented by unlabeled NH_2OH. In experiment IV the adapted ^{15}N tracer model was also applied for released N_2 using the determined mole fractions of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ as well as the current ^{15}N mole fraction of NO_2^-. However, it has to be considered that an N_2 formation based on the unlabeled source (i.e. NH_2OH) cannot be calculated exactly, because it is indistinguishable from atmospheric N_2 contamination. In addition it should be noted here that if the mass distribution of N_2O is determined via the species $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$ a mass correction concerning the natural occurrence of ^{17}O ($^{14}N_2{}^{17}O = 45$; $^{14/15}N_2{}^{17}O = 46$) and ^{18}O ($^{14}N_2{}^{18}O = 46$) should be conducted.

In spite of the developed ^{15}N tracer model described above (Equations 5.4 to 5.6) the verification of hybrid N-N gas production in a soil still remains a heavy challenge. This is mainly related to the fact that the ^{15}N abundance of the labelled N source (e.g. NO_2^-) might be in some cases difficult to determine, in particular with respect to non-suspended soils incubated under low oxygen or anaerobic conditions. Therefore, we developed the novel characteristic factor R_{binom} (ranging from 0 to 2), which indicates hybrid N gas formation only by using the mass distribution of an analysed N-N gas (e.g. α_{44}, α_{45}, and α_{46}). As shown in chapter 5.7.1 an R_{binom} value of >1 can be only achieved by an occurrence of a hybrid N-N gas species (e.g. N_2O formation by codenitrification).

5.4. Results

5.4.1. $^{15}NO_3^-$ treatment (Experiment I)

Prior to the tracer application no native NO_3^- was detected in the soil suspension. This indicates a complete depletion during pre-incubation and explains the absence of N_2O formation before $^{15}NO_3^-$ was applied. After tracer application $^{15}N_2O$ as well as $^{15}N_2$ was produced immediately. The measured ^{15}N abundance of NO_3^- in the soil suspension was ≈30 at% (both replicates). This perfectly corresponds with the calculated ^{15}N abundance of the N source of N_2 (30.1 ±0.5 at%; n = 12, both replicates) and N_2O (30.2 ±0.4 at%; n = 10, both replicates) and hence, clearly indicate that both gases were solely produced by $^{15}NO_3^-$ utilization. As expectable under these conditions the R_{binom} value (see chapter 5.7.1) of released N_2O was always constant at 1.00 (1.002 ±0.004; n = 10, both replicates). The N_2O release reached maximum rates of only 0.08 µmol h^{-1}, while N_2 exceeded N_2O by two orders of magnitude (up to 15 µmol h^{-1}) (Figure 5.1). Furthermore, N_2O release showed rather constant rates, while N_2 release constantly increased. 6 to 7 h after $^{15}NO_3^-$ application N gas release decreased dramatically and fell below the determination limit. Since NO_3^- was already undetectable at the second solution sampling (6 h after tracer application) it can be assumed that this occurred due to a complete depletion of the N substrate. Thus it appears that N_2O and N_2 formation was directly related to NO_3^- conversion by denitrification.

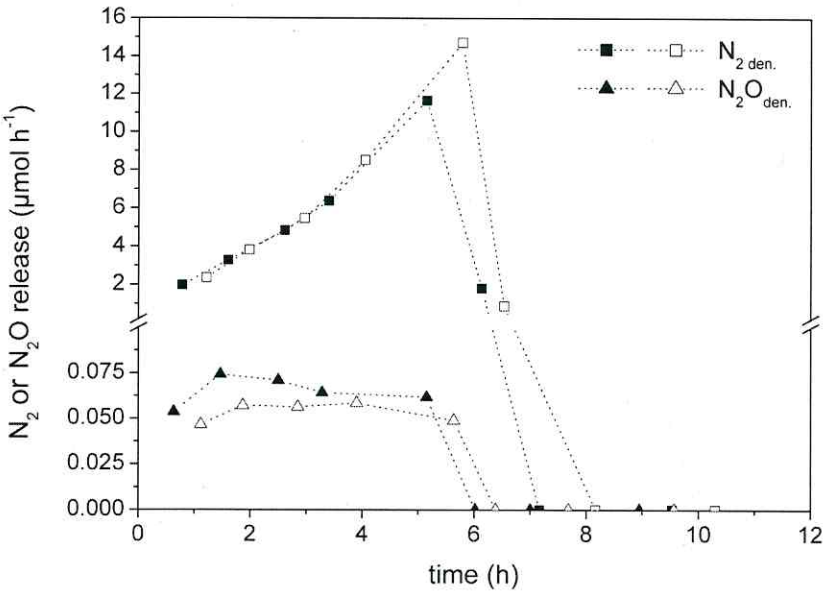

Figure 5.1 *N_2 and N_2O release by denitrification in the $^{15}NO_3^-$ treatment (experiment I) (black and white symbols refer to replicate 1 and 2, respectively; time = 0 represents $^{15}NO_3^-$ application).*

5.4.2. $^{15}NH_2OH$ treatments (Experiment II and III)

Non-autoclaved soil suspension (Experiment II) – Before the application of $^{15}NH_2OH$ no N_2O release occurred and also no native NO_3^- or NO_2^- was detected in the soil suspension. This indicates that all denitrifiable N sources were completely depleted during pre-incubation. After $^{15}NH_2OH$ application a tremendous, peak-like release of $^{15}N_2O$ occurred, which afterwards remained constantly low with a slight decrease towards the end of incubation (Figure 5.2a). The ^{15}N abundance of released N_2O was always constant at around 30 at% (30.3 ±0.3 at%; n = 26, both replicates). Correspondingly, the calculated ^{15}N abundance of the N_2O source compound was also 30 at% (30.1 ±0.1 at%; n = 26, both replicates) (Figure 5.3d). Hence, N_2O formation occurred solely due to $^{15}NH_2OH$ conversion. This observation was also confirmed by a constant R_{binom} value of 1.00 (1.002 ±0.004; n = 26, both replicates) during the whole incubation (Figure 5.3c). The calculated N_2O release rates ranged between 27 µmol h^{-1} after tracer application and 1.2 µmol h^{-1} at the end of incubation. Concerning N_2 a similar ^{15}N abundance for the N source compound was calculated (30.0 ±0.1 at; n = 26, both replicates) (Figure 5.3d). It thus follows that also N_2 was formed based on $^{15}NH_2OH$ conversion. The calculated N_2 release rates revealed a very similar pattern in comparison to N_2O. However, observed rates (0.6 to 6 µmol h^{-1}) always remained below that of N_2O (Figure 5.2a). Directly after $^{15}NH_2OH$ application N_2O accounted for around 80 % of total N-N gas release. Subsequently the composition of released N-N gas rapidly changed in favor of N_2 resulting in

a rather constant emission ratio of $^2/_3$ N_2O and $^1/_3$ N_2 (Figure 5.2a). NO_3^- and NO_2^- were detectable after $^{15}NH_2OH$ application, but occurred at very low concentrations (not shown) near to the determination limit (20 µM and 1µM for NO_3^- and NO_2^-, respectively) of the SPINMAS system (Stange et al., 2007). Nonetheless, both compounds showed an increased ^{15}N abundance of up to 30 at% which indicates $^{15}NH_2OH$ as the precursor.

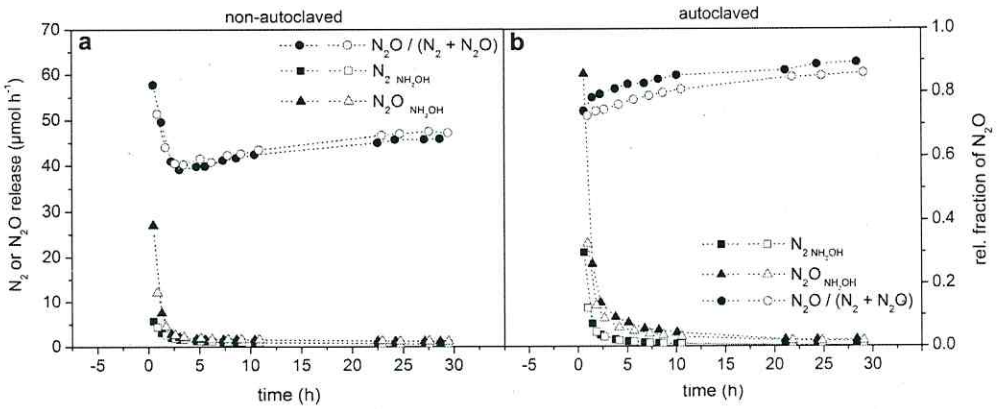

Figure 5.2 *N_2 and N_2O production based on $^{15}NH_2OH$ conversion and relative fraction of N_2O on total N-N gas production using non-autoclaved (left; experiment II) and autoclaved (right; experiment III) soil suspension (black and white symbols refer to replicate 1 and 2, respectively; time = 0 represents NH_2OH application).*

Autoclaved soil suspension (Experiment III) – No N_2O release occurred before the application of $^{15}NH_2OH$, even if native NO_3^- and NO_2^- were still detectable in the soil suspension (not shown). Thus it appears that microbial N_2O production by denitrification was completely prevented by autoclaving. After $^{15}NH_2OH$ application a tremendous, peak-like $^{15}N_2O$ release occurred, which afterwards remained constantly low (Figure 5.2b). The ^{15}N abundance of released N_2O was constant at 30 at% (30.3 ±0.1 at%; n = 22, both replicates). Correspondingly, also the calculated ^{15}N abundance of the N_2O source compound was found to be constant at 30 at% (30.1 ±0.1 at%; n = 22, both replicates) (Figure 5.3d). Both results clearly identify $^{15}NH_2OH$ as the source of N_2O formation. This is also confirmed by a constant R_{binom} value of 1.00 (1.003 ±0.002; n = 22, both replicates) during the whole incubation (Figure 5.3c). During the first 10 h N_2O release due to $^{15}NH_2OH$ conversion ranged between 2 and 60 µmol h^{-1} and then remained constantly low at around 1.3 µmol h^{-1}. Concerning N_2 a similar ^{15}N abundance of the N source compound was calculated (30.1 ±0.3 at%; n = 22, both replicates) (Figure 5.3d), which indicates that also N_2 was formed based on $^{15}NH_2OH$ utilization. The calculated N_2 release (0.2 to 21 µmol h^{-1}) always remained below that of N_2O, but happened very similarly compared to the pattern of N_2O (Figure 5.2b). Directly after $^{15}NH_2OH$ application N_2O release accounted for around 75 % of total N-N gas

release. Afterwards it slightly changed in favor of N_2O and resulted in an emission ratio of $^{9}/_{10}$ N_2O and $^{1}/_{10}$ N_2 (Figure 5.2b). NO_3^- and NO_2^- analyses revealed that they were also affected by $^{15}NH_2OH$ conversion. Both compounds showed an increase in the ^{15}N abundance of up to 8 and 1 at%, respectively. This indicates that also under autoclaved conditions some $^{15}NH_2OH$ was converted to NO_3^- and NO_2^-. However, while NO_2^- showed a constantly low concentration (≈16 µM), NO_3^- concentration was 2 orders of magnitude higher and slightly decreased from 2100 to 1600 µM.

5.4.3. $^{15}NO_3^-$ + NH_2OH treatments (Experiment IV and V)

Non-autoclaved soil suspension (Experiment IV) – Prior to a NH_2OH application N_2 and N_2O release via $^{15}NO_3^-$ utilization was found to be very similar compared to experiment I. N_2 release increased to up to ≈1.6 µmol h^{-1}, while N_2O release (0.06 µmol h^{-1} in maximum) was two orders of magnitude below N_2 (Figure 5.4a and 5.5a). The ^{15}N abundance of released N_2O was similar to the ^{15}N abundance of added $^{15}NO_3^-$ (≈31 at%) (Figure 5.4b). Correspondingly, the calculated ^{15}N abundance of the N source of N_2O was also at around 30 at%. A similar result was also found for N_2 (Figure 5.3b). Hence, before NH_2OH application the observed N-N gas release occurred solely due to denitrification of added $^{15}NO_3^-$.

After NH_2OH application the ^{15}N abundance of NO_3^- and NO_2^- showed a rather constant decrease to around 14 and 20 at%, respectively (Figure 5.4b). Hence, NH_2OH acted as an N source for NO_3^- and NO_2^- formation. In addition, a constant decrease in NO_3^- (≈800 to ≈100 µM) and NO_2^- concentration (≈100 to ≈20 µM) was observed (not shown). A considerable effect of NH_2OH application was also found concerning the ^{15}N abundance of released N_2O and N_2. Both gases first showed a rapid decrease, which was then followed by a short increase and a second weak decrease (Figure 5.4b). The pattern was similar for both gases, but strongly pronounced only concerning N_2O. The measured ^{15}N abundance of N_2O and N_2 was always below that of NO_3^- and NO_2^-, respectively (Figure 5.4b). The calculated ^{15}N abundance of the N_2O and N_2 source compound also showed a rapid decrease down to values <5 at%. However, these values neither coincided with NO_3^- nor NO_2^- (Figure 5.3b and 5.4b) (please note that unlabeled N-N gas gained from NH_2OH is taken into account during this calculation; see chapter 3.3.4). This is in clear contrast to experiment I, II, and III where N_2O and N_2 formation was solely related to the added N tracer ($^{15}NO_3^-$ or $^{15}NH_2OH$) and always in agreement with the ^{15}N tracer model described in chapter 3. In contrast to experiment II, III, and V the calculated R_{binom} values of N_2O rapidly began to exceed 1.00 after NH_2OH was applied. After 4 to 5 h they remained fairly constant at around 1.06 (1.061 ±0.005; n = 24, both replicates) until the end of incubation. This indicates a considerable release of hybrid N_2O (see chapter 5.7.1). Correspondingly, the present ^{15}N tracer model also calculated a hybrid N_2O formation (NH_2OH + $^{15}NO_2^-$; Equation 5.6). Furthermore, the model calculated a simultaneous non-hybrid N_2O formation based on NH_2OH (Equation 5.4) and $^{15}NO_2^-$

(Equation 5.5), respectively. The revealed pattern of non-hybrid N_2O release due to NH_2OH conversion appeared very similar to the pattern found in experiment II, III, and V (see Figure 5.2 and 5.4c). Nonetheless, the initial N_2O release rates due to NH_2OH conversion were distinctively lower (maximum release rates of up to 7 µmol h^{-1}, Figure 5.4a). Concerning non-hybrid N_2O formation due to $^{15}NO_2^-$ utilization (i.e. denitrification) a significant increase by one order of magnitude was caclulated by the model. The modeled N_2O release rates ranged between 0.3 and 0.8 µmol h^{-1} (Figure 5.4a). A maximum N_2O release occurred 1 to 2 h after NH_2OH was applied. Concerning hybrid N_2O formation a similar pattern compared to denitrification was calculated. However, release rates of hybrid N_2O were one order of magnitude higher (1.0 and 2.3 µmol h^{-1}) than found for denitrified N_2O (Figure 5.4a). The maximum hybrid N_2O release occurred 3 to 4 h after NH_2OH application.

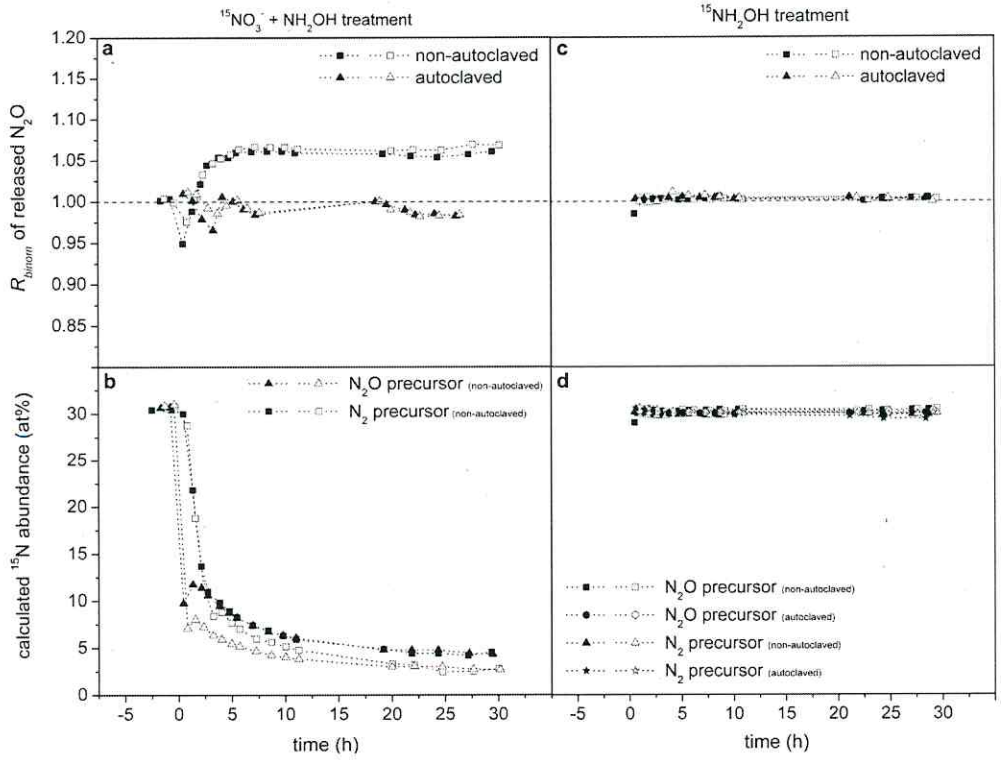

Figure 5.3 *R_{binom} values (see chapter 5.7.1) of released N_2O and predicted ^{15}N abundance of the N_2 and N_2O source in the $^{15}NO_3^-$ + NH_2OH treatment (left; experiment IV and V) and in the $^{15}NH_2OH$ treatment (right; experiment II and III) (black and white symbols refer to replicate 1 and 2, respectively; time = 0 represents NH_2OH application;in Fig. 5.3a and 5.3b NO_3^- was added 2–3 h prior to NH_2OH application).*

The application of the present ^{15}N tracer model on released N_2 revealed a non-hybrid N_2 formation based on $^{15}NO_2^-$ (i.e. denitrification) as well as a hybrid N_2 formation based on a combination of NH_2OH-N and $^{15}NO_2^-$-N. After NH_2OH application the calculated non-hybrid N_2 formation due to denitrification decreased by two orders of magnitude (rates of <0.2 µmol h^{-1}) (Figure 5.5a). By contrast, the quantified hybrid N_2 formation increased to a maximum (0.3 µmol h^{-1}) 3 to 5 h after NH_2OH application and then dropped to around 0.1 µmol h^{-1} (Figure 5.5a).

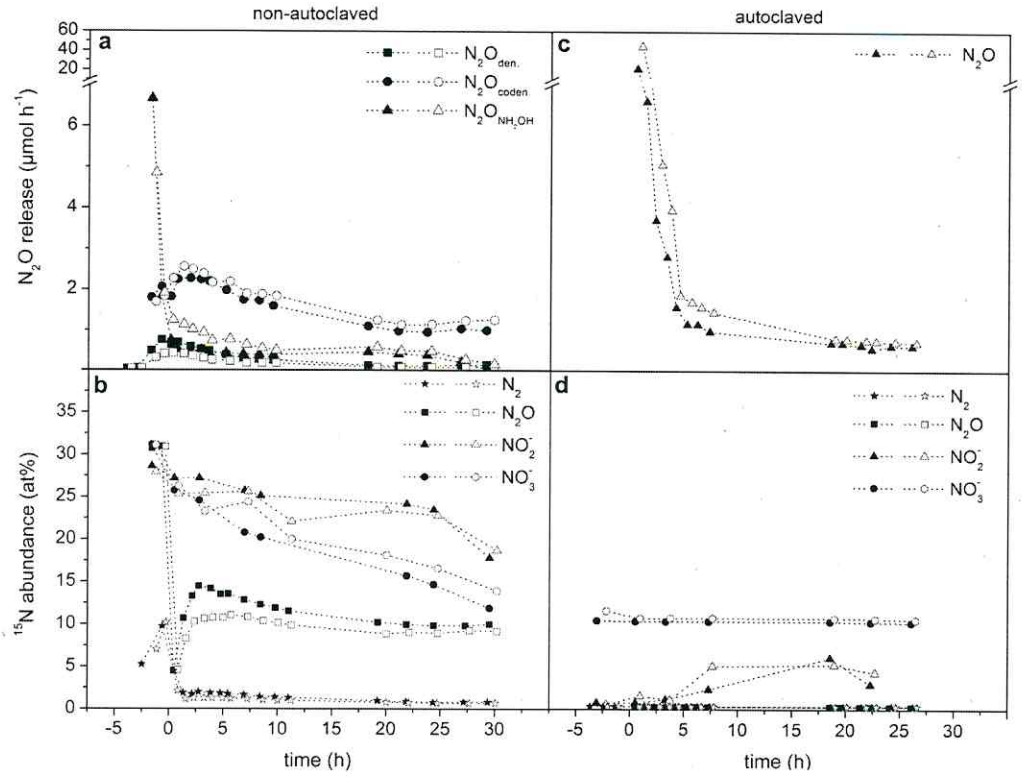

Figure 5.4 *N_2O release (please note the discontinuity of the y-axis) and ^{15}N abundance of all determined nitrogen compounds in the $^{15}NO_3^-$ + NH_2OH treatment using non-autoclaved (left; experiment IV; after t = 0 total N_2O release was partitioned according to Equation 5.4 to Equation 5.6) and autoclaved (right; experiment V) soil suspension (black and white symbols refer to replicate 1 and 2, respectively; time = 0 represents NH_2OH application, while NO_3^- was added 2–3 h prior to NH_2OH application).*

Hence, the modeled pattern of hybrid N_2 formation occurred very similar compared to the pattern found for hybrid N_2O formation. A comparison of N_2O and N_2 concerning the contribution of denitrification and simultaneous hybrid N-N gas formation revealed a very similar composition. In general hybrid N-N gas prevailed and accounted for 75 to 95 %

(Figure 5.5b). A clear difference between N_2O and N_2 occurred only during the first 3 h after NH_2OH application, where the fraction of hybrid N_2 was clearly lower compared to the fraction of hybrid N_2O. A direct NH_2OH conversion to non-hybrid N_2 could not be exactly quantified, because of its natural ^{15}N abundance which is indistinguishable from atmospheric N_2 contamination.

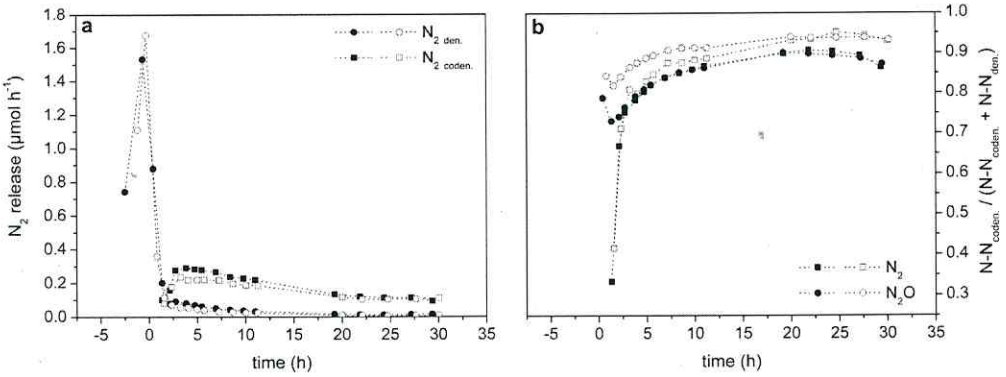

Figure 5.5 *Microbial N_2 release (a) and composition of biogenic N_2 and N_2O (b) in the $^{15}NO_3^-$ + NH_2OH treatment using non-autoclaved soil suspension (experiment IV; N-N gas release was partitioned according to Equation 5.5 and Equation 5.6) (black and white symbols refer to replicate 1 and 2, respectively; time = 0 represents NH_2OH application, while NO_3^- was added 2–3 h prior to NH_2OH application).*

Autoclaved soil suspension (Experiment V) – After the application of $^{15}NO_3^-$ no N_2O or N_2 release was observed, which indicates that denitrification did not occur after autoclaving. In contrast, the subsequent application of NH_2OH resulted in a tremendous, peak-like N_2O release with maximum rates of 45 µmol h^{-1} (Figure 5.4c). After 5 h N_2O release remained constantly low at 0.7 µmol h^{-1}. Released N_2O showed absolutely no ^{15}N enrichment and hence neither $^{15}NO_3^-$ nor $^{15}NO_2^-$ was involved in N_2O production (Figure 5.4d). In contrast to experiment IV (non-autoclaved) the calculated R_{binom} value of N_2O was always 1.0 (0.99 ±0.01; n = 28, both replicates) without considerable changes during the incubation (Figure 5.3a). Thus, NH_2OH can be assumed as the only source of the observed N_2O release. With respect to N_2 a similar release pattern was observed (not shown). However, the contribution of NH_2OH to total N_2 release cannot be exactly calculated due to the equality of the ^{15}N abundance of NH_2OH and ambient atmospheric N_2. The measured ^{15}N abundance of NO_3^- (10 at%) was distinctively below the ^{15}N abundance of the applied tracer (30 at%), which indicates the presence of native NO_3^- unutilized due to autoclaving (Figure 5.4d). Determined NO_3^- concentrations ranged from 1600 to 1800 µM. Both ^{15}N abundance and concentration of NO_3^- remained constant during incubation. Hence, under autoclaved conditions no relevant production of NO_3^- based in NH_2OH conversion took place. In contrast to NO_3^-, determined

NO_2^- showed extremely low concentrations (<10 µM) with a slight increase of the ^{15}N abundance (up to 6 at%) (Figure 5.4d).

5.5. Discussion

5.5.1. Hybrid N_2O due to codenitrification of NH_2OH

It was confirmed by experiment I (only $^{15}NO_3^-$) that under the given incubation conditions only denitrified N_2O is formed when only NO_3^- is delivered. This perfectly corresponds with the current knowledge of denitrification (Knowles, 1982; Zumft, 1997). However, when NH_2OH was additionally applied during an active denitrification of NO_3^- (experiment IV) a considerable change in the N_2O release pattern was observed (Figure 5.4a). While in experiment I N_2O release was only related to added $^{15}NO_3^-$, in experiment IV the N_2O release revealed a clear incorporation of an unlabeled N source (i.e. NH_2OH). It was already reported by others (Bremner et al., 1980; Nelson, 1985; Bremner, 1997) that NH_2OH can be rapidly converted to N_2O due to an abiotic redox reaction with oxidized metal species (e.g. Mn). A similar N_2O formation was found in experiment III (only $^{15}NH_2OH$; autoclaved) and V ($^{15}NO_3^-$ + NH_2OH; autoclaved). When NH_2OH was applied to an autoclaved soil suspension a rapid and peak-like N_2O formation occurred (Figure 5.2b and 5.4c). Moreover, even in the presence of $^{15}NO_3^-/^{15}NO_2^-$ (experiment V) no ^{15}N labeling was found in released N_2O (Figure 5.4d), which proves that abiotic N_2O was solely produced by NH_2OH. These results correspond well with data from Bremner et al. (1980). They reported that, independently from the presence of NO_2^-, NH_2OH was rapidly converted to N_2O (most of it within the first hours after NH_2OH application) when applied to a sterilized soil. Thus, we can conclude that in the presence of NO_3^- and NH_2OH under non-autoclaved conditions (experiment IV) the former species was denitrified to N_2O, while simultaneously the latter species was chemically converted to N_2O.

The ^{15}N abundance of a labeled N_2O source (e.g. $^{15}NO_2^-$) can be calculated by the $^{14}N/^{15}N$ distribution within released N_2O (i.e. α_{44}, α_{45}, and α_{46}) (Equation 3.1), as long as only an unlabeled N source (e.g. NH_2OH) simultaneously contributes to the total soil N_2O release. Hence, in experiment IV a reliable calculation of the ^{15}N abundance of the labeled N_2O source (i.e. $^{15}NO_3^-/^{15}NO_2^-$) can be expected, if only denitrification and abiotic NH_2OH conversion would have taken place. However, the calculated ^{15}N abundance of the labeled N_2O source was always distinctively below the measured ^{15}N abundance of $^{15}NO_3^-$ and $^{15}NO_2^-$ (Figure 5.3b and 5.4b). If denitrified N_2O would have been produced directly by $^{15}NO_3^-$ or $^{15}NO_2^-$, then the calculated ^{15}N abundance of the labeled N_2O source should be equal to either $^{15}NO_3^-$ or $^{15}NO_2^-$. If we assume a denitrification directly based on $^{15}NO_3^-$ and $^{15}NO_2^-$, then the calculated ^{15}N abundance of the labeled N_2O source should stay in between. Since both scenarios do not match our calculations, the observed N_2O formation in experiment IV can not be explained by considering only denitrification and simultaneous abiotic NH_2OH

conversion. However, if hybrid N_2O formation due to a co-metabolic NH_2OH utilization during denitrification (i.e. codenitrification) is taken into account (as reported by e.g. Garber and Hollocher, 1982; Kim and Hollocher, 1984; Aerssens et al., 1986; Hulse et al., 1989), the calculated ^{15}N abundance of the labeled N_2O source should be systematically underestimated. In fact this was found in experiment IV (Figure 5.3b and 5.4b). A similar systematic underestimation was reported by Laughlin and Stevens (2002) associated with hybrid N_2 formation by codenitrification. Thus it appears well-founded that the observed underestimation was caused by a hybrid N_2O formation due to codenitrification of NH_2OH.

As shown in chapter 5.7.1 the novel characteristic factor R_{binom} was derived to improve the identification of such a hybrid N-N gas production. If only one N source forms the N_2O the R_{binom} value will be 1.00. This was clearly found in experiment I, II, III, and V where only NO_3^- or NH_2OH acted as the substrate of N_2O formation. By contrast, only in the presence of $^{15}NO_3^-$ and NH_2OH under non-autoclaved conditions (experiment IV) the R_{binom} values of N_2O were clearly different to 1.00. While R_{binom} remained at 1.00 as long as only $^{15}NO_3^-$ was present, it rapidly increased to a rather constant value of 1.06 immediately after NH_2OH was additionally applied (Figure 5.3a). First, this clearly contradicts the assumption of a single N_2O source (see chapter 5.7.1). And second, it also contradicts the assumption of only non-hybrid N_2O formation by abiotic NH_2OH conversion and simultaneous denitrification, since in this case R_{binom} would be <1.00 (see chapter 5.7.1). An R_{binom} value of >1.00, as only observed in the presence of $^{15}NO_3^-$ and NH_2OH under non-autoclaved conditions, can only be achieved by a considerable release of hybrid N_2O (see chapter 5.7.1). Hence, it can be concluded that the addition of NH_2OH in fact resulted in a formation of hybrid N_2O. The rather small deviation from 1.00 is mainly caused by the low ^{15}N abundance of the labeled N sources (Figure 5.4b). Even if we assume only hybrid N_2O formation by $^{15}NO_2^-$ and NH_2OH the maximum R_{binom} value would range from 1.16 at the beginning to 1.09 at the end of incubation.

As no labeled N_2O was formed, when $^{15}NO_3^-$ and NH_2OH were added to an autoclaved soil suspension (experiment V; Figure 5.4d), it appears evident that the hybrid N_2O formation observed under non-autoclaved conditions was mediated by a microbial process. An abiotic production of hybrid N_2O based on a reaction of NH_2OH and nitrous acid (HNO_2) appears therefore extremely unlikely. Such acid catalyzed reactions were reported (Mirvish, 1975; Kim and Hollocher, 1984; Calmels et al., 1985) to occur at an optimum pH between 2 and 3, which is, however, in a clear contrast to the slightly alkaline conditions found in the soil suspension (7.8 ±0.1; n = 5). In addition, hybrid N_2O was formed not at all when only NH_2OH was applied under non-autoclaved conditions (experiment II; Figure 5.3c and 5.3d). However, hybrid N_2O formation was observed under non-autoclaved conditions, when NO_3^- and NH_2OH were applied (experiment IV). In addition, it was shown that NO_3^- was actively utilized for N_2O and N_2 formation by denitrification immediately prior to NH_2OH application. Thus it appears that hybrid N_2O formation, as detected only in experiment IV (NH_2OH +

$^{15}NO_3^-$, non-autoclaved), was dependent on a microbial process which utilized NH_2OH only in the presence of active denitrification, i.e. a microbial process which appears to perfectly coincide with the definition of codenitrification as given by Tanimoto et al. (1992). Furthermore, it also goes hand in hand with results of microbial studies on N_2O formation due to co-metabolic utilization of NH_2OH during denitrification (Iwasaki and Mori, 1958; Renner and Becker, 1970; Matsubara, 1970; Garber and Hollocher, 1982; Kim and Hollocher, 1984; Aerssens et al., 1986; Hulse et al., 1989). Therefore, it can be concluded that hybrid N_2O formation, as indicated by R_{binom} and calculated by the present ^{15}N tracer model (Figure 5.3a and 5.4a), did in fact occur due to codenitrification.

5.5.2. Considerations on NH_2OH utilization as quantified by the ^{15}N tracer model

The present ^{15}N tracer model (Equations 5.4 to 5.6) was applied to the data set of experiment IV to take into account the formation of hybrid N_2O as indicated by the characteristic factor R_{binom}. The model revealed that three different pathways contributed simultaneously to the total N_2O release. Two different phases of N_2O formation were found: (i) a short but intensive N_2O production phase largely caused by NH_2OH conversion and (ii) a subsequent phase mainly dominated by hybrid N_2O formation (Figure 5.4a).

The calculated N_2O release due to NH_2OH conversion (Figure 5.4a) tended to correspond very well with the abiotic N_2O formation by NH_2OH conversion observed under autoclaved conditions (experiment III and V; Figure 5.2b and 5.4c). Hence, it can be assumed that NH_2OH conversion to N_2O, as calculated by the model, occurred due to an abiotic process. Bremner et al. (1980) reported that up to 83 % of NH_2OH added to a sterilized soil was abiotically converted to N_2O within 2 h by a redox reaction with oxidized metal species (e.g. Mn). Therefore, the modeled N_2O formation due to abiotic NH_2OH conversion may be explained by a rapid and strong reaction of NH_2OH at an initial incubation state, followed by a gradual decline due to a depletion of oxidized metal species. However, the initial N_2O peak was clearly lower than the initial N_2O peaks measured under autoclaved conditions (Figure 5.2b and 5.4c). As reported by others (e.g. McNamara et al., 2003; Berns et al., 2008) a sterilization of soils, in particular concerning autoclaving, can alter the availability and composition of soil N and C compounds. Hence, the found differences might be explained to some extent by the impact of autoclaving. Moreover, it has to be considered that abiotic N_2O formation by NH_2OH conversion was impeded by the simultaneous utilization of NH_2OH for hybrid N_2O formation (Figure 5.4a). Furthermore, abiotic N_2O formation was apparently also hindered by simultaneous nitrification. As shown in Figure 5.4b the ^{15}N abundance of NO_3^- and NO_2^- continuously decreased after NH_2OH was applied, which indicates that the latter compound was contributing to the NO_3^- and NO_2^- pool. A similar ^{15}N dilution was not observed under autoclaved conditions (Figure 5.4d) and hence, lends support to the assumption of a biogenic origin. As reported elsewhere (e.g. Arp and Stein, 2003) some nitrifying species can oxidize ammonia (NH_3) to NO_2^- via NH_2OH also under anaerobic

conditions by using for example nitrogen dioxide (NO_2) as the electron acceptor instead of O_2. However, in experiment IV the observed ^{15}N abundance of $^{15}NO_2^-$ always followed the decrease observed for $^{15}NO_3^-$ (Figure 5.4b), implying that the latter species was the precursor of the former compound (i.e. reductive pathway). Even if the detailed mechanism of the observed microbial NH_2OH oxidation remains unclear yet, it appears that only NO_3^- was produced without NO_2^- as a "free" intermediate.

The rapid abiotic conversion of NH_2OH indicates that under oxygen limited conditions NH_2OH released during nitrification may rather be abiotically converted to N_2O than co-metabolically utilized in the denitrification process (i.e. codenitrified). Moreover, this also supports the idea that soil N_2O production during nitrification (e.g. Bollmannn and Conrad, 1998; Khalil et al., 2004) may be caused by an abiotic NH_2OH conversion than by a microbially mediated NH_2OH conversion. An abiotic N_2O formation by NH_2OH gained from the nitrification of NH_3 was already suggested by some authors (Stüven et al., 1992; Moir et al., 1996; Bremner, 1997; Otte et al., 1999; Arp and Stein, 2003) and even demonstrated by Schmidt et al. (2004b). The latter authors found that during NH_3 oxidation by mutant strains of *Nitrosomonas europaea* high quantities of NH_2OH are released into the medium and then chemically converted to N_2O. Since the dynamic of N_2O formation due to NH_2OH conversion, as revealed by the present ^{15}N tracer model, corresponds to the abiotic N_2O formation found in experiment III and V (Figure 5.2b, 5.4a, and 5.4c), it appears unlikely that NH_2OH was microbially converted to N_2O. From this it can be concluded that a considerable N_2O production by nitrification did not occur.

Concerning microbial N_2O formation the present ^{15}N tracer model revealed a significant change in the N_2O release after NH_2OH was applied additionally to NO_3^-. While only negligible amounts of N_2O were released by denitrification before an application (Figure 5.4a), a clear increase of denitrified N_2O (1 order of magnitude) after NH_2OH application was calculated by the model. In addition, a simultaneous release of hybrid N_2O due to codenitrification of NH_2OH was also calculated. The modeled contribution of codenitrified N_2O always exceeded the modeled release of denitrified N_2O (up to 95 % of total biogenic N_2O release; Figure 5.5b). Similar results were also reported by Garber and Hollocher (1982) who incubated anaerobically single denitrifying species in the presence of $^{15}NO_2^-$ and NH_2OH. It was shown that hybrid N_2O accounted for 47 to 98 % depending on the incubated denitrifier. The revealed dynamic of both biogenic pathways (Figure 5.4a) is also consistent with the assumed interrelation of denitrification and N-nitrosation (i.e. codenitrification) as proposed by e.g. Weeg-Aerssens et al. (1988). They reported that during the denitrification of NO_2^- different nucleophiles (e.g. NO_2^- and N_3^-) compete for an enzyme-bound mono-nitrogen intermediate, which occurs after NO_2^- in the denitrification pathway. Thus one can assume that the predominance of hybrid N_2O was largely caused by the high concentration of NH_2OH compared to NO_2^-. In addition, Garber and Hollocher (1982) reported that hybrid N_2O formation by N-nitrosation behaves kinetically similarly to denitrification. This would also

reasonably explain the similarity of the dynamic of codenitrified and denitrified N_2O formation as calculated by the present ^{15}N tracer model.

Besides hybrid N_2O the present ^{15}N tracer model also revealed a release of hybrid N_2 after NH_2OH was additionally applied to $^{15}NO_3^-$ (experiment IV) (Figure 5.5a). To our best knowledge a hybrid N_2 formation by codenitrification of NH_2OH has never been reported so far. However, as shown in Figure 5.3b the calculated ^{15}N abundance of the labeled N_2 source compound (Equation 3.1; see chapter 3.3.4) was similarly underestimated as already found for N_2O. The underestimation clearly supports the modeled release of hybrid N_2 inasmuch as it contradicts with the assumption of only abiotic NH_2OH conversion and simultaneous denitrification (as discussed in the previous section). However, since this underestimation is almost equal to the underestimation of the labeled N_2O source (Figure 5.3b), it appears most likely that hybrid N_2 was formed just by a reduction of hybrid N_2O due to conventional denitrification. If we consider N_2O as the direct precursor of N_2 this assumption appears even more reasonable, since then N_2 simply reproduces the ^{15}N distribution of N_2O (please note that the underestimation is only dependent on the ^{15}N abundance of the labeled N source and the denitrification/codenitrification ratio). Furthermore, the calculated denitrified and codenitrified N_2 appear to only reproduce the composition of biogenic N_2O (Figure 5.5b). Thus it follows that the observed formation of hybrid N_2 was apparently caused by a reduction of hybrid N_2O rather than a result of codenitrification of NH_2OH. It was recently underlined by Chapuis-Lardy et al. (2007) that soils have to be considered as an important N_2O sink due to their widespread ability of a microbial N_2O reduction to N_2.

5.6. Conclusions

The present ^{15}N tracer model was shown to allow the quantification of non-hybrid and hybrid N_2O formation in the course of microbial N transformation. Moreover, the implementation of the novel characteristic factor R_{binom} permitted a simple verification of hybrid N_2O formation. Both approaches revealed that soil N_2O formation involves a considerable complexity of different reaction pathways interacting with each other and that in fact codenitrification of NH_2OH has to be taken into account. Even if high quantities of NH_2OH were used to provoke a significant formation of hybrid N_2O, it can be concluded that the Haplic Chernozem soil in general exhibits microbial species which are capable to perform codenitrification. Hence, the present ^{15}N tracer approaches could be applied as a general screening tool for studies on the capability of soils to perform codenitrification.

The observed utilization of NH_2OH allows us to draw the conclusion that (i) chemical N_2O formation, (ii) biogenic N_2O formation due to codenitrification, and (iii) nitrification has to be in principle considered as competing pathways for NH_2OH utilization in soils. The present ^{15}N tracer approaches should be therefore also used for studies on N_2O production by nitrification, denitrification, and codenitrification. For that purpose NH_4^+ could be applied along with $^{15}NO_3^-$. This appears particularly expedient inasmuch as NH_4^+ was also shown to

be involved in hybrid N_2O formation by codenitrification (Tanimoto et al., 1992; Immoos et al., 2004; Su et al., 2004). Moreover, a soil N_2O production due to nitrification and simultaneous denitrification was already reported by plenty of studies (e.g. Bollmann and Conrad, 1998; Stange et al., 2009).

Even if hybrid N-N gas formation due to codenitrification is already known since several decades (Garber and Hollocher, 1982; Kim and Hollocher, 1984; Tanimoto et al., 1992), a tremendous gap of knowledge concerning its impact on N cycling exists. It was recently pointed out by Jungkunst et al. (2006) that the observed heterogeneity of soil N_2O production at the field scale is largely related to different microbial communities which react differently to similar soil conditions. They underlined that new additional model concepts and process studies are urgently required to deepen knowledge of microbial N_2O production in soils. As demonstrated here the application of a ^{15}N tracer technique which considers the total $^{14}N/^{15}N$ mass distribution within N_2O (i.e. $^{44}N_2O$, $^{45}N_2O$, and $^{46}N_2O$) appears to be a promising way. Nonetheless, an application of the present approach on unsuspended soil samples or under in-situ conditions has to be evaluated yet. Its main restrictions are directly related to soil heterogeneity, which hinders a homogenous ^{15}N labeling and also impedes the required ^{15}N analysis of soil N species (i.e. NO_2^-). However, it was reported elsewhere (Garber and Hollocher, 1982) that in the presence of codenitrification NO production by denitrification is increased as a kind of abortive reaction. Hence, the question rises to what extend a ^{15}NO gas analysis might be suitable to replace the more difficult $^{15}NO_2^-$ analysis and thus simplifies the experimental application of the present ^{15}N tracer model.

5.7. Appendix

5.7.1. Introducing R_{binom} as characteristic factor of hybrid N-N gas formation

The R_{binom} factor permits an identification of hybrid N-N gas formation solely based on the mass distribution of an N-N gas species (e.g. α_{44}, α_{45}, and α_{46}). In order to improve the understandability of this section the application of R_{binom} is described based on $N_2{}^{16}O$ (nonetheless, all of the following explanations are also valid for N_2).

The combinability of ^{14}N and ^{15}N atoms within N_2O allows the formation of three different masses according to the following combinations: [$^{14}N^{14}N$], [$^{14}N^{15}N$], and [$^{15}N^{15}N$]. If we consider these masses as relative fractions of the total amount of N_2O the mass distribution within N_2O can be expressed by their respective mole fractions termed here as α_{44}, α_{45}, and α_{46} ($\alpha_{44} + \alpha_{45} + \alpha_{46} = 1$). The ^{15}N mole fraction of an N_2O gas species ($^{15}N/[^{14}N+^{15}N]$ = relative ^{15}N abundance; here referred to as c) can then be generally expressed using the mole fractions of the N_2O masses according to Equation 5.7,

Equation 5.7 $$c = \frac{\alpha_{45}}{2} + \alpha_{46}$$

The mass distribution within N_2O derived from one N source with the ^{15}N mole fraction a (e.g. non-hybrid N_2O from abiotic NH_2OH decomposition) will follow a random mass distribution (Hauck et al., 1958; Hauck and Bouldin, 1961). The random distribution within this non-hybrid N_2O species can be simply described by a binomial function which only requires the ^{15}N mole fraction of the related N_2O source: (i) $\alpha_{44} = (1\text{-}a)^2$; (ii) $\alpha_{45} = 2(1\text{-}a)a$; (iii) $\alpha_{46} = a^2$; and (iv) $(1\text{-}a)^2 + 2(1\text{-}a)a + a^2 = 1$. Since the ^{15}N mole fraction of formed N_2O will be equal to the ^{15}N mole fraction of the related N source (i.e. $c = a$), the mole fractions α_{44}, α_{45} , and α_{46} of a binomial distributed N_2O species can be equally expressed by its ^{15}N mole fraction c.

Russow et al. (1996) have shown that the ^{15}N mole fraction of an N_2O species with a binomial mass distribution can also be expressed by only using the ratio of α_{45} to α_{44} as follows,

Equation 5.8
$$c_{binom} = \frac{\frac{\alpha_{45}}{\alpha_{44}}}{(2 + \frac{\alpha_{45}}{\alpha_{44}})}$$

where c_{binom} represents the ^{15}N mole fraction of an N_2O species which is assumed to exhibit a binomial mass distribution. Hence, the ratio of Equation 5.8 to Equation 5.7 (= R_{binom}) would be 1, if the mass distribution within N_2O is binomial. Thus it appears that non-hybrid N_2O generated by one N source with the ^{15}N mole fraction a exhibits an R_{binom} value of 1. This can be easily proven if the ratio of Equation 5.8 to Equation 5.7 is further simplified to Equation 5.9.

Equation 5.9
$$R_{binom} = \frac{c_{binom}}{c} = \frac{\alpha_{45}}{2c(1-c)}$$

It follows that R_{binom} can be expressed as the measured α_{45} divided by the expected α_{45} when a binomial mass distribution within N_2O is assumed (= $2c[1\text{-}c]$). Since here the measured mole fraction of α_{45} will correspond to the expression $2c(1\text{-}c)$, the resulting R_{binom} value will be 1.

However, if non-hybrid N_2O is generated simultaneously by two different N sources (e.g. N_2O formation by NH_2OH conversion and simultaneous denitrification of NO_2^-) with the ^{15}N mole fraction a and b ($a \neq b$), respectively, the resulting mass distribution within the mixture of both N_2O species will be non-binomial (non-random) (Hauck et al., 1958; Hauck and Bouldin, 1961). Hence, if Equation 5.9 is applied to this N_2O gas mixture the R_{binom} value will be different to 1. To estimate the impact of mixing both N_2O species on the R_{binon} value Equation 5.9 was rearranged as the difference of the numerator and the denominator.

Equation 5.10
$$\alpha_{45} - 2c(1-c) \begin{cases} > 0 \\ = 0 \\ < 0 \end{cases}$$

Thus it appears that R_{binom} will be (i) >1, if Equation 5.10 is >0, (ii) <1, if Equation 5.10 is <0, and (iii) =1, if Equation 5.10 is 0 (i.e. indicates binomial mass distribution). Since prior to mixing both non-hybrid N_2O species are generated based on a binomial mass distribution (as described above) the required α_{45} and c of the N_2O gas mixture can be expressed as follows.

Equation 5.11 $$\alpha_{45} = 2x(1-a)a + 2(1-x)(1-b)b$$

Equation 5.12 $$c = x[(1-a)a + a^2] + (1-x)[(1-b)b + b^2]$$

The term x and 1-x represent the fraction (0 to 1) of the two non-hybrid N_2O species on the total N_2O gas mixture and a and b represent the ^{15}N mole fraction of the two different N sources. If α_{45} and c of the N_2O gas mixture (Equation 5.11 and 5.12) are introduced into Equation 5.10 the resulting Equation 5.13

Equation 5.13 $$2x(b-a)^2(x-1) \leq 0$$

proves that the difference of the measured α_{45} and the expected binomial α_{45} (= 2c[1-c]) will be always <0 if two non-hybrid N_2O species simultaneously arise (i.e. 0 < x < 1) from two differently labeled N sources (i.e. $a \neq b$). Hence, the R_{binom} value of such a non-hybrid N_2O gas mixture will be always <1. Otherwise, R_{binom} will be 1 (i.e. indicates binomial mass distribution), if only one N source contributes (x = 1 or x = 0) or the ^{15}N mole fraction of both N sources are equal ($a = b$).

If a hybrid N_2O species is generated by two different N sources (e.g. $NH_2OH + NO_2^-$) with the ^{15}N mole fraction a and b ($a \neq b$), respectively, α_{44}, α_{45}, and α_{46} can be expressed as follows: (i) $\alpha_{44} = (1-a)(1-b)$; (ii) , $\alpha_{45} = (1-a)b + (1-b)a$; (iii) $\alpha_{46} = ab$; and (iv) $(1-a)(1-b) + (1-a)b + (1-b)a + ab = 1$ (Clough et al., 2001). Thus it appears that a hybrid N_2O species will generally exhibit a non-binomial (non-random) mass distribution as long as the ^{15}N mole fraction of the related N sources are different (i.e. $a \neq b$). Hence, if Equation 5.9 is applied the resulting R_{binom} value will be also different to 1. Based on the analytical expressions given by Clough et al. (2001) α_{45} and c of a hybrid N_2O gas species can be expressed as follows.

Equation 5.14 $$\alpha_{45} = (1-a)b + (1-b)a$$

Equation 5.15 $$c = \frac{(1-a)b + (1-b)a}{2} + ab$$

Introducing both equations into Equation 5.10 results in Equation 5.16,

Equation 5.16 $$\frac{1}{2}(a-b)^2 \geq 0$$

which proves that in a hybrid N_2O species the difference of the measured α_{45} and the expected binomial α_{45} (= $2c[1\text{-}c]$) will always be >0 as long as the ^{15}N mole fraction of the related N sources are different (i.e. $a \neq b$). Hence, the R_{binom} value will always be >1, if hybrid N_2O is generated by two differently labeled N sources (maximally 1.99, assuming a = 0.00366 and b = 0.99).

If hybrid N_2O, originating from two N sources with the ^{15}N mole fraction a and b ($a \neq b$), is simultaneously released with non-hybrid N_2O, originating from the N source with the ^{15}N mole fraction b (e.g. codenitrified N_2O from NH_2OH and $^{15}NO_2^-$ and denitrified N_2O from $^{15}NO_2^-$), then α_{45} and c of the resulting N_2O gas mixture can be expressed as follows,

Equation 5.17 $$\alpha_{45} = x[(1-a)b + (1-b)a] + 2(1-x)(1-b)b$$

Equation 5.18 $$c = x[\frac{(1-a)b + (1-b)a}{2} + ab] + (1-x)[(1-b)b + b^2]$$

where x and 1-x represent the fraction (0 to 1) of hybrid and non-hybrid N_2O on the total N_2O gas mixture and a and b represent the ^{15}N mole fraction of the two related N sources. Introducing both equations into Equation 5.10 results in Equation 5.19,

Equation 5.19 $$\frac{1}{2}x^2(b-a)^2 \geq 0$$

which proves that the difference of the measured α_{45} and the expected binomial α_{45} (= $2c[1\text{-}c]$) will always be >0 as long as the ^{15}N mole fractions of both related N sources are different and x (fraction of hybrid N_2O) is >0. Hence, R_{binom} will always be >1 if a non-hybrid N_2O species is mixed with a hybrid N_2O species (if $a \neq b$).

Recapitulatory, it can be concluded that non-hybrid N_2O generated by one N source will always exhibit a R_{binom} value of 1 and that a mix of non-hybrid N_2O gas simultaneously generated by two differently labeled N sources will always exhibit an R_{binom} value of <1. On the other hand, the R_{binom} value of a hybrid N_2O species generated by two differently labeled N sources will always be >1, even if a non-hybrid N_2O species simultaneously generated by one of these N sources is mixed in. From this it follows that the R_{binom} value of an N_2O gas mixture, which arises from two differently labeled N sources (e.g. NH_2OH and $^{15}NO_2^-$) by non-hybrid N_2O formation and simultaneous hybrid N_2O formation (e.g. non-hybrid N_2O formation by NH_2OH conversion, non-hybrid N_2O formation by denitrification of $^{15}NO_2^-$, and hybrid N_2O formation by codenitrification of NH_2OH and $^{15}NO_2^-$), (i) tend to shift towards <1 when both non-hybrid N_2O species prevail or (ii) tend to shift towards >1 when the hybrid

N_2O gas species dominates or one of the non-hybrid N_2O species is marginal. From this it follows that an R_{binom} value of >1 clearly proves a contribution of a hybrid N_2O species, while a value of <1 does not exclude a hybrid N_2O species.

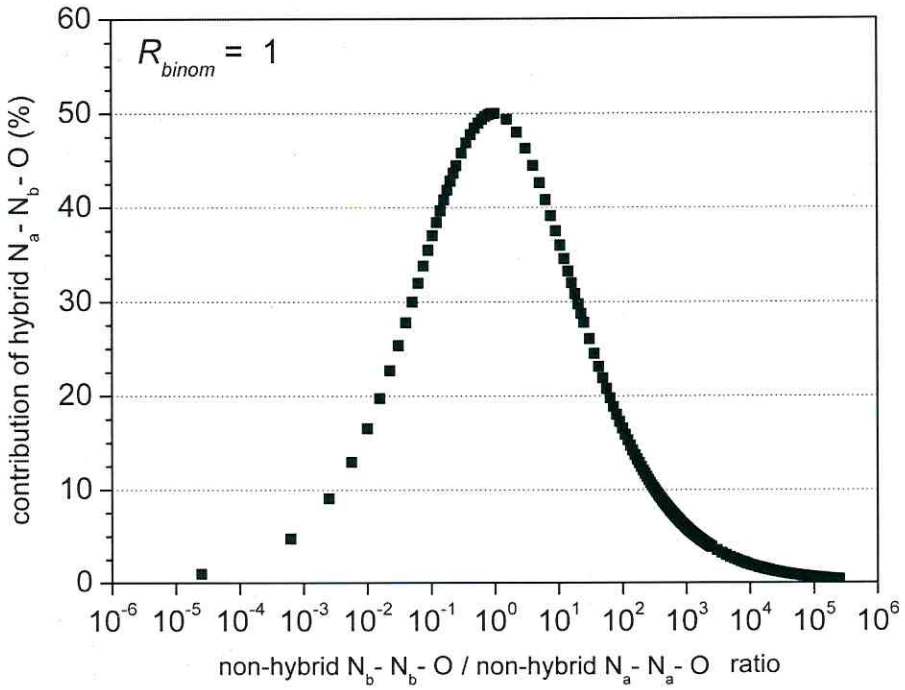

Figure 5.6 *Composition of an N_2O gas mixture with an a R_{binom} value of 1 that consists of one hybrid N_2O species and two non-hybrid N_2O species formed by two nitrogen sources with the ^{15}N mole fraction a and b.*

However, this also implies that under particular conditions a mixture of two non-hybrid N_2O species and one hybrid N_2O species (as described) can also exhibit an R_{binom} value of 1 (i.e. their mass distribution is binomial). In this case the R_{binom} value of the gas mixture would simulate a non-hybrid N_2O species formed by only one N source. Empirically it was found that the mass distribution within such a N_2O gas mixture follows a binomial function (R_{binom} = 1), if the relation of the absolute gas amounts delivered by each of the three pathways is according to Equation 5.20,

Equation 5.20 $$R_{binom} = 1 \quad ; \text{if} \quad XY = \frac{1}{4}Z^2$$

where X and Y, respectively, represent the amount of the two non-hybrid N_2O species and Z represents the amount of hybrid N_2O (total gas amount of the N_2O gas mixture = X + Y + Z). Setting Y to a fixed value of 1 and using a theoretical amount for Z allows the calculation of X

and hence, the determination of the individual contribution of each pathway on the total N_2O gas mixture required to retain a binomial mass distribution (i.e. R_{binom} = 1). In order to visualize this interrelation a wide range of Z values (10^{-2} to 10^{+3}) was applied. Plotting the calculated contribution of hybrid N_2O versus the ratio of X/Y reveals a log normal relation (Figure 5.6). The curve describes the composition of an N_2O gas mixture which exhibits a binomial mass distribution (R_{binom} = 1), but actually consists of two non-hybrid and one hybrid N_2O gas species generated by two differently labeled N sources ($a \neq b$) as described above. The R_{binom} value will always be >1, if the contribution of hybrid N_2O gas stays above the curve. Otherwise, R_{binom} will always be <1, if the contribution of hybrid N_2O gas remains below the curve. Thus it appears that a hybrid N_2O formation can always be proven, if its current contribution to the total N_2O gas mixture accounts for >50 %. In addition, it can be concluded that hybrid N_2O production can also be proven at a contribution ≤50 %, if the ratio of non-hybrid N_2O formed by the two differently labeled N sources ($a \neq b$) differs from 1.

6. Concluding discussion

The process of codenitrification appears to be one of the most interesting processes of microbial nitrogen transformation inasmuch as it permits a variety of organic (e.g. amines) and inorganic (e.g. NH_4^+) nitrogen species to be co-metabolised to N_2O and N_2 in the course conventional denitrification. The thorough literature review given in chapter 2 revealed that codenitrification has to be considered as a ubiquitous pathway of microbial nitrogen transformation. It was found that codenitrification is not only significant for fungal denitrification, as originally assumed by Tanimoto et al. (1992), but is performed by a variety of denitrifying species of all three domains: *archaea*, *bacteria*, and *eukarya* (kingdom *fungi*). Hence, one would expect that codenitrification plays a significant role for microbial nitrogen cycling in the biosphere. However, in spite of the fact that codenitrification was already described two decades ago (Shoun et al., 1992; Tanimoto et al., 1992) it remained almost unconsidered by the scientific community. Even today a tremendous gap of knowledge exists and thus, profound research is required to further elucidate its impact on microbial nitrogen cycling in the biosphere. The author hopes that the present doctoral thesis gives new perspectives for future research on codenitrification and may help to deepen our knowledge of this intriguing microbial process.

6.1. ^{15}N-aided analysis of hybrid N_2O/N_2 formation by codenitrification

Investigations on microbial N_2O and N_2 production due to denitrification and concomitant codenitrification are in general restricted by two major constraints: (i) a quantification of N_2 formation related to microbial nitrogen transformation and (ii) a differentiation of denitrified non-hybrid N-N gas and simultaneously codenitrified hybrid N-N gas.

It was recently pointed out by Groffman et al. (2006) that (in contrast to N_2O) the microbial production of N_2 is extremely difficult to quantify. While N_2O occurs only in trace amounts within the earth's atmosphere, the latter species represents its major component (78.08 vol.% N_2). Thus, a determination of microbial N_2 formation is still today one of the main methodological challenges concerning a comprehensive investigation of nitrogen cycling in the biosphere. Direct measurements of microbial N_2 formation can only be carried out in an artificial atmosphere "free of" atmospheric N_2. This type of N_2 determination was realised by a few studies, which applied a gas flow soil core technique (steady state condition) with an artificial atmosphere for sample incubation (Swerts et al., 1995; Scholefield et al., 1997a; Butterbach-Bahl et al., 2002; Cardenas et al., 2003; Mander et al., 2003). However, in order to differentiate denitrified non-hybrid N-N gas from simultaneously codenitrified hybrid N-N gas an additional application of a ^{15}N tracer technique is required. Therefore, in the present doctoral thesis a gas flow soil core technique was coupled online to a GC-MS system (GC 5890 Series II; HP – Agilent, USA / ConFlow IRMS - delta S; Thermo-Finnigan MAT, Bremen, Germany) to allow an isotopic analysis of released N-N gases (see chapter 3). To permit a simultaneous gas sampling for N_2O and N_2 a cryoenrichment technique was used for

both gases. While N_2O was sampled by freezing out with liquid dinitrogen as already described elsewhere (e.g. Speir et al., 1995; Sich and Russow, 1998), N_2 was sampled by a new approach using the dinitrogen selective molecular sieve Köstrolith SX6® cooled with liquid dinitrogen. The novel incubation system exhibits a number of advantages which are important for detailed studies on microbial N_2O and N_2 production. First, the isotopic analysis of N_2O and N_2 allows an application of the ^{15}N tracer technique and thus a process-related study of nitrogen gas production (e.g. by denitrification and concomitant codenitrification) by using ^{15}N tracer models. Second, the current detection limits concerning N_2 flux determination are significantly decreased due to the ^{15}N aided differentiation from atmospheric N_2 contamination. In contrast to a ^{15}N assisted offline steady state approach as e.g. published by Ruser et al. (2006), an online determination of soil N_2 release allows a distinctively higher sampling frequency. In contrast to non-steady state approaches (e.g. Malone et al., 1998; Bergsma et al., 2001; Stevens and Laughlin, 2001a) the system also allows a continuous determination of soil N_2 and N_2O release for long incubation periods without disturbing the gas headspace of incubated samples. The cryoenrichment sampling of N_2O and N_2 permits a very precise determination of the gas release rates based on a single gas analysis (sampled N amount per time of capturing). However, due to the fact that the developed gas flow soil core technique (including ^{15}N isotope analysis) is rather complex and difficult to automate it suffers from a high work load. Nonetheless, it can be clearly concluded from chapter 3 that the developed incubation system represents an adequate laboratory method for comprehensive studies on N_2O and N_2 formation in the course of different but simultaneously occurring microbial nitrogen transformation processes (e.g. denitrification and concomitant codenitrification).

The ^{15}N tracer technique has been used since decades in order to elucidate the pathways of nitrogen transformation in different environments (e.g. Faust et al., 1981; Faust, 1993), in particular concerning the formation of gaseous nitrogen species such as N_2O and N_2 (e.g. Siegel et al., 1982; Russow et al., 2008). Based on the principles of random and non-random isotope distribution within a dinitrogen gas species (Hauck et al., 1958; Hauck and Bouldin, 1961) a variety of ^{15}N approaches has been developed for a process related determination of N_2O and N_2 formation in the course of common denitrification (e.g. Mulvaney and Boast, 1986; Arah, 1992; Russow et al., 1996; Khalil et al., 2004). However, until now a concomitant release of hybrid N_2O or hybrid N_2 in the course of denitrification was never considered by these former ^{15}N tracer models. It is demonstrated in chapter 4 (see Figure 4.3) that these former approaches will in fact fail when hybrid N_2O/N_2 is formed simultaneously with non-hybrid N_2O/N_2. As shown in chapter 2 a simultaneous release of non-hybrid and hybrid N-N gas due denitrification and concomitant codenitrification was already reported for a variety of denitrifying species. Thus a hybrid N-N gas formation should be always taken into account when a nitrogen gas release under denitrifying conditions has to be studied. Moreover, it appears obvious that the tremendous gap of knowledge concerning hybrid N_2O

and hybrid N_2 formation is mainly due to a lack of applicable methods which would in general allow the study of codenitrification. Thus, a novel type of ^{15}N tracer model was urgently required and was therefore developed within this doctoral thesis (see chapter 4). This type of analytical ^{15}N tracer model was originally designed for an investigation of hybrid N_2 formation in course of anaerobic ammonium oxidation (van de Graaf et al., 1990), which was also shown to occur simultaneously with non-hybrid denitrified N_2 (Thamdrup and Dalsgaard, 2002; Dalsgaard et al., 2003; Kuypers et al., 2003; Rysgaard et al., 2004; Dalsgaard et al., 2005). However, as demonstrated in chapter 4 this ^{15}N tracer model can also be used for an investigation of hybrid N-N gas formation in the course of codenitrification. For that purpose the model was adapted (chapter 5) to consider two different nitrogen sources (e.g. NH_2OH and NO_2^-), where each source separately generates non-hybrid N-N gas (e.g. by NH_2OH decomposition and denitrification) and simultaneously both sources are combined to hybrid N-N gas species (e.g. by codenitrification). Based on the developed soil incubation system (chapter 3) and the adapted ^{15}N tracer model (chapter 4 and 5) an experimental study was conducted to evaluate the applicability of both approaches concerning studies on hybrid N-N gas production due to codenitrification. A suspended soil (Haplic Chernozem, Agricultural Research Site Bad Luchstädt) was incubated under conditions favourable for denitrification and tested for a hybrid N_2O production by codenitrification of NH_2OH (chapter 5). Until now, a hybrid N_2O production by codenitrification of NH_2OH was reported by only a few studies, which have been conducted on a microbial species level or by using purified enzymes (Iwasaki and Mori, 1958; Renner and Becker, 1970; Matsubara, 1970; Garber and Hollocher, 1982b; Kim and Hollocher, 1984; Aerssens et al., 1986). It was found within the present doctoral thesis that hybrid N_2O formation due to codenitrification of NH_2OH can be in fact occur in a (suspended) soil. The hybrid N_2O production was predicted by the adapted analytical ^{15}N tracer model.

However, even if codenitrification is considered by the novel type of analytical ^{15}N tracer model, a verification of hybrid N-N gas production still remains a tremendous challenge. This occurs mainly due to the fact that the ^{15}N abundance of the labelled nitrogen source utilised during e.g. denitrification and simultaneous codenitrification (e.g. NO_2^-) are difficult to determine. This appears particularly important concerning non-suspended soils considering (i) soil heterogeneity, (ii) sampling procedures, and (iii) a lack of knowledge concerning the effective ^{15}N abundance of microbially available nitrogen compounds. Therefore, the novel characteristic factor R_{binom} was additionally developed within this thesis (see chapter 5.7.1.). The R_{binom} factor permits an identification of hybrid N-N gas formation solely based on the mass distribution of an N-N gas species (e.g. α_{44}, α_{45}, and α_{46}). Hence, based on the same experimental ^{15}N data of a released N-N gas species the R_{binom} value allows an easy identification of hybrid N-N gas production without the need of delivering additional data concerning the ^{15}N abundance of related nitrogen sources (e.g. NH_2OH and NO_2^-). As shown in chapter 5 the R_{binom} value was successfully applied to identify hybrid N_2O production and

hence, it can be easily used to pre-evaluate the results of a ^{15}N tracer experiment concerning an occurrence of hybrid N-N gas production during soil incubation.

Even if codenitrification was provoked by artificially high NH_2OH concentrations it can be clearly concluded that the used Haplic Chernozem soil exhibits the capability to perform codenitrification, i.e. inhabits microbial species which allow a hybrid N_2O formation due to codenitrification. Therefore, it is proposed here that the approach presented in chapter 5 could be applied as a general screening tool for evaluating the capability of a soil to perform codenitrification.

Recapitulatory, it can be concluded that all developed experimental and mathematical methods (chapter 3 to 5) represent promising tools for future studies on hybrid N_2O and hybrid N_2 formation due to codenitrification.

6.2. Considerations on codenitrification in the biosphere

6.2.1. Terrestrial environments

The literature review (see chapter 2) reveals that the process of codenitrification should be considered as a ubiquitous pathway of microbial N transformation in terrestrial environments. However, until now only two studies effectively demonstrated N-N gas production due to codenitrification beyond a microbial species level. Laughlin and Stevens (2002) reported that soil N_2 formation in a Typic Dystrochrept (acidic brown earth) was clearly dominated by fungi species, which largely produced hybrid N_2 due to codenitrification. In addition, it was shown in this doctoral thesis (see chapter 5) that codenitrified hybrid N_2O is produced in a soil (suspension of a Haplic Chernozem) when high quantities of NH_2OH are simultaneously supplied with NO_3^- under conditions favourable for denitrification. Besides this, a few studies reported N transformation phenomena, which have been discussed by the authors as caused by codenitrification. Kammann et al. (2008) reported a higher soil N_2O release in a permanent grassland soil when treated with an artificially elevated atmospheric CO_2 concentration. It was assumed that the increased CO_2 level enhanced the formation of labile organic N compounds and resulted in a shift towards a more organic N based N transformation. They concluded that the increased N_2O formation was due to enhanced codenitrification activity caused by a continuously growing fungal biomass. Roobroeck et al. (2010) recently reported "excess" N-N gas production in a peat soil (highly productive sedge monolith fen ecosystem) after nitrate addition, which was interpreted by them as apparently caused by codenitrification. Beyond that studies have revealed that the intramolecular distribution of ^{15}N in soil N_2O (i.e. $^1\delta^{15}N$–$^2\delta^{15}N$–O) can exhibit considerable differences (Yamulki et al., 2001; Perez et al., 2001; Bol et al., 2003) and thus may provide evidences that the N-N linkage within N_2O originates from two different N compounds (i.e. formation of hybrid N_2O gas). Wilson et al. (2006) observed considerable differences in the intramolecular isotopic signature of N_2O released from a field of maize irrigated by piggery waste. They concluded that the most likely mechanism was a bacterial/fungal codenitrification in the presence of both NO_3^- and NH_3 under anaerobic,

nutrient rich conditions. Meijide et al. (2010) reported isotopomer anomalies within N_2O released from an arable soil (Chromic Luvisol), which was also considered to be related to codenitrification activity. However, as recently pointed out by Well and Flessa (2009) the intramolecular distribution of ^{15}N in soil N_2O is affected by a variety of N transformation processes and therefore, the interpretation of these data has to be done with caution.

In order to gets an estimate concerning the relevance of codenitrification in terrestrial ecosystem, natural availability of suitable nucleophiles (e.g. NH_2OH, primary amines) and reaction kinetics of denitrification/codenitrification can be used. According to the sequential reaction scheme of denitrification (see chapter 2.1.3) N-N gas production by denitrification and simultaneous codenitrification can be considered as a competing reaction on the enzymatic nitrosyl intermediate between NO/NO_2^- on the one side and codenitrification related nucleophiles (e.g. primary amines) on the other side (e.g. Weeg-Aerssens et al. 1987/1988). As reported elsewhere (e.g. Kim and Hollocher, 1984; Conrad, 1996; Zumft, 1997; Su et al. 2004) K_m values of NO_2^- and NO reduction are largely in the nM to µM range, while microbial nitrosation of codenitrification related nucleophiles exhibit K_m values in the mM range (see chapter 2.3.4). Significant codenitrification rates should be therefore only occur if the concentration of natural nucleophiles is at least one or more orders of magnitude higher as compared to their competitors NO_2^- and NO, respectively. In fact, in most studies summarised in Table 2.1 co-metabolisable nucleophiles have been supplied at very high concentrations (10^0 to 10^3 mM). However, since NO_2^- and NO generally occur at very low concentrations in soils (nM to µM range) (Van Cleemput and Samater 1996; Ludwig, et al., 2001; Rammon and Peirce, 2002), hybrid N-N gas production via codenitrification can be considered as of ecological relevance if suitable nucleophiles occur in µM to mM range.

The nucleophile NH_2OH appears to be particularly interesting inasmuch as it represents an already well known intermediate compound of aerobic ammonia oxidation (i.e. nitrification) (e.g. Lees, 1952; Verstraete and Alexander, 1972). In addition, NH_2OH has also been proven to be directly involved in soil N_2O formation via nitrification (Khalil et al., 2004) as well as via abiotic NH_2OH decomposition (Bremner et al., 1980; Nelson, 1982). Regarding studies where NH_2OH has been artificially added to microorganisms (e.g. Garber and Hollocher, 1982b; Kim and Hollocher, 1984; Weeg-Aerssens et al. 1987/88; Goretski and Hollocher 1991) or soil (see chapter 5) codenitrification contributed with up to 98 % to the total microbial N_2O gas release. Considering NH_2OH as a naturally occurring nucleophile, however, a hybrid N_2O production by codenitrification in terrestrial environments appears to be rather unlikely for obvious reasons. NH_2OH is a highly reactive species and assumed to occur naturally at only very low concentrations (≤nM range) in soils. To the best of our knowledge no study dealing with soil NH_2OH content is currently available. Our own analytical efforts to determine the natural NH_2OH content in a Haplic Chernozem soil have been unsuccessful (unpublished). It is known that NH_2OH remains stable only in the cationic form (i.e. at pH < 5), while in soils at around neutral pH (optimal for microbial N-nitrosation)

it decomposes rapidly (von Breymann et al., 1982; Seike et al., 2004). However, as shown by Weeg-Aerssens et al. (1988) at a decreasing availability of organic substrates (required as electron donors for heterotrophic denitrification) the codenitrification/denitrification ratio increases significantly. Since NH_2OH has been demonstrated to act as electron and N donor during microbial N-nitrosation (Iwasaki and Mori, 1958; Matsubara, 1970; Garber and Hollocher, 1982b, Weeg-Aerssens et al., 1988; Su et al., 2004) hybrid N_2O production in soils by codenitrification of NH_2OH might be therefore of higher relevance in C-limited environments.

Besides NH_2OH, primary amine compounds (e.g. amino acids) appear to be a far more relevant source of hybrid N-N gas production in soils. According to Stevenson (1982) amino acids represent one of the major pools of soil organic N. Thorn and Mikita (2000) proved that at least abiotic nitrosation is directly related to the quality of soil organic matter. While N-nitrosation occurred in a peat humic acid with a considerable elemental N content (≈4%), in humic or fulvic acids with a significantly lower N content (<1%) N-nitrosation did not occur at all. Jones et al. (2002) reported that across 6 different soil types in Ireland (e.g. Placic podzol, Dystric histosol) free amino acids accounted for 10–40% of the total soluble N in the soil solution. The average soluble N concentrations of amino acids were 24 ± 8 µM. Senwo and Tabatabai (1998) found that the N content of amino acids extracted from different cultivated soils (e.g. Typic hapludalf, USDS Soil Taxonomy) ranged from 32% to 50% of total organic N, whereby total amino acid N accounted for up to 300 mg kg^{-1} soil. Similar results have been published by McLain and Martens (2005) who reported high contents of primary amines (amino acids and amino sugars) in a semiarid soil (Typic Torrifluvents, USDS Soil Taxonomy). Considering the average content of e.g. NO_2^- in soils (0.1 mg NO_2^--N kg-1 soil; Van Cleemput and Samater, 1996) a formation of hybrid N_2 by codenitrification is therefore very likely from a kinetic point of view. Hence, it appears less surprising that hybrid N-N gas production in a soil without adding co-metabolisable nucleophiles, as only shown by Laughlin and Stevens (2002), revealed hybrid N_2 formation (92 % of total soil N_2 release), while simultaneously released N_2O resulted from denitrification only. Besides primary amines also NH_3 is already known to undergo microbial N-nitrosation resulting in hybrid N_2 formation (Kim and Hollocher, 1984). Inasmuch as NH_3 is already well known to act as the key substrate of the nitrification process (e.g. Arp and Stein, 2003), it has to be equally considered as a suitable nucleophile for hybrid N_2 gas production in soils. However, based on its equilibrium reaction with its hydrogenated form NH_4^+ (Da Silva et al., 2007) it occurs at only small concentrations in soils with neutral pH conditions. Even if NH_4^+ is known to occur at considerable concentrations in soils (µM range e.g. Jones et al., 2002) significant hybrid N_2 production via NH_3 will be hampered by its low equilibrium concentration. A few studies demonstrated that also NH_4^+ is apparently utilised during codenitrification resulting in hybrid N_2O formation (Tanimoto et al., 1992; Immoos et al., 2004; Su et al., 2004). Although the biochemical origin of hybrid N_2O/N_2 gas production via NH_4^+/NH_3 still needs to be clarified,

it appears that NH_4^+ might represent a more relevant N compound for hybrid N-N gas production in soils than NH_3.

Even if a few studies imply that codenitrification could be a significant process of microbial N turnover, the effective contribution of codenitrification on N-N gas release from terrestrial environments still remains hidden. Müller et al. (2006) suggested that codenitrification might be important in ecosystems characterised by established community structures of fungi and bacteria and a closed N cycle, as for instance occurring in permanent grassland or undisturbed forest soils.

6.2.2 Aquatic environments

To the best of our knowledge codenitrification has never conclusively been proven to occur in aquatic environments. However, inasmuch as denitrification is already known to be widely distributed within marine, lacustrine, and fluvial ecosystems (e.g. Pina-Ochoa and Alvarez-Cobelas, 2006), one could assume that codenitrification occurs equally in aquatic environments. For example Bol et al. (2004) revealed that the intramolecular isotopic distribution of ^{15}N in N_2O (i.e. $^1\delta^{15}N$–$^2\delta^{15}N$–O) released from estuarine soil under flooded conditions significantly differed from non-flooded conditions. While the $^2\delta^{15}N$ value revealed almost no distinct differences between flooded (-11.6 to 16.0‰) and non-flooded conditions (-14.6 to 9.6 ‰), the $^1\delta^{15}N$ value ranged from -52.4 to -7.3‰ under flooded and -16.7 to -6.1‰ under non-flooded conditions, respectively. This was interpreted by them as an unidentified pathway of N_2O production, possibly related to nitrification or nitrifier-denitrification. However, considering the hybrid character of codenitrified N_2O the observed intramolecular differences could equally reflect the isotopic abundance of two different N sources (e.g. NO_2^- and NH_2OH). More recently, Erler et al. (2008) reported unusually high production of $^{14,15}N_2O$ relative to $^{15,15}N_2O$ when an intact sediment core (surface flow constructed wetland) was labelled with $^{15}NO_3^-$. They concluded that nitrifier-denitrification (see Poth and Focht, 1985) was responsible for the observed anomaly. However, another possible explanation might be N_2O production via biotic N-nitrosation, where e.g. $^{15}NO_2^-$ (from denitrification of $^{15}NO_3^-$) reacts with $^{14}NH_2OH$ gained from $^{14}NH_3$ oxidation (i.e. nitrification). It was in fact already demonstrated by Schmidt et al. (2004b) that during the oxidation of NH_3 by a mutant of *Nitrosomonas europaea* high quantities of NH_2OH are released. Moreover, the present doctoral thesis demonstrated hybrid N_2O production by codenitrification in a soil suspension, when high quantities of NH_2OH are added during active denitrification (see chapter 5). Even if these reports do not prove codenitrification in aquatic ecosystems, they at least provide some hints that it may occur also here.

As already discussed in the former section most promising nucleophiles for hybrid N-N gas formation under natural conditions are NH_2OH, NH_4^+/NH_3, and primary amines (e.g. amino acids naturally occurring in organic matter). Considerable NH_2OH concentrations (up to 50 $\mu g\ N\ L^{-1}$; µM range) have been at least reported for aquatic environments such as reed beds

(Seike et al., 2004). In general, concentrations of NH_2OH in aquatic ecosystems occur at the nM range (e.g. Pittwell, 1975, Schweiger et al., 2007) and hence, NH_2OH might be less significant for hybrid N_2O formation in most aquatic environments. Considering amino compounds, however, a variety of aquatic ecosystems (largely marine) have been shown to exhibit suitable concentrations (µM range) for microbial N-nitrosation reactions via codenitrification (e.g. Henrichs and Farrington, 1979; Lee, 1988; Suttle et al. 1991; Munster, 1993). Most of it originates from organic matter which is formed within the surface water and then deposits on the sediment. Lee (1988) pointed out that amino acids represent one quarter of the particulate organic carbon and half of the particulate organic nitrogen in marine surface waters. According to Henrichs and Farrington (1979) within marine sediment pore water the amino acid concentration can rise to 1 mg L^{-1}. Therefore, in aquatic environments naturally occurring primary amines appear to be of high significance as potential substrates for a hybrid N_2 production via codenitrification. Similarly, also NH_4^+/NH_3 have to be considered as biorelevant nucleophiles for hybrid N-N gas formation via codenitrification. Instead of codenitrification, however, the process of anaerobic ammonium oxidation (anammox) was shown to be a major NH_4^+ utilising process within aquatic ecosystems (Jetten et al., 2005; Op den Camp et al., 2006). During the anammox process a NH_4^+-N and a NO_2^--N are microbially linked and finally converted to hybrid N_2 gas (van de Graaf et al., 1995). The principle reaction scheme was already postulated in 1977 by Broda and proceeds in the sense of an N comproportionation (see chapter 2.1.1). From this it follows that N_2 gas production by the anammox process appears to be similar to the codenitrification process. In contrast to codenitrification, however, the anammox process was shown to be only performed by particular non-denitrifying species of the phylum *Planctomycetes* (Kuenen, 2008). Nonetheless, Strous et al. (2006) recently reported that anammox bacteria (e.g. *Kuenenia stuttgartiensis*) exhibit an enzyme type of cytochrome cd_1 NIR, which is in fact already known to perform biotic N-nitrosation in the course of denitrification (Averill, 1996; Kim and Hollocher, 1984; Weeg-Aerssens et al., 1988). Based on the denitrifying enzyme a novel reaction pathway for the anammox process has been postulated by Strous et al. (2006) (Figure 6.1). First, NO_2^- is reduced to NO by the cd_1 NIR and then a hydrazine synthase is assumed to catalyse the hybrid N-N linkage between NO and NH_4^+ resulting in the production of N_2H_4. Second, N_2H_4 is oxidised to N_2 gas by a hydrazine dehydrogenase (Karlsson et al., 2009; Strous et al., 2006). If we assume that the N-N linkage during anammox occurs due to biotic N-nitrosation of NH_4^+ by NO, the reaction should actually occur via an enzymatic metal-NO-complex formed by the hydrazine synthase (Figure 6.1).

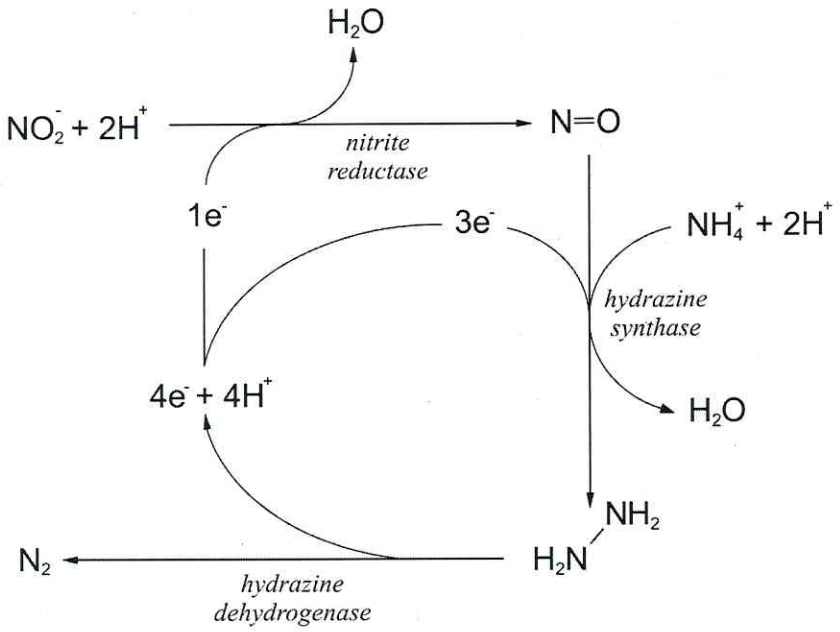

Figure 6.1 *Postulated reaction scheme of anaerobic oxidation of NH_4^+ with NO_2^- to N_2 (after Fuchs and Schlegel, 2007; modified).*

However, according to former reports on biotic N-nitrosation via denitrification (Averill, 1996; Kim and Hollocher, 1984; Weeg-Aerssens et al., 1988) a *cd*$_1$ NIR can equally act as a nitrosating agent by either using NO_2^- or NO as the nitrosyl donor. Moreover, hybrid N_2 formation by a cytochrome *cd*$_1$ NIR (purified from *Pseudomonas aeruginosa*) due to an N-nitrosation of NH_3 with NO_2^- was in fact already demonstrated by Kim and Hollocher (1984). By contrast, however, an N-nitrosation of its protonated form (i.e. NH_4^+) during codenitrification was reported to result in hybrid N_2O production (Tanimoto et al., 1992; Immoos et al. 2004; Su et al., 2004). Even if the anammox pathway is already thoroughly studied (Francis et al., 2007; Strous et al., 2006; van der Star et al., 2008), it appears promising to further elucidate to what extent anammox and codenitrification can be viewed as analogue processes of microbial N transformation.

6.2.3. Nitrogen immobilisation due to biotic nitrosation

As pointed out by Williams (2004) abiotic nitrosation reactions via NO_2^- are in general rather complex, but always result in either N gas liberation or N immobilisation. Thorn and Mikita (2000) demonstrated that abiotic nitrosation of soil humic substances (e.g. secondary aliphates, aromates, and amides) by NO_2^- can occur on aromatic C, methylene C, or an N atom. Depending on the reacting nucleophile and its specific binding site, NO_2^--N is either released in the form of N gas or immobilised by fixation onto soil organic compounds. Also

Van Cleemput and Samater (1996) reported the build up of soil organic N species (e.g. nitro- and nitroso compounds) due to abiotic nitrosation of phenolic soil compounds via NO_2^-. Similar results have also been published earlier by Bremner and Fuhr (1966) and Smith and Chalk (1980).

As suggested by Garber and Hollocher (1982b) biotic metal-nitrosyl species (e.g. denitrifying enzymes; see chapter 2.1.2 and 2.1.3), which catalyse the codenitrification process, can also act as nitrosyl donors to a variety of N-, O-, S-, and C-nucleophiles. Hence, one could expect that during microbial nitrosation also N immobilisation might occur (e.g. by C-nitrosation of phenols). In fact, a variety of microbial species have already been proven to perform nitrosation reactions without N gas formation, but with different nitrosamine species as the reaction products (Mills and Alexander, 1976; Calmels et al., 1985; Leach et al., 1987; Stamler et al., 1992). It was shown (e.g. Leach et al., 1987; Calmels et al., 1996) that biotic nitrosation of heterocyclic amines (e.g. morpholine) by *Echerichia coli* and *Pseudomonas aeruginosa* resulted in immobilisation of NO_2^--N in the form of nitroso compounds. Mills and Alexander (1976) reported a formation of dimethylnitrosamine upon biotically mediated nitrosation of dimethylamine by *P. stutzeri* and *E. coli*, respectively. Kunisaki and Hayashi (1979) proved an enzymatically initiated N immobilisation due to a nitrosation of secondary amines (e.g. piperidine) by *E. coli*. Thus it appears that during N cycling in the biosphere biotic nitrosation reactions mediated by denitrifying species might even contribute to N immobilisation. Davidson et al. (2003) already underlined that our understanding of the mechanism by which inorganic N in form of NO_3^- and NO_2^- is immobilised in soils still appears to be insufficient. They pointed out that at least under acidic conditions (e.g. forest soils) abiotic nitrosation might significantly contribute to N immobilisation in soils. Since biotic nitrosation was shown to occur optimally around neutral pH conditions (see chapter 2.3.2), it appears likely that codenitrification may act as an important pathway of N immobilisation within many ecosystems.

Nitrogen immobilisation due to biotic nitrosation was also observed under aerobic conditions in the course of conventional nitrification (e.g. Azhar et al., 1986; Verhaegen et al., 1988; Azhar et al., 1989a; Azhar et al., 1989b). Despite the fact that until now no conclusive evidence of hybrid N-N gas formation via nitrification was delivered, observed N immobilisation seems to indicate the existents of a co-metabolic nitrification process (i.e. co-nitrification) as an aerobic analogue to codenitrification. Azhar et al. (1986) reported that during nitrification N bonding onto soil organic matter occurred due to a nitrosation reaction via the nitrification intermediate NO_2^- with phenolic compounds (e.g. 1-naphtol). Up to 55% of applied NH_4^+ was bound on organic matter (within 60 days of incubation) resulting in the build up of plenty of organic species such as amine-, nitroso-, nitro-, azido-, and oxime compounds. By contrast, however, no N immobilisation onto soil organic matter occurred when a nitrification inhibitor (nitrapyrin) was applied or NH_4^+ was replaced with NO_3^-. Azhar et al. (1989a) pointed out that in the nitrification mediated nitrosation process NO_2^- forming

microorganisms are obviously of predominant importance. They even suggested that this microbial process might be an essential pathway for the formation of humic materials in soils due to a coupling of phenols and aromatic amines. Thus, we could draw the conclusion that soil N immobilisation by biotic nitrosation might also occur among nitrifying species which are capable of mediating nitrosation reactions. Nonetheless, there is an urgent need to clarify the impact of codenitrification and even "co-nitrification" on N immobilisation during biospheric N cycling.

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