Microbial denitrification control in acid and neutral soils with implications for greenhouse gas emissions and atmospheric chemistry

Mikroorganismer kontrollerer denitrifikasjon i sur og neutral jord med implikasjoner for veksthusgassutslipp og atmosfærekjemi

Philosophiae Doctor (PhD) Thesis

Lim Yen Nee, Natalie

Faculty of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences

Ås 2017

Thesis number 2017:51
ISSN 1894-6402
ISBN 978-82-575-1450-1

PhD supervisors

Prof. Åsa Frostegård (main supervisor)
Faculty of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences
1432 Ås, Norway
asa.frostegard@nmbu.no

Prof. Lars R Bakken (co-supervisor)
Faculty of Environmental Sciences and Natural Resource Management
Norwegian University of Life Sciences
1432 Ås, Norway
lars.bakken@nmbu.no

Dr. Linda Bergaust (co-supervisor)
Faculty of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences
1432 Ås, Norway
linda.bergaust@nmbu.no

Thesis evaluation committee

Univ.-Prof. Dipl.-Biol. Dr. Christa Schleper (Opponent 1)
Department of Ecogenomics & Systems Biology
University of Vienna
1090 Vienna, Austria
christa.schleper@univie.ac.at

Prof. Dr. Marcus Andreas Horn (Opponent 2)
Institute for Microbiology
Leibniz Universität Hannover
30419 Hannover, Germany
horn@ifmb.uni-hannover.de

Dr. Phillip Pope (Coordinator)
Faculty of Chemistry, Biotechnology and Food Science
Norwegian University of Life Sciences
1432 Ås, Norway
phil.pope@nmbu.no

Table of Contents

Acknowledgements

The present work was performed at the Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, with financial support from the university. Additional bioinformatics work was performed during a visit to the Department of Microbiology, Cornell University.

First and foremost, my sincerest thanks to my supervisor and co-supervisor, Åsa Frostegård and Lars Bakken, for their invaluable guidance and support since I had first started as a Masters student in your group. The countless opportunities you have provided me with for scientific research and conference networking are much appreciated. My experience here has allowed me to grow both as a scientist and as a person, and for that alone, you have my utmost gratitude.

I am immensely grateful to all the members of the NMBU Nitrogen Group for providing me with my home-away-from-home. I will always treasure the countless (and sometimes random) discussions I have had with you guys. Special thanks to Else and Rannei – the fact that the labs still stand and none of us has burnt anything down is testament to your science monkey-herding prowess. ☺

Last but not least, thank you to my friends and family, both in Norway and abroad, for their support regardless of their physical proximity. In particular, my boundless gratitude to mum and dad for listening to my many complaints with kind words of encouragement, and Jonathan for helping me to de-stress (with Assassin's Creed discussions, no less!). To my friends, thanks a million for dragging me out of the lab for a short chat/coffee/ice-cream, going dancing, or just playing the piano – I really needed those all-too-seldom breaks from my work.

I will forever be grateful to all who have given me assistance, big or small, during my time here in Norway. This has truly been a life-changing experience.

Ås (Norway), June 2017

Lim Yen Nee, Natalie

This page intentionally left blank

Summary

As with the ancient philosophy of *ex nihilo, nihil fit* (out of nothing, nothing is produced: nothing comes from nothing), the law of the conservation of mass dictates that no atoms in a chemical reaction may be created or destroyed. Such conservation of Earthly nitrogen makes up the global nitrogen cycle – a series of biological processes continuously recycling nitrogen. Of special interest is denitrification, an anaerobic process which contributes to the sizeable yearly production of environmentally-important nitrite, NO and N_2O. In itself, N_2O is a powerful greenhouse gas with a global warming potential >300 times that of CO_2. Nitrite and NO, in contrast, may influence atmospheric chemical reactions via gaseous nitrous acid (HONO) production, and may also wreak havoc at a molecular level within microorganisms. Nitrite is potentially toxic because of its propensity to form aqueous nitrous acid (HNO_2), which is able to pass freely through cell membranes; whereas NO is a key signalling molecule in regulating the transcription of various genes, further demonstrating the biological and chemical impact of denitrification intermediates.

Despite its key role in producing environmentally important compounds, there is still considerable ignorance surrounding denitrification in an environmental setting. Not to say our current knowledge is insignificant, quite the contrary: Denitrification is a modular process primarily mediated by bacteria, where nitrate/nitrite is reduced to N_2O/N_2 via NO; under anoxia denitrifiers shift from respiring O_2 to nitrate and other N-oxides; denitrification is adversely affected by O_2 levels and decreasing pH (most sensitive being N_2O reduction to N_2); and many, if not all, of the genes involved in denitrification are controlled by transcriptional regulators influenced by NO. Nevertheless, much of what we currently know has been elucidated from pure culture studies, and precious little is understood in mixed communities or in the environment. Further complicating this, commonly-used investigation methods (e.g. primer-based analysis of genes and transcripts) have been restricted by their unsuitability for community-wide application due to inherent biases. Additionally, relatively few studies have attempted to reconcile genetic/transcription studies with phenotypic observations of substrate consumption/production, leading to a disconnect between proposals of molecular systems/responses and real-world effects.

Thus, the aim of this thesis was to identify pH-dependent, anaerobic biological and chemical N-redox transformations in soils. The sub-goals therein were to:

- Develop improved protocols for co-extracting DNA and mRNA from inhibitor-rich soils for metagenomic/metatranscriptomic analyses
- Understand the pH-dependent regulatory mechanisms of denitrification controlling nitrite, NO and N_2O accumulation
- Map the genetic potential (metagenome) and transcriptional response (metatranscriptome) related to N-transformations by different organism groups, and the realised metabolism (process measurements)
- Determine the extent of abiotic reactions controlling nitrite levels in soils of different acidity

Soils of contrasting pH (pH 3.8 and 6.8) were assessed for their denitrification ability by monitoring nitrate, nitrite, and N-gas kinetics in microcosm experiments during anoxia (**Paper III**). Soil pH had the expected effect on N_2O reduction: there was immediate reduction at pH 6.8, while pH 3.8 showed negligible reduction during the first 35-40 h. Although both soils produced nitrite and NO transiently, nitrite was kept low at pH 3.8, unlike pH 6.8 where approximately half of added nitrate-N accumulated as nitrite before further reduction. Despite this high total nitrite concentration at pH 6.8, concentrations of un-dissociated HNO_2 were two orders of magnitude lower than at pH 3.8. Such information is important for understanding HONO emissions to the atmosphere.

To identify the reasons behind these phenotypes, we sequenced the DNA and mRNA of both soils obtained using our **Paper I** co-extraction method for inhibitor-rich samples. Classification revealed contrasting gene and transcript taxonomic profiles, indicating widespread modularity of denitrification potential and activity across microbial guilds in soils. Regardless, both soils had similar denitrification genetic potential, with a clear dominance of *nirK* and *qnor* over *nirS* and *cnor*. Transcription of *nap+nar* > *nirK+nirS*, potentially explaining the accumulation of nitrite at pH 6.8, but not the low nitrite levels at pH 3.8 which were attributed instead to combined chemodenitrification and metabolic control. Curiously, N_2O reductase (N2OR) gene transcription at pH 3.8 was detected without corresponding N_2O reduction. This is the first time to our knowledge that N2OR gene transcripts from multiple bacterial lineages have been confirmed in the absence of consequent N_2O reduction. This suggests that N2OR non-functionality is an overarching phenomenon across microorganisms

in acid environments, strengthening the hypothesis of post-transcriptional N2OR gene regulation.

Abiotic degradation of the same soils was modelled using sterilised soils (**Paper II**) to clarify the control of nitrite reductases on nitrite levels at different pH. Predictably, chemical decomposition at pH >6 was negligible, but comparable to biological reduction at pH <6. However, under highly acidic conditions (pH <4), abiotic decomposition was overshadowed by enzymatic reduction during most of the incubation period, indicating strong biological suppression of nitrite levels. This non-linear chemical response contends for more careful consideration of abiotic N-kinetics in soils.

Collectively, these results present a convincing argument for pH-dependent N_2O management in the presence of strong biologically-driven control of potentially toxic and environmentally harmful denitrification intermediates (nitrite and NO). Additionally, this thesis challenges predictions of NO, N_2O, and N_2 emissions from genetic potential and/or transcriptional activity without relevant phenotypic data.

This page intentionally left blank

Sammendrag

Parmenides' utsagn «*de nihilo quoniam fieri nil posse videmus*» (gresk; fra ingenting kommer ingenting) har sitt motstykke i loven om massens konstans som sier at intet atom i en kjemisk reaksjon dannes eller ødelegges. Det gjelder selvsagt også for nitrogen, som gjennom nitrogensyklusen endrer binding og oksidasjonstrinn gjennom et mangfold av biologiske reaksjoner. Det transporteres mellom biosfære, lithosfære, og atmosfære, men mengden nitrogen forblir konstant. Denitrifikasjon inntar en nøkkelrolle i nitrogensyklusen fordi den resirkulerer nitrogen fra biosfære til atmosfærisk N_2. Men i tillegg til N_2 produseres også N_2O, som er en klimagass med >300 ganger sterkere klimapådriv (pr kg) enn CO_2. Videre kan denitrifikasjon gi utslipp av NO og HONO (HNO_2), som begge påvirker troposfærens kjemi. NO og HNO_2 har også biologiske effekter på andre organismer i jord; de er giftige for noen organismer, og de påvirker genregulering hos andre.

Gitt denitrifikasjonens nøkkelrolle i nitrogensyklusen, og dens mangfold av biologiske og økologiske bivirkninger, vet vi mindre enn vi burde om prosessen og organismene. Ikke så å forstå at vi er helt uvitende: vi vet at det er en «modulær» prosess, som reduserer nitrogen trinnvis fra nitrat/nitritt til N_2 via NO og N_2O, og at de gjør dette for å opprettholde respiratorisk metabolisme i fravær av oksygen. Vi vet at oksygen er en universell repressor av de genene som koder for denitrifkasjons-enzymene, og vi kjenner mange av de andre komponentene i det genregulatoriske nettverket som kontrollerer de enkelte genene. Mye av denne kunnskapen er imidlertid basert på studier av noen få modellorganismer, gjerne studert i renkultur (ikke i samliv med andre organismer), og det råder usikkerhet med hensyn til relevansen av denne kunnskapen for forståelsen av hvordan prosessen reguleres i komplekse mikrobesamfunn. Forsøk på å studere genregulering i slike samfunn har avdekket mange metodiske problemer. Standard-verktøy i slike undersøkelser har vært å kvantifisere gener og gen-ekspresjon basert på Polymerase Chain Reaction (PCR), men svakheten ved denne teknikken er at vi kan få misvisende resultater fordi 1) metodene for ekstraksjon av DNA/RNA er dårligere (bias) og 2) «primerne» fanger kun opp en liten andel av de sekvensene som finnes, for eksempel i jord. En annen svakhet med mange slike molekylærbiologiske studier av denitrifikasjon i jord har vært mangelfull analyse av «fenotypen», det vil si prosesshastigheter og kinetikk. I verste fall har man nøyd seg med å kvantifisere gener og gen-transkripter, og tatt det for gitt at dette er uttrykk for potensiell og faktisk metabolsk aktivitet.

Dette er bakteppet for mitt doktorgradsarbeid, som i hovedsak har dreid seg om å bestemme hvordan pH i jord påvirker anaerobe biologiske og kjemiske nitrogen redoks-transformasjoner i jord. Delmålene har vært

- Utvikle en bedre metode for effektiv og representativ ekstraksjon av både DNA og RNA fra jord for å muliggjøre troverdige metagenomiske og metatranskriptomiske analyser
- Forstå hvordan pH påvirker regulering av denitrifikasjon i jord, og derigjennom regulerer utslippet av NO og N_2O
- Kartlegge det genetiske potensialet for nitrogentransformasjoner (metagenom), organismenes forsøk på å uttrykke dette potensialet (metatranskriptom), og i hvilke grad de faktisk lykkes (prosess-måling)
- Bestemme betydningen av biotisk versus kjemisk transformasjon av nitritt, som funksjon av pH i jord.

Jord med pH 6.8 og 3.8 fra et langvarig kalkingsforsøk ble undersøkt med hensyn til denitrifikasjons-kinetikk, deriblant transient akkumulasjon av mellomproduktene nitritt, NO og N_2O. Eksperimentene viste den forventede effekten av lav pH på jordens evne til å redusere N_2O (forsinket N_2O-reduksjon ved lav pH gir høyt utslipp av N_2O). Nitritt-akkumulasjon viste det motsatte mønster: jord med pH 6.8 akkumulerte store mengder nitritt, mens nitrittakkumulasjon i sur jord var marginal. På tross av dette var konsentrasjonen av udissosiert nitritt langt høyere i sur enn i basisk jord. Dette belyser pH-virkning på emisjon av HNO, som antas å være proporsjonal med konsentrasjonen av udissosiert nitritt.

Metagenomiske ag metatranskriptomiske analyser ble anvendt for å forstå disse fenomenene. Det var klare forskjeller mellom jordtypene, både med hensyn til genetisk sammensetning av denitrifikasjonsfloraen (metagenom) og dens genuttrykk (metatranskriptom), men den totale mengden av gener som koder for de enkelte stegene i denitrifkasjon viste mindre avhengighet av pH, og kunne bare i noen grad forklare de klare forskjellene i prosesshastighet. Dette illustrerer naiviteten i å anta at tilstedeværelsen av et gen (eller et transkript) er ekvivalent med aktivitet. Spesielt viktig er observasjonen av at bakteriene i sur jord åpenbart prøvde å uttrykke genet som koder for N_2O-reduktase (de transkriberte *nosZ* i like stor grad som i basisk jord), men ingen lyktes (ingen aktivitet). Dette er i og for seg observert tidligere, både i renkulturer og i jord, og tilskrives en post-transkripsjonell effekt av lav pH. Mine resultater har imidlertid stor betydning fordi transkripsjon ble undersøkt ved analyse av meta-transkriptomet. Tidligere undersøkelser i jord har alle vært basert på PCR, med de mangler og usikkerheter som er knyttet til dette. På bakgrunn av mine resultater kan vi med

langt større sikkerhet fastslå at den post-transkripsjonelle blokkeringen av *nosZ*-uttrykket ved lav pH er et universelt fenomen.

De lave nitritt-konsentrasjonene i sur jord kan i teorien skyldes rask kjemisk nedbrytning av nitritt ved lav pH, det vil si at dette ikke skyldes regulering på cellenivå. For å belyse dette ble den kjemiske nedbrytningskinetikken av nitritt bestemt i gammasterilisert jord, og denne første-ordens kinetikken ble brukt til å modellere nitrittkinetikken i levende jord. Resultatet viste at nitritt holdes lavt i sur jord først og fremst på grunn av bakterienes regulering, og i mindre grad på grunn av kjemisk nedbrytning. De er med andre ord en regulatorisk respons på lav pH.

Samlet har resultatene gitt sterk støtte til hypotesen at N_2O emisjon kan begrenses ved å juster pH i jord, fordi dette skyldes en post-transkripsjonell effekt av lav pH. Videre er det klart at nitritt-kinetikk i sur jord i all hovedsak er styrt av mikro-organismenes regulering av balansen mellom nitratreduktase og nitrittreduktase.

This page intentionally left blank

Abbreviations

AMO	Ammonia monooxygenase
Anammox	Anaerobic ammonium oxidation
AOA	Ammonia-oxidising archaea
AOB	Ammonia-oxidising bacteria
ATP	Adenosine triphosphate
Comammox	Complete ammonia oxidisers
DNRA	Dissimilatory nitrate reduction to ammonium
FPKM	Fragments per kilobase million
gDNA	Genomic DNA
HAO	Hydroxylamine dehydrogenase
HH	Hydrazine hydrolase
HZO	Hydrazine oxidoreductase
MG	Metagenome
MT	Metatranscriptome
N2OR	Nitrous oxide reductase
NAR	Nitrate reductase
NIF	Nitrogenase
NIR	Nitrite reductase
NOB	Nitrite-oxidising bacteria
NOR	Nitric oxide reductase
NXR	Nitrite oxidoreductase
qPCR	Real-time PCR
RPKM	Reads per Kilobase Million
RPM	Reads per Million
TNA	Total nucleic acids
TPM	Transcripts per Million

This page intentionally left blank

List of papers

Paper I

Lim NYN, Roco CA, Frostegard A (2016) Transparent DNA/RNA co-extraction workflow protocol suitable for inhibitor-rich environmental samples that focuses on complete DNA removal for transcriptomic analyses. *Front Microbiol* 7:1588.

Paper II

Lim NYN, Frostegård Å, Bakken LR. Soil pH dependent nitrite kinetics during anoxia; the role of abiotic reactions versus microbial reduction. (Under review in *Soil Biology and Biochemistry*)

Paper III

Lim NYN, Shapleigh JP, Bakken LR, Frostegård Å. Linking meta-omics to the kinetics of denitrification intermediates reveals pH-dependent causes of N_2O emissions and nitrite accumulation in soil. (Manuscript in preparation)

This page intentionally left blank

1 Introduction

1.1 The Nitrogen Cycle

Nitrogen is one of the six essential elements of life (carbon, hydrogen, nitrogen, oxygen and sulphur), and is a key element in a large number of molecules involved in a variety of biological processes. Given that it constitutes the bulk of the atmosphere in the form of dinitrogen gas (N_2) making up 78 %, the cycling of nitrogen is an important and well established field of study. Several interlinked aerobic and anaerobic processes make up the biological pathways of the nitrogen cycle, with nitrite and nitric oxide (NO) being two central molecules involved in almost all processes except nitrogen fixation (Fig. 1). Often, these processes can be observed in the same environment, and may either vie for the same nitrogenous molecules or are favoured under contrasting conditions (such as under oxic and anoxic conditions) e.g. simultaneous nitrification and denitrification (Burns et al., 1996; Russow et al., 2009).

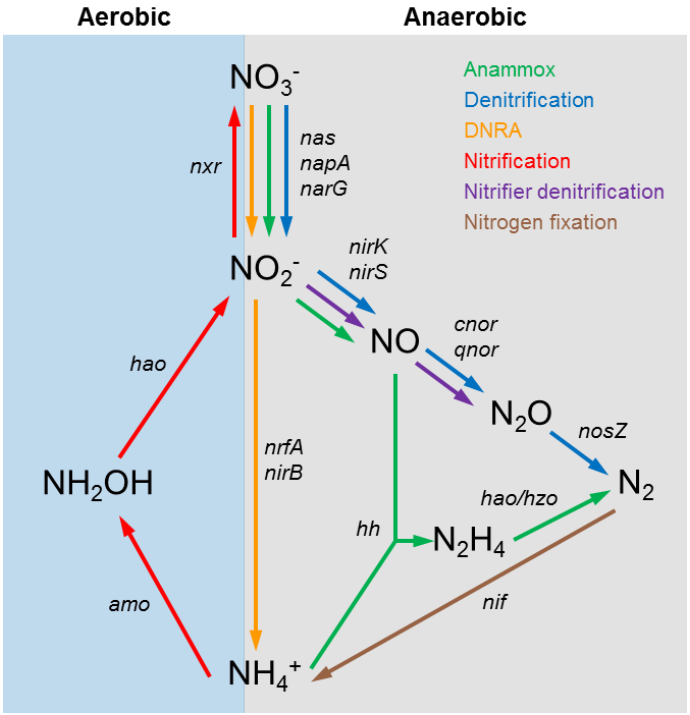


Fig. 1 Aerobic and anaerobic nitrogen cycle pathways. Genes encoding the enzyme related to each pathway are listed next to the respective arrow: nitrate reductases (*nas*, *nar*, *nap*), nitrite reductases producing nitric oxide (*nirK*, *nirS*), nitrite reductases producing ammonium (*nrfA*, *nirB*), nitric oxide reductases (*cnor*, *qnor*), nitrous oxide reductase (*nosZ*), nitrite oxidoreductase (*nxr*), hydroxylamine dehydrogenase (*hao*), ammonia monooxygenase (*amo*), hydrazine hydrolase (*hh*), hydrazine oxidoreductase (*hzo*), and nitrogenase (*nif*). The reduction of N_2O to N_2 by nitrifiers has not yet been confirmed and is thus not included in this figure. Figure adapted from Canfield et al. (2010) and Pauleta et al. (2013).

1.1.1 Aerobic processes

Nitrification is the main aerobic biological process in the nitrogen cycle, and involves the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and nitrate (NO_3^-). Nitrification can be further split into the two processes: ammonium oxidation ($NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^-$) and nitrite oxidation ($NO_2^- \rightarrow NO_3^-$), using the enzymes ammonia monooxygenase (AMO) and hydroxylamine dehydrogenase (HAO), and nitrite oxidoreductase (NXR) respectively (Fig. 1). Since nitrification preserves nitrogen in the soil and provides a key connection between decomposing matter and denitrification, soil nitrifiers play an important role in the nitrogen cycle. Nitrifiers make up three very different group of organisms: (i) ammonia-oxidising archaea (AOA) fall within the phylum Thaumarchaeota; (ii) ammonia-oxidising bacteria (AOB) are comprised of a monophyletic cluster within the gammaproteobacteria and a few clusters in betaproteobacteria; whereas (iii) nitrite-oxidising bacteria (NOB) are the most diverse, spread out over four phyla (Brochier-Armanet et al., 2008; Daims et al., 2016; Purkhold et al., 2000). Since ammonia-oxidation is generally considered the rate-limiting step in nitrification and AOA are known to dominate the ammonia-oxidising population in soils, there has been much interest in AOA in the environment (Daims et al., 2016; Leininger et al., 2006; Lüke et al., 2016; Prosser and Nicol, 2012). However, aside from these three classical nitrifier groups, there is also the recently discovered comammox (complete ammonia oxidisers) bacteria that possess homologues of AMO, HAO, and NXR, and are able to perform the complete nitrification process within a single organism (Daims et al., 2016).

1.1.2 Anaerobic processes

Nitrogen fixation may be regarded as the most important biological process in the nitrogen cycle, since the fixing of N_2 gas as ammonia introduces biologically-accessible nitrogen into the environment. An exclusively prokaryotic process (eukaryotes involved in nitrogen fixation do so with prokaryotic symbionts), nitrogen-fixing organisms utilise the nitrogenase enzyme (NIF) to perform a very energy-costly process that requires the hydrolysis of 16 ATP molecules per N_2 molecule (Barney et al., 2006; Seefeldt et al., 2009). However, in recent decades anthropogenic processes such as the Haber-Bosch process contribute to 45% of yearly fixed nitrogen globally, introducing large quantities of biologically-available nitrogen into terrestrial and marine environments that are not completely offset by the major N_2-emitting process, denitrification (Canfield et al., 2010).

Denitrification has been studied extensively for many decades, because it is a major loss of biologically-available nitrogen (Canfield et al., 2010; Wijler and Delwiche, 1954). *Sensu stricto*, classical denitrification is the stepwise reduction of NO_3^- or NO_2^- → NO → nitrous oxide (N_2O) or N_2 (Mahne and Tiedje, 1995; Shapleigh, 2013), using the enzymes nitrate reductase (NAR), nitrite reductase (NIR), NO reductase (NOR), and N_2O reductase (N2OR), respectively (Zumft, 1997). Denitrifiers may be bacterial or fungal, but only prokaryotes are, as far as we know today, able to perform the final step of N_2O reduction to N_2 because fungi do not possess the N2OR enzyme (Thomson et al., 2012). Moreover, while fungal denitrification is not necessarily insignificant, bacteria are known to play the more important and influential role in denitrification (Herold et al., 2012; Long et al., 2013). Thus, many environmental studies concentrate solely on bacterial denitrifiers (Brenzinger et al., 2015; Henderson et al., 2010). Being a major part of this thesis, classical denitrification is further elaborated below (*Section 1.2 below*).

Nitrifier denitrification, the reduction of nitrite to N-gas by nitrifiers, is a separate process from classical denitrification, and is thus different from nitrification-coupled-denitrification. Nitrifier denitrification is performed solely by nitrifiers possessing both nitrification- and denitrification-related enzymes, whereas the coupled processes simply refer to the use of nitrifier-generated nitrate/nitrite by denitrifiers (Wrage et al., 2001; Zhu et al., 2013). While nitrifier denitrification appears to be identical to denitrification (in terms of enzymes and reaction intermediates), there is no nitrate generated in this process – nitrite oxidised from ammonia is followed by the reduction to NO, N_2O, or even N_2 (Cantera and Stein, 2007; Muller et al., 1995; Wrage et al., 2001). However, studies on the effect of O_2 on these processes have noted that while nitrifier denitrification is capable of producing significant quantities of NO and N_2O at low O_2 concentrations (0.5 % O_2), only classical denitrification is active under anoxic conditions (Zhu et al., 2013). To complicate matters, despite the evidence of N_2-production by nitrifier denitrification and finding NIR, NOR and N2OR gene homologues in different nitrifiers, no complete set of denitrification enzymes has been found in a single organism, nor has an N2OR gene homologue been found in the nitrifier that had produced N_2 gas (Hu et al., 2015; Muller et al., 1995). Furthermore, some believe that the term "nitrifier denitrification" is a misnomer because there is little evidence that the process is dissimilatory, and may thus simply be a nitrite detoxification mechanism (Schreiber et al., 2012).

Codenitrification produces N_2O or N_2 from two separate N sources (resulting in the formation of hybrid N-gases), and has been regarded as the pathway through which fungi, which

lack N2OR, produce N_2 (Spott et al., 2011). However, it bears remarkable similarities to chemodenitrification (which involves chemical nitrosation reactions that also produce hybrid N-compounds, *Section 1.3*), and there have been suggestions that codenitrification may be better known as "bionitrosation" (Spott et al., 2011). Despite apparent evidence of hybrid N_2 from codenitrification in the environment (Laughlin and Stevens, 2002; Long et al., 2013), chemical nitrosation alone is capable of producing comparable quantities as detected in codenitrification studies (Kumon et al., 2002; Mania et al., 2014; Stieglmeier et al., 2014). A recent report has also questioned the reliability of hybrid N-gas measurements as evidence of codenitrification, doubting fungal codenitrification as a true biological process (Phillips et al., 2016).

Anaerobic ammonium oxidation (anammox) is the other process capable of producing hybrid N_2 from two distinct sources of N (Fig. 1). Believed to be the predominant pathway for N-loss from marine environments and aquifer soils (Lam et al., 2009; Wang et al., 2017), anammox is an anaerobic process performed by a slow-growing monophyletic cluster within the phylum Planctomycetes (Jetten et al., 2001). Anammox bacteria utilise NAR and NIR to reduce $NO_3^- \rightarrow NO_2^- \rightarrow NO$, then use hydrazine hydrolase (HH) to produce hydrazine (N_2H_4), and either HAO or hydrazine oxidoreductase (HZO) to form N_2. Because HAO and HZO are both capable of oxidising N_2H_4 and have similar gene sequences (HZO is believed to be the HAO equivalent in anammox bacteria), they are often collectively referred to in the anammox process as HAO/HZO (Jetten et al., 2001; Kraft et al., 2011).

Dissimilatory nitrate reduction to ammonium (DNRA) is the other main anaerobic process in the nitrogen cycle aside from denitrification, and involves the reduction of nitrate to NH_4^+ via nitrite (Fig. 1). Unlike denitrification, DNRA keeps N in biologically accessible forms, thus making it an important N-cycling process. The reduction of nitrite to NH_4^+ is performed by NrfA or NirB, although the former is more commonly associated with DNRA and the latter with nitrite detoxification (Decleyre et al., 2016; Mania et al., 2016; Moreno-Vivián et al., 1999). However, DNRA organisms are also known to produce N_2O (possibly as a detoxification mechanism) despite not necessarily possessing NOR, thus the relationship between DNRA and N_2O emissions is not entirely clear (Hu et al., 2015; Rütting et al., 2011; Stremińska et al., 2012).

1.1.3 Simultaneous anaerobic processes

Aside from the previously mentioned nitrification-coupled-denitrification, many of the biological processes are known to occur simultaneously in mixed communities (Bleakley and Tiedje, 1982; Dalsgaard et al., 2014; Dong et al., 2009; Long et al., 2013). In the environment, denitrification and DNRA coexist and compete for nitrate, resulting in the production of both N_2O/N_2 and NH_4^+ (Bleakley and Tiedje, 1982; Rütting et al., 2011). However, DNRA appears to be more efficient and competitive at higher C/NO_3^- ratios (Bleakley and Tiedje, 1982; Rütting et al., 2011; Stevens et al., 1998; Stremińska et al., 2012; Strohm et al., 2007), and is likely favoured under high C-decomposition conditions, although high quantities of NO_3^- may swing in favour of denitrification (Hardison et al., 2015). Similar observations have been made in pure cultures of *Bacillus vireti*, where the transcription of DNRA- or denitrification-related genes were favoured under low or high nitrate levels respectively, resulting in different accumulation of intermediates and end-products (Mania et al., 2016). Thus, the ratio of N_2O/N_2 and NH_4^+ produced from nitrate differs depending on environmental conditions. Despite this, the importance of DNRA compared to denitrification is uncertain: DNRA is believed to yield more energy than denitrification, but has historically been regarded as a minor process in the environment although recent studies seem to indicate otherwise (Smith et al., 2015; Strohm et al., 2007; Wijler and Delwiche, 1954). It has also been suggested that DNRA activity may have been exaggerated in laboratory experiments due to experimental design (the use of soil slurries apparently favours DNRA), possibly due to DNRA organisms being more competitive under aerobic or fluctuating O_2 conditions (Rütting et al., 2011). Moreover, isotope (^{15}N) tracing field experiments have not yielded clear results, due to competition with alternative transformation pathways (involving plants, or immobilisation and remineralisation) of the same biologically-available N-compounds involved in DNRA (Rütting et al., 2011).

In contrast, there is strong evidence that anammox is insignificant in most soils when compared to either DNRA or denitrification (Hardison et al., 2015; Hu et al., 2015; Long et al., 2013), despite its clear dominance elsewhere in water-rich environments (Lam et al., 2009; Wang et al., 2017). Additionally, evidence suggests that anammox is not a conventional terrestrial process: (i) Anammox is less affected by oxygen than the other processes; (ii) Anammox NIR genes are distinct from denitrifier NIR ($\leq$ 63% sequence identity); and (iii) NAR and NOR in anammox organisms may be used in unconventional ways, where NAR acts as a nitrite oxidoreductase producing nitrate from nitrite and NOR converts $2NO \rightarrow N_2 + O_2$ (Dalsgaard et al., 2014; Lam et al., 2009).

1.2 Denitrification

As aforementioned, classical denitrification is the stepwise enzymatic reduction of nitrate/nitrite to N_2O/N_2 via NO. While it is generally regarded as an anaerobic process, only the final step of N_2O reduction to N_2 by N2OR is strongly affected by O_2 (Qu et al., 2016), and denitrification under fully oxic conditions (known as "aerobic denitrification", generating only NO and N_2O as end-products) has been observed and studied for many decades (Meiklejohn, 1940; Mørkved et al., 2007).

Since denitrification *sensu stricto* does not always include the reduction of nitrate to nitrite nor N_2O to N_2, these two processes are sometimes known separately as "nitrate reduction" and "N_2O reduction", respectively. Complicating this, while the full set of denitrification enzymes are sometimes present in its entirety in some organisms, different organisms within a shared community may possess the enzymes necessary for each reduction reaction, creating the potential of the existence of a denitrifying environment in the absence of "complete" denitrifiers (Jones et al., 2008). Furthermore, even individual strains of complete denitrifiers are known to regulate the denitrification process differently, creating distinctive denitrification regulatory phenotypes (DRP) such as "progressive onset" and "rapid complete onset", where there is an accumulation then subsequent utilisation of each denitrification intermediate in the former but not in the latter (Liu et al., 2013). However, for simplicity DRP is disregarded in this thesis (since pure cultures are not discussed), and the term "complete denitrification" henceforth refers to the reduction of nitrate to N_2 (NO_3^- → NO_2^- → NO → N_2O → N_2), and "incomplete denitrification" refers to the reduction of nitrate to N_2O (NO_3^- → NO_2^- → NO → N_2O), unless otherwise specified. Due to the complications surrounding nitrite chemistry at low pH (*Section 1.3 below*), truncated forms of denitrification that start from nitrite (instead of nitrate) are expressly specified.

1.2.1 Nitrogenous compounds in denitrification

The main compounds involved in denitrification are nitrate, nitrite, NO, N_2O and N_2. Although other compounds such as methyl nitrite (CH_3ONO) and nitrogen dioxide (NO_2 gas: different from aqueous nitrite, NO_2^-) have been observed in relation to biotic and abiotic processes related to denitrification, they are generally regarded as unimportant in classical denitrification and are thus not be discussed here (Magalhães and Chalk, 1987; McKenney et al., 1990; Nelson and Bremner, 1970; Nömmik and Thorin, 1972).

1.2.1.1 Nitrate

Nitrate, one of the two main forms of inorganic N taken up by plants, is chemically stable and degradation is very slow even in highly acidic environments (van Cleemput, 1998). However, many microorganisms are capable of reducing nitrate to nitrite, making it a process that is known to dominate over others such as denitrification, DNRA, or anammox in the environment (Zumft, 1997). Microorganisms that are only capable of nitrate reduction and no other steps in denitrification are known as nitrite accumulators, and are estimated to outnumber denitrifiers 4:1 in soil environments (Gamble et al., 1977). Additionally, there is evidence that nitrate may be preferentially used over other nitrogenous compounds (Burns et al., 1996; Mania et al., 2016), which may also explain early observations that high levels of nitrate delays N_2O reduction in denitrification (Blackmer and Bremner, 1978).

1.2.1.2 Nitrite

Unlike nitrate, nitrite is far less stable chemically and readily decomposes without the aid of biological processes especially under acidic conditions (Porter, 1969; Stevenson et al., 1970) (further elaborated in *Section 1.3 below*). Further complicating matters, measuring nitrite at low pH can be difficult, since the methods used to extract and analyse nitrite may themselves increase decomposition rates (Homyak et al., 2015). Nevertheless, nitrite has been observed to accumulate during denitrification, with increasing levels detected with increasing pH (Glass and Silverstein, 1998; Henderson et al., 2010). This accumulation is potentially problematic, because nitrite can be toxic to organisms by passing through membranes in the form of HNO_2 (Kaiser and Heber, 1983; Samouilov et al., 2007). Nitrite toxicity is especially apparent at acidic pH, and has been observed to have either a transitory (Bancroft et al., 1979) or complete (Meiklejohn, 1940) inhibition effect on denitrification, possibly differentiated by the extent of nitrite build-up.

Additionally, nitrite plays a major role in atmospheric chemistry through the abiotic formation of aqueous and gaseous nitrous acid (Fig. 2, differentiated as HNO_2 and HONO respectively), and is able to do so even in neutral or basic pH soils (Oswald et al., 2013; Su et al., 2011). In turn, HONO is an important player in tropospheric chemistry, acting as a major source of OH and catalytically destroying ozone, thereby potentially contributing to climate change (Jacob, 2000; Kulmala and Petäjä, 2011; Spataro and Ianniello, 2014).

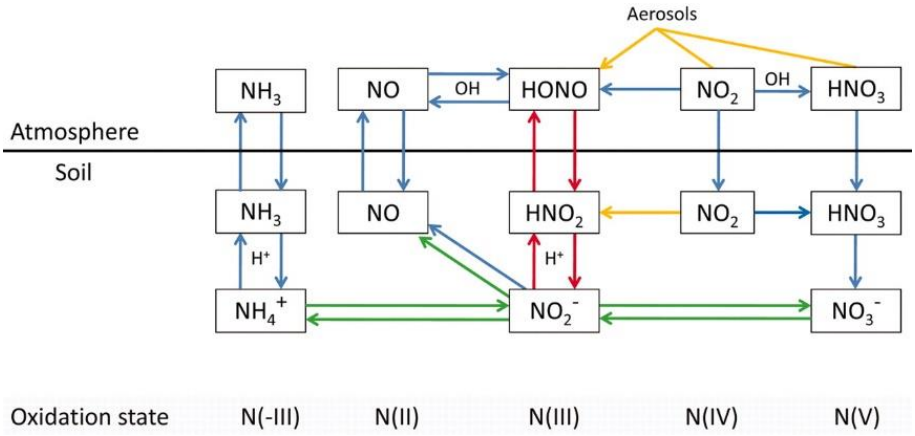


Fig. 2 Soil nitrite and atmospheric nitrous acid (HONO). Red arrows represent HONO emissions from soil nitrite, green arrows represent biological processes, orange arrows represent chemical conversion of NO_2 and HNO_3 to HONO, and blue arrows represent other processes. From Su et al. (2011). Reprinted with permission from AAAS.

1.2.1.3 Nitric oxide (NO)

Nitric oxide is a very important molecule, playing large roles in biological signalling and atmospheric chemistry (Bowman et al., 2011; Su et al., 2011). Due to its effects on gene activation via regulators such as the Crp-Fnr superfamily of transcriptional regulators and NsrR (further analysed in *Section 1.2.3 below*), NO is capable of activating a large number of denitrification-related genes (Rodionov et al., 2005). Abiotically, NO may interact with OH in the atmosphere to form HONO (Su et al., 2011), thus contributing to the atmospheric HONO pool (Fig. 2). Moreover, direct HONO emissions (originating from soil nitrite) and NO emissions can be comparable under some circumstances, meaning that the denitrification process creates two potential sources of the atmosphere-altering HONO (Oswald et al., 2013).

1.2.1.4 Nitrous oxide (N_2O) and dinitrogen gas (N_2)

Aside from the undesired effect of losing biologically-available N from terrestrial and marine environments, the two main denitrification end-products (N_2O and N_2) have two different effects on the environment. Dinitrogen gas is, being a triple-bonded molecule (N≡N) is chemically-demanding to break, relatively chemically inert and has little effect on the environment (Barney et al., 2006; Canfield et al., 2010). In contrast, N_2O is a very powerful greenhouse gas with a global warming potential that is approximately 300 times that of the more commonly known CO_2 (Lashof and Ahuja, 1990), and has recently been recognised as the "single most important ozone-depleting emission" from anthropogenic sources (Ravishankara et al., 2009). Thus, much research has focussed on the potential of N_2O sinks in

mixed communities and pure cultures alike, in the hopes of reducing atmospheric N_2O to harmless N_2 (Butterbach-Bahl et al., 2013; Jones et al., 2014; Mania et al., 2014; Palmer and Horn, 2012).

1.2.2 Enzymes related to the denitrification process

Each step in denitrification uses one of four reductases that are encoded for by different genes (Fig. 1): NAR is encoded for by the genes *nar* (membrane-bound) and *nap* (periplasmic), NIR by the genes *nirK* (copper-containing) and *nirS* (cytochrome cd_1), NOR by *cnor* (cytochrome *c* dependent) and *qnor* (quinol-dependent), and N2OR by *nosZ* (Spiro, 2012; Zumft, 1997). These genes are evolutionarily distinct and separate from one another, though there is some indication that some genes may be higher correlated with the coexistence of others (Graf et al., 2014). However, unlike nitrifiers and anammox organisms, denitrifiers are not restricted to specific microbial guilds, strongly suggesting that horizontal gene transfer or other evolutionary phenomena likely played a role in the spread of denitrification genes (Jones et al., 2008; Shapleigh, 2013).

1.2.2.1 Nitrate reductase (NAR)

Not strictly a denitrification enzyme, NAR exists in a variety of oxic and anoxic environments because they do not necessarily require anaerobiosis for function (Bergaust et al., 2008; Dendooven and Anderson, 1995). Although there are other types of NAR (e.g. the assimilatory nitrate reductase Nas), only the aforementioned membrane-bound Nar and periplasmic Nap enzymes have been linked to denitrification (Moreno-Vivián et al., 1999; Richardson et al., 2001; Zumft, 1997). These two NAR may be differentiated by their association with other anaerobic processes (Nap has been associated with the periplasmic nitrite reductase NrfA in the DNRA process), as well as their sensitivity to O_2: Nap is unaffected by O_2 concentrations and has been associated with aerobic denitrification; whereas cytoplasmic Nar not only requires an O_2-inhibited nitrate-porter to function, but is also upregulated by the transcriptional regulator Fnr under anoxic conditions (Moreno-Vivián et al., 1999). There has also been some evidence of Nap and Nar being favoured under low and high levels of nitrate respectively (Wang et al., 1999), but the effect of nitrate as compared to O_2 has not been determined.

1.2.2.2 Nitrite reductase (NIR)

Nitrite reductases are important enzymes, both due to their role in controlling the levels of potentially toxic nitrite, and because nitrite is a central molecule in almost all biological

processes in the nitrogen cycle (Fig. 1). Similar to NAR, there are other types of non-denitrification-related NIR. Of particular interest is NrfA and NirB, both of which have been associated with DNRA and have been linked to Nap and Nar (Cole and Richardson, 2008). Evidence indicates that NrfA (associated with Nap) is favoured in substrate limiting conditions, whereas NirB (associated with Nar) is favoured in excess conditions (Wang et al., 1999; Wang and Gunsalus, 2000). It has thus been suggested that the latter is involved in nitrite detoxification or the storage of N (Malm et al., 2009; Mania et al., 2016; Wang and Gunsalus, 2000). In contrast, both denitrification-related NIR, NirK and NirS, are respiratory enzymes (Zumft, 1997).

Although NirK and NirS perform the same function in denitrifiers, the genes encoding the two enzymes appear to be evolutionarily distinct (Jones et al., 2008; Zumft, 1997). While the genes *nirK* and *nirS* have recently been found within a single organism, there has not yet been evidence that both genes are active and functional (Graf et al., 2014). Perhaps because of their evolutionary distinctiveness as well as NirS having been more extensively studied, NirS has been the predominant enzyme detected and isolated (Coyne et al., 1989; Gamble et al., 1977; Palmer and Horn, 2012) until recent years (Brenzinger et al., 2015; Coyotzi et al., 2017; Maeda et al., 2010). Thus the frequency and magnitude of NirK occurrence may be severely underestimated, in part due to poor primer constructs.

1.2.2.3 Nitric oxide reductase (NOR)

Nitric oxide reductases are the major contributor to N_2O production within the nitrogen cycle. Although there are three types of NOR (cNor, qNor, and qCu_ANor), the last type is not as well-characterised, other than that it appears to be a hybrid of the former two NOR (it may be capable of accepting electrons from either menaquinol or *c*-type cytochrome), and that it has a binuclear Cu_A centre (Spiro, 2012; Suharti et al., 2004; Zumft, 2005). Regardless, all three NOR perform the same process (reducing NO to N_2O) using similar active centres and are thus functionally equivalent for the purposes of denitrification (Zumft, 2005).

At a process level, there have been conflicting reports as to whether or not a relationship between NOR and denitrification potential exists (Chen et al., 2012; Yu et al., 2014). On one hand, NOR controls the levels of the signalling molecule NO, in turn regulating all denitrification genes via the Crp-Fnr superfamily (see *Section 1.2.3 below*). On the other hand, many of the other denitrification enzymes are shared by non-classical denitrifiers (e.g. nitrifiers, DNRA organisms, etc.), complicating statistical correlations. Furthermore, the type of NOR

analysed and method used (primer-based or otherwise) likely introduces its own bias to such sweeping conclusions. Thus, whether or not NOR may be considered a key indicator for denitrification remains to be seen.

1.2.2.4 Nitrous oxide reductase (N2OR)

Nitrous oxide reductases can be divided into two structurally similar variants, referred to as Z-type (or "typical" Nos, zNos) and c-type (or "atypical" Nos, cNos), and are mainly differentiated by their export pathway (zNos uses the Tat pathway whereas cNos uses the Sec secretory system) as well as their electron transfer pathways (Pauleta et al., 2013; Spiro, 2012). Interestingly, correlations have been drawn between cNos and zNos abundance with an environment's N_2O sink capacity (Jones et al., 2014). However, although there has been much evidence that cNos is the most abundant environmentally and may have been overlooked due to primer-bias (Jones et al., 2012; Orellana et al., 2014; Sanford et al., 2012), a very recent study has disabused this notion: cNos indeed overshadowed zNos in the genetic potential, but microbial activity was strongly dominated by zNos-carrying organisms instead (Coyotzi et al., 2017). Considering that the previous studies were based on DNA and metagenomes, this is indicative that transcriptional control and/or posttranscriptional phenomena may play a large role in N2OR function.

Startlingly, multiple reports have appeared to put transcriptional analyses to doubt, where *nosZ* transcripts were detected without corresponding N_2O reduction or N_2 production (Brenzinger et al., 2015; Henderson et al., 2010; Liu et al., 2010). However, these may be explained by the presence of O_2 which is known to suppress N2OR activity (Betlach and Tiedje, 1981; Zumft, 1997), or the common use of acetylene to determine N_2 production – suspected to be inconclusive due to the interference of acetylene with NO and N_2O (Nadeem et al., 2013). Nevertheless, pure culture and extracted cell experiments have revealed that low pH may cause the failure of a yet unknown post-transcriptional process: enzymes made at pH 7 were able to reduce N_2O at pH 5.7, when enzymes synthesised at the latter pH could not (Bergaust et al., 2010; Liu et al., 2014).

1.2.3 Gene regulation

Denitrification genes are controlled by a number of different transcriptional regulators. The Crp-Fnr superfamily of transcriptional regulators in bacteria is an extremely large group of regulators that control a wide range of functions including biological N-processes, and include members such as Fnr, NnrR, and Dnr (Körner et al., 2003). In response to anaerobiosis, the

Crp-Fnr superfamily is known to activate the transcription of all denitrification- and DNRA-related genes: *nap*, *nar*, *nir* (B, K and S), *nrfA*, *nor* (C and Q), and *nosZ* (Cole and Richardson, 2008; Dalsgaard et al., 2014; Rodionov et al., 2005). Additionally, NO also signals the Crp-Fnr superfamily, which in turn influences the transcription of entire gene clusters, although the exact mechanism through which NO acts upon the transcriptional regulator is not fully understood (Bergaust et al., 2012; Spiro, 2012; Vollack and Zumft, 2001; Zumft, 2005).

Superfamilies aside, regulators such as Hcp, NarXL, NarQP, and NsrR are also involved in the transcriptional regulation of multiple denitrification enzymes, in response to molecules such as nitrate/nitrite or NO (Bergaust et al., 2012; Medinets et al., 2015; Rodionov et al., 2005; Spiro, 2012). For example, there is evidence that NasST regulates both Nap and NosZ under a yet uncertain nitrate-mediated process, likely related to its function as a regulator for nitrate/nitrite-sensing (Luque-Almagro et al., 2013; Sánchez et al., 2014). At the level of individual operons, gene-specific regulators such as NirI, NorR, and NosR regulate the *nir*, *nor*, and *nos* gene clusters respectively, although there is indication that they may ultimately do so via the Crp-Fnr superfamily (Cuypers et al., 1992; Medinets et al., 2015; Pauleta et al., 2013; Saunders et al., 1999).

1.2.4 Environmental variables affecting denitrification

While not actual transcription regulators, environmental variables are known to affect denitrification activity and community composition. The presence of oxygen, as seen in the previous sections, plays a large role in affecting the denitrification process. Carbon has long been known to be important in denitrification (Burford and Bremner, 1975), and its limitation may result in the preferential use of more oxidised electron acceptors over N_2O (Betlach and Tiedje, 1981). This in turn may explain the accumulation of N_2O in some environments (Schalk-Otte et al., 2000). Similarly, nitrogen availability is known to affect the accumulation of denitrification intermediates possibly by repressing the transcription or activity of certain enzymes (Dendooven and Anderson, 1995; Mania et al., 2016). Thus high concentrations of nitrate or nitrite may result in large quantities of N_2O produced, sometimes even appearing to inhibit N_2O reduction (Blackmer and Bremner, 1978; Burns et al., 1996; Firestone et al., 1980).

One "master variable" of denitrification is pH. Globally, most soils are below neutral pH (Fig. 3), thus the effect of low pH environments on denitrification (and the potential loss of biologically-available N) is important to agricultural and forestry industries alike. It is generally recognised that pH towards neutral allows for more efficient denitrification, with acidic soils

producing a higher N_2O:N_2 ratio (Bakken et al., 2012; Firestone et al., 1980; Raut et al., 2012). This may be partially because pH affects nitrate and nitrite accumulation and degradation, which in turn affects all downstream denitrification steps (Shen et al., 2003; Wang et al., 2015). However, the optimum pH for denitrification is generally soil dependent and the maximum denitrification rate may not always be close to neutral (Herold et al., 2012; Šimek et al., 2002). This likely reflects a community of environmentally-selected microorganisms, such as acid-tolerant denitrifiers (Van Den Heuvel et al., 2010; Lycus et al., 2017).

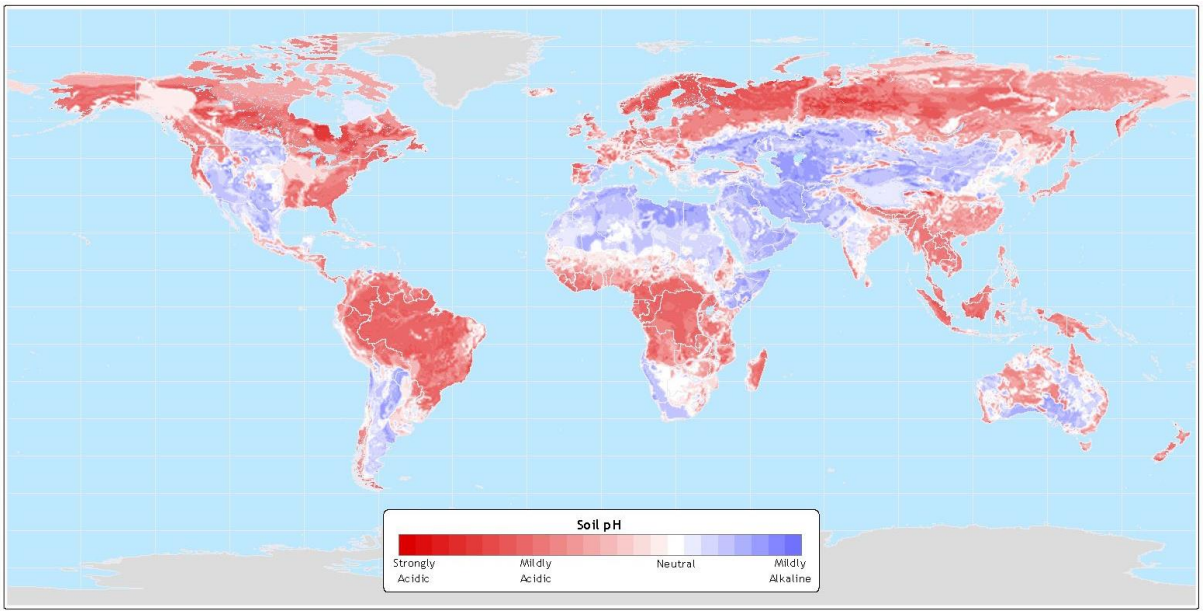


Fig. 3 Global soil pH. Used by permission of The Center for Sustainability and the Global Environment, Nelson Institute for Environmental Studies, University of Wisconsin-Madison [data obtained from the SoilData System developed by the International Geosphere-Biosphere Program Data and Information System (IGBP-DIS, 1998)].

1.3 Chemical decomposition

Commonly known as "chemodenitrification", the abiotic decomposition of N-compounds is long-known to have complicated studies on the biological processes of nitrogen cycling. In particular, the fast chemical transformations of nitrite (especially at low pH) is the most familiar to biologists, especially when compared to nitrate, which is highly resistant to chemical decomposition (van Cleemput, 1998). In high organic environments (e.g. peat), the loss of nitrite can be very fast and severe, resulting in a failure to recover nearly all extractable nitrite within 70 minutes of addition (Stevens and Laughlin, 1995).

Abiotic nitrite decomposition can result in a range of gases in mixture or alone, some of which are also observed in classical denitrification: NO, N_2O, N_2, and even CO_2 (Nelson and

Bremner, 1970; Porter, 1969; Stevenson et al., 1970; Stevenson and Swaby, 1964). Of these gaseous compounds, NO is the most abundantly produced and commonly observed gas, regardless of pH (Bremner, 1997; McKenney et al., 1990; Nömmik and Thorin, 1972; Porter, 1969).

Due to its reaction speed and similar gaseous products, chemodenitrification has generally been regarded as indistinguishable from classical denitrification in environments of pH <6 (Spott et al., 2011). However, not all products of chemical decomposition are typical of the biological nitrogen cycle, and these chemically-nitrosated organic compounds in the soil may not be available to biological processes (Nömmik and Thorin, 1972). Unfortunately, despite decades-old knowledge of its existence (Wijler and Delwiche, 1954), little is known of the exact chemistry of such chemodenitrification in soils, other than that nitrite is readily lost, likely due to the nitrosation of organic matter (such as humus) in the soil to form stable organic N compounds (Nömmik and Thorin, 1972; Thorn and Mikita, 2000). However, there is evidence that not all quickly immobilised N in soil environments is also chemically decomposed, and may be extractable in its original form (Dail et al., 2001).

1.4 Meta-omic analysis

With the ever-lowering costs of sequencing, there has been a shift from traditional fingerprinting methods such as DGGE, to amplicon-sequencing, and now to metagenomics/metatranscriptomics. This has spawned a slew of studies reanalysing (or improving) existing nucleic acid extraction methods (Arbeli and Fuentes, 2007; Krsek and Wellington, 1999; Peršoh et al., 2008; Vishnivetskaya et al., 2014), as well as generated a mass of bioinformatics methods (Bolger et al., 2014; Kim et al., 2016; Narayanasamy et al., 2016; White et al., 2016), with the resulting data spanning almost all fields of biology (Franzosa et al., 2014; Orellana et al., 2014; Tveit et al., 2015; Twin et al., 2013). Yet sequencing and bioinformatics are still far from perfect, and a number of reviews have warned of the challenges that remain, as well as the follies of inadvertently replacing proper, sounded out hypotheses with these modern analytics (Franzosa et al., 2015; Moran et al., 2013; Nesme et al., 2016; Prosser, 2015). Nevertheless, meta-omic analysis offers scientists the possibility of in-depth community analysis that crosses the boundaries of specific taxonomic guilds and metabolic pathways, as well as the use of published data for entirely novel multi-study analyses.

Previously available tools for the analysis of nucleic acids were largely primer-based, and were thus highly susceptible to biases caused by gene sequences that did not match the

conserved regions of such "universal" primers (Throbäck et al., 2004). This led to the repeated search for, construction of, and comparison of primers that were sadly still short of true "universality" (Chen et al., 2010b; Jones et al., 2012; Jung et al., 2012). Advances in high-throughput sequencing technologies in recent years have paved the way for analysis of the metagenome (MG) that is independent of the existence of primers and conserved sequences, and has allowed for more detailed analyses and the capture of previously undetectable microorganisms (Mason et al., 2012; Orellana et al., 2014; Tveit et al., 2013). However, MG only reflects the potential of any given community, and does not necessarily reflect the activity or the response. Some studies have thus utilised DNA stable-isotope probing to help differentiate between the actively growing and inactive portions of the community (Chen et al., 2010a; Coyotzi et al., 2017), but this is still unable to supply information on gene expression. Additionally, DNA is known to survive extracellularly in the environment, and may thus complicate any conclusions drawn about genetic potential (Levy-Booth et al., 2007). Accordingly, the metatranscriptome (MT) is important for the true differentiation between that which is simply present, and that which actively responds to an environmental signal. An additional benefit to the MT is that it is a more sensitive and responsive bioassay than the MG or metaproteome due to the short half-life of mRNA compared to DNA and proteins, making it more suitable for phenome-linked meta-omic studies where the immediate biological response to environmental changes is of interest (Moran et al., 2013). Hence, studies with both MG and MT are able to draw conclusions on genetic potential vs. response to environmental stimuli, granting insights to biological processes (Franzosa et al., 2014; Mason et al., 2012; Narayanasamy et al., 2016).

1.4.1 Obtaining material for analysis

The first step in all nucleic acid analysis, be it meta-omic or otherwise, is the acquisition of nucleic acid material for said purpose. Ever since the earliest days of nucleic acid extraction, scientists have sought better and increasingly efficient methods to obtain the "best" nucleic acids – that which is plentiful and of high quality. As a result, there is a wide variety of extraction and purification methods available (Cullen and Hirsch, 1998; Griffiths et al., 2000; Nicolaisen et al., 2008; Tan and Yiap, 2009), and also many studies assessing the efficiency and effectiveness of such methods (Bakken and Frostegård, 2006; Krsek and Wellington, 1999; Mahmoudi et al., 2011). These studies often assess the effects of altering individual aspects of the extraction process such as nucleic acid precipitation (Arbeli and Fuentes, 2007), pre-extraction of cells prior to lysis (Courtois et al., 2001; Lindahl and Bakken, 1995), removal

of enzymatic inhibitors (Cullen and Hirsch, 1998; Peršoh et al., 2008), as well as the usefulness of method modularity (Lever et al., 2015; Lim et al., 2016). While the volume of research into extraction methods alone may seem like senseless nit-picking, the importance of suitable methods cannot be stressed enough. Being the foremost step in nucleic acid analyses, extraction methods affect all downstream analyses – contamination in extraction materials will lead to vastly different conclusions, and different extraction methods can lead to false assumptions about changes in community composition (Salter et al., 2014; Vishnivetskaya et al., 2014).

A complicating factor in nucleic acid extraction from environmental samples, is the presence of enzymatic inhibitors. Although these inhibitors are known to affect a wide range of enzymes including restriction enzymes, DNases, RNases and polymerases, the mode of inhibition is still not entirely clear (Sutlovic et al., 2008; Tebbe and Vahjen, 1993). Furthermore, these same inhibitors have been found to affect fluorometric methods for nucleic acid measurement, and there is some evidence that this interference may be possible even in the absence of enzymatic inhibition (Bachoon et al., 2001; Sidstedt et al., 2015; Zipper et al., 2003). This lack of enzymatic interference may however be explained by the observation that different variants of the same enzyme may have different inhibitor sensitivities, and that not all studies utilise the same variants (Abu Al-Soud and Rådström, 1998; Albers et al., 2013). Nevertheless, the issue of inhibitors in soils is major, since the effect may also be primer-dependent (Albers et al., 2013; Huggett et al., 2008; Tebbe and Vahjen, 1993). Moreover, while increasing the quantity of fluorophores or DNA molecules may help to relieve the inhibition, it can also result in self-competition (fluorophore with fluorophore, or DNA with DNA), thereby reducing enzyme activity (Tebbe and Vahjen, 1993; Zipper et al., 2003). Thus, it is of utmost priority to choose a suitable nucleic acid extraction method to bypass this issue whenever possible.

1.4.2 Sequencing and analysis

A wide variety of amplicon-free sequencing options are available, ranging from short reads of several tens of basepairs (e.g. ABI SOLiD), to a few hundred basepairs (e.g. Illumina technologies), to over a thousand basepairs (e.g. Moleculo, aka TruSeq Synthetic Long-Read technology), or even several tens of thousands basepairs (e.g. PacBio technologies) [from Mardis (2017) and manufacturers' websites referred therein]. However, there is no “perfect” sequencer, and each has its own issues, in terms of read accuracy, cost, sequencing depth or read length (Quail et al., 2012). Ultimately, the type of technology chosen is dependent upon

researcher preference, which is often based on a mixture of belief of reliability, available resources, and financial capability.

Downstream, bioinformatics analysis is a complex field of its own, with an ever-increasing quantity of tools and pipelines for the analysis of nearly any type of sequencing data. Each tool or pipeline claims to be better than its peers (Bray et al., 2016; Buchfink et al., 2015; Narayanasamy et al., 2016; Wood and Salzberg, 2014), and very often scientists are at a loss as to which is the "best" method to use for their own dataset. Given that most biologists are neither computer scientists nor full-time statisticians, declarations of "reducing the demands on main memory bandwidth" (Buchfink et al., 2015) pale in comparison to our perceived importance of "correct" sequence matches and alignments ("correct", ironically, being a statistically determined decision made by said tool). If we were to assume that the tools were otherwise computationally and statistically perfect, then the single unifying issue all bioinformatics analyses face is the completeness of the databases we use. Poor databases are known to exist, and multiple studies (both laboratory- and *in silico*-based) have had to manually-curate their databases in order to properly perform their analyses (Graf et al., 2014; Jones et al., 2012; Orellana et al., 2014). Without good databases, read-assigning tools are unable to assign proper protein and/or taxonomic lineages, which in turn complicates meta-omic studies. To paraphrase Donald Rumsfeld, "We cannot know what we do not know," which is fair critique of the current state of public databases.

Another major issue in bioinformatics, is the normalisation of datasets (for comparisons across studies). There are a variety of traditionally used units, ranging from normalising reads/copy numbers to the weight of sample, extracted DNA/RNA, or housekeeping genes (for DNA). However, none of these methods are perfect: The weight of soil is not common across soil types and moisture contents, and dry/wet weight is not directly comparable; extracted DNA may be affected by contaminating extracellular DNA, and being a separate molecule with a different half-life is not entirely suited to normalising RNA quantities; total RNA values are largely affected by rRNA, not mRNA; and no housekeeping transcript (one that is constantly expressed at the same level) exists for RNA. Another oft-used normalisation factor is the quantity of 16S rRNA genes in a sample. However, 1-15 copies of 16S rRNA genes exist per genome, and the number of bacteria (as well as what species possessing how many copies) is unknown (Větrovský and Baldrian, 2013). Alternatively, spiking samples with alien DNA or RNA has been used to correct for both extraction efficiencies and sequencing depth, as well as a means of absolute quantification. However, given the complexities of environmental samples,

spiking experiments need to be performed for different samples and treatments to validate its use and suitability. The introduced nucleic acids may also act as preferential adsorption-site competitors in the environment (Frostegård et al., 1999; Paulin et al., 2013), thereby complicating extraction efficiency and absolute quantification calculations. Despite this, such suitability confirmation or method validation has rarely been performed when used (Huggett et al., 2005).

Unique to MG and MT analyses, the normalisation units Reads per Million (RPM), Reads/Fragments per Kilobase Million (RPKM/FPKM), and Transcripts per Million (TPM) are commonplace. These normalise for the sequencing depth of each reaction, and the latter units (RPKM/FPKM and TPM) also normalise for gene lengths. Although TPM is favoured by bioinformaticians (RNA-Seq Blog, 2015) since it allows for comparison across samples (the total TPM of all samples are the same, so all values can be directly compared), it requires prior knowledge of the gene lengths of all reads in a sequencing reaction. Since knowing the length of the unknown is impossible especially in complex environments such as soils, RPM and its variations (without normalising for gene length) is still often used (Orellana et al., 2014).

1.4.3 Comparing meta-omic data with the phenome

Even with all the information provided by sequencing analyses, taxonomy and metabolic activity of communities are not necessarily directly correlated: The metabolic redundancy is often large in complex communities, thus organisms of taxonomically-divergent lineages may be metabolically similar and fulfil the same functions within an ecosystem. Such evidence is mounting, and so now the aim is often to determine the microbial/molecular cause behind the phenotype, without necessarily being concerned with the exact species present (Taxis et al., 2015; Tveit et al., 2015). However, such determinations can be difficult if one were to do so "blindly" without simultaneous phenotypic data, especially if one considers the interlinked complexities of potential post-transcriptional/translational modifications and community metabolism.

Taking denitrification as an example, complete denitrification to N_2 of an environment is not restricted to the presence of complete denitrifiers since a community of organisms that perform only one or two steps of denitrification may achieve the same effect of complete nitrate reduction to N_2. Since single bacteria may possess a diverse combination of denitrification genes and the environmental conditions faced by bacteria are both felt and affected by the overall community, accurate predictions of denitrification response based purely on genetic

potential are difficult to achieve. Transcription profiles often paint a clearer picture, since we are able to see community responses in real-time based on the changing environment, but variations in translation ratios, as well as post-translational phenomena, reduce the accuracy of absolute enzyme presence and activity predictions (Gingold and Pilpel, 2011). While it is not wholly incorrect to draw conclusions from just the MG or MT, much care should be taken when inferring conclusions on phenotypic activity based purely on molecular results. Thus, it is important to link MG and MT with phenomics: The genome would provide the potential of a community and may reveal past selection pressures, the transcriptome reveals the "intended response" of the community, and the phenome is the actual result of their response. Only by linking all three are we able to understand how potentials are linked to possible responses, and how expression patterns affect what actually happens in the environment. This is even more important for complex environmental communities, where the domino effect is in play: The response of one microorganism may directly change an environmental variable, affecting other microorganisms, thereby triggering a cascade response.

1.4.4 Other types of meta-omic data

Aside from the MG and MT, the metaproteome and meta-metabolome have also garnered interest for their potential to inform about the real-time situation within a cell. Unfortunately, metaproteomics suffers from low extraction scales and struggles with the complexity of mixed communities, and is still largely regarded as a descriptive rather than analytical approach towards environmental samples (Keiblinger et al., 2016). Furthermore, protein responses to environmental stimuli are relatively slow (compared to RNA) and may last for some time after the event, complicating the correlation of protein presence with pulse events (Moran et al., 2013). Metabolomics fares even poorer, being a notoriously difficult field of study, mostly due to the complexity involved in its analysis. Although the metabolome is undoubtedly useful in determining exactly what happens at a cellular level, there is no single technology capable of analysing, characterising, and identifying the complete metabolome of a single cell to date, let alone that of a community as would be required in meta-metabolomics (Roessner and Bowne, 2009). Together, these likely explain why MG and MT studies are still the most dominant in the field of meta-omics, with an increasing number of studies attempting to link the two.

This page intentionally left blank

2 Main approaches and rationales of thesis

Even in the mid-20th century, NO, N_2O, and N_2 were known as enzymatic products of denitrification stemming exclusively from nitrate/nitrite (and does not involve NH_4^+), that could be further reduced given sufficient time and an enclosed atmosphere (Wijler and Delwiche, 1954). It was also then suggested that the heterogeneity of soil together with soil moisture could disrupt O_2 diffusion, creating anoxic "pockets" (microsites) within which denitrifying activity could be higher than in the bulk soil, thereby generating a mix of N_2O and N_2 even under apparently non-anoxic conditions (Wijler and Delwiche, 1954). Since then, many studies have confirmed that incomplete denitrification resulting in the accumulation or emission of denitrification intermediates (nitrite, NO, and/or N_2O) is not uncommon in the environment (Abed et al., 2013; Mørkved et al., 2007; Palta et al., 2013; Raut et al., 2012). Our interest in denitrification arises from the impact it has on global agricultural practices and climate change: N-fertilisers are often used to boost crop yields, yet long-term or overuse can cause soil acidification (Guo et al., 2010; Qu et al., 2014; Raut et al., 2012), which in turn drives NO production by chemodenitrification and increases biologically-produced N_2O, both of which are major players in driving climate change. Conversely, liming soils in response to such soil acidification may result in the accumulation of nitrite and increased HONO emissions (Glass and Silverstein, 1998; Oswald et al., 2013), which also contributes to climate change.

Thus, the main aims of this thesis were to characterise the biological and chemical fates of exogenous N-addition at different pH by monitoring the nitrogenous compounds involved in denitrification in a highly acidic and a neutral-pH soil, and to link phenotypic observations to molecular information (genes and transcripts), in an attempt to determine patterns and correlations between the "macro", "micro", and "molecular" scale. The soil chosen for use in this thesis had been shown in field studies to exhibit a strong pH effect even within a small pH interval, making it a perfect case study for chemical and biological denitrification processes under highly acidic and neutral conditions (Russenes et al., 2016).

As was alluded to in the Introduction, biological and chemical processes in the nitrogen cycle can be difficult to differentiate, given the interlinking nature of multiple compounds. Biotic nitrite reduction at pH <6 is generally considered indistinguishable to abiotic nitrite decomposition and the resultant NO production is supposedly dominated by chemodenitrification (van Cleemput, 1998; Spott et al., 2011). However, given the potential toxicity of nitrite and NO at uncontrolled levels, we postulated that microbial processes also participated in the control of these two molecules even at low pH, and that the resultant low

concentrations of nitrite is caused by a mix of both abiotic and biotic processes (instead of sole reliance on chemical degradation). **Paper II** tackles this hypothesis by attempting to separate the chemical and biological processes involving nitrite in three soils of varying pH (pH 3-7), via laboratory experiments and the construction of models, to better understand abiotic vs. biotic nitrite consumption. Due to the speed of nitrite "disappearance" in acidic environments (<10 min), a method to quickly and accurately determine nitrite concentrations from non-slurry soil was also designed.

Chemical processes aside, biological nitrification and denitrification can also be hard to disentangle, especially with the existence of nitrifier denitrification (Burns et al., 1996). However, since nitrification requires the presence of O_2, and there is evidence that nitrifier denitrification does not occur under fully anoxic conditions (Zhu et al., 2013), conducting experiments under complete anoxia removes any potential interference from nitrifiers. As such, **Paper III** focuses purely on the anoxic processes of nitrogenous compounds common to soil environments (denitrification and DNRA), with a particular focus on denitrification kinetics and the genes/transcripts involved under such conditions. Although the most acid soil (pH 3.8) could potentially reduce all produced N_2O to N_2 (**Paper II**), it was only able to do so after ≥2 days. Unlike in our air-tight microcosm experiments, gaseous N_2O produced in the environment would not persist in the soil matrix for further microbial reduction to N_2, and would be lost to the atmosphere. Thus to maintain environmental relevance, the incubation experiment in **Paper III** was restricted to the first 45 h of anoxia.

As was briefly alluded to in the Introduction, the evolutionary distinctiveness and apparent horizontal gene transfer of denitrification genes renders well-defined analysis methods of the 16S rRNA gene useless. These reasons have also plagued primer constructions, with most primers targeting denitrification-related enzymes favouring specific groups of organisms over others (Coyotzi et al., 2017; Penton et al., 2013; Throbäck et al., 2004). Thus, meta-omic analyses were applied in **Paper III** to remove primer-related biases and to secure a wider coverage of the community. Because this is a unique study interlinking the MG, MT, and phenome of an intact (non-slurry) complex soil denitrification community, manually-curated datasets (this term is used to differentiate from "official" databases) were employed in concert with custom-designed bioinformatics pipelines and data analysis/visualisation methods. Using these tools, our aim was to characterise the denitrification transcription of the soils, and determine the links between observed phenotypes and biological response. In particular, we aimed to establish if our soils depended on (or had the potential for) complete denitrifiers or a

community of organisms to perform denitrification as a whole. We also hypothesised that both NIR and NOR transcription under acidic conditions have been especially underestimated due to poor available primers, and that the transcriptional response is comparable to that of the neutral pH soil. Additionally, we postulated that *nosZ* transcripts could be detected at low pH in the absence of apparent N2OR activity [in an intact soil (non-slurry) system without the use of acetylene], due to post-transcriptional phenomena affecting N2OR activity under acidic conditions.

To obtain the nucleic acid material for sequencing in **Paper III**, a suitable method was necessary to obtain total nucleic acids (TNA). Previous attempts to extract DNA and RNA from these soils had resulted in exceedingly low yields that, in some cases, remained undetectable even after amplification (Liu et al., 2010). Thus, **Paper I** aimed to provide an in-depth examination of a variety of TNA-extracting kits and methods when applied to a known inhibitor-rich, high organic content, and low TNA-yielding soil, as well as to construct a transparent and easily optimisable modular method that resulted in high quality and quantities of inhibitor-free DNA and RNA via systematic trial-and-error. Method modularity and transparency were the top priorities in the construction of this method to allow maximum applicability to environmental samples of differing types, since universally applicable extraction methods are unlikely to be found.

This page intentionally left blank

3 Main results and discussion

3.1 Nucleic acid extraction for downstream meta-omic analyses

Although the main aims of the thesis pertained to analysing biological denitrification at two contrasting pH levels (one near neutral, and the other highly acidic), there was persistent difficulty in extracting usable nucleic acids, especially from the highly acidic soil. Despite some previous success using a previously published method (Liu et al., 2010), the extracted nucleic acids were often of poor quality, and the RNA from the low pH soil was often contaminated with residual genomic DNA (gDNA). Extended treatments of the RNA (purification and digestion) resulted in RNA that was undetectable even post-amplification (Liu et al., 2010). Hence, **Paper I** details the extensive testing of multiple TNA extraction kits and methods, including baseline quality/quantity assessments based on soil-specific DNA-only extraction kits (**Paper I, Table 1 and S2**). Total nucleic acids were desired because soil environments are highly heterogeneous with hotspots of microbial activity in microsites, and we wished to reduce potential variation between extraction reactions by co-extracting DNA and RNA from the same sub-samples.

Upon failing to locate a suitable TNA extraction kit, the method (Nicolaisen et al., 2008) originally used in the previously-published article (Liu et al., 2010) was broken down into its key steps and systematically tested using other previously published alternatives. A variety of extraction buffers, lysis methods, nucleic acid precipitants, and downstream purification kits were examined for improvements to TNA quality and/or quantity. Interestingly, when investigating different enzymes used downstream for their resilience to residual inhibitory compounds, we discovered that enzyme resilience did not play as great a role as we had previously expected. The conclusion was that for complete gDNA digestion to take place, maximal upstream removal of inhibitors was necessary, and that individual enzyme variability was not a strong factor. Although this appears to be contrary to previous reports that select enzymes can retain its activity in the presence of inhibitors, those studies were performed on DNA polymerases (Albers et al., 2013; Kermekchiev et al., 2009) – it is important to remember that while inefficient amplification still results in amplified products, inefficient digestion results in residual gDNA, defeating the very purpose of digestion.

With these findings, we were able to construct a standard workflow protocol (**Paper I, Fig. 2**) that emphasised the importance and order of specific processes rather than individual chemical components, techniques, or enzymes. In particular, the maximal removal of inhibitors

prior to all enzymatic steps (including nucleic acid digestion) was of utmost importance, the failure of which likely being the explanation for unsuccessful nucleic acid extractions from other kits and methods. The resultant modularity and transparency of the protocol gives users the freedom to choose their individual method and/or kit of choice without sacrificing quality.

During our investigations into enzymatic inhibition, we discovered that enzymatic activity was potentially uneven across extraction replicates (**Paper I, Fig. 3**). This may have been caused by differing inhibitor contents in the original sample (owing to the heterogeneity of soil), or from human error resulting from the handling of a large number of samples. Regardless of the reason, this highlights the importance of checking all RNA samples for the complete removal of gDNA, and not simply using "representative samples" to determine gDNA absence.

Additionally, we provided evidence to the consequence of inappropriate gDNA assessments: We were able to detect residual gDNA in RNA samples via amplification when direct analysis of unamplified nucleic acids (agarose gel visualisation or Nanodrop/Qubit quantification) showed an absence of gDNA. Despite being fully aware of the potential problems arising from contaminating gDNA in RNA samples, proper indication and appropriate determination of complete gDNA removal is deeply lacking in studies published during the past few years in selected journals that are dominating in the field of microbial ecology (briefly reviewed in **Paper I**).

Aside from gDNA removal assessments, a less serious but equally important oversight by much of the scientific community involves the reporting of real-time PCR (qPCR) efficiencies. Despite the clear guidelines available in the literature (Bustin et al., 2009; Gadkar and Filion, 2013) and free-to-use tools that assist in checking extraction and amplification efficiencies (Beller et al., 2002; Ruijter et al., 2009), many still fail to do so in a sample-appropriate manner. Proof of poor correlation between "standard amplification efficiency" and "cycle threshold quantification" calculations has been acknowledged for many years (Ruijter et al., 2013; Smith and Osborn, 2009; Töwe et al., 2010), and alternative tools that analyse individual PCR reactions without the need of external standards are freely available (Ruijter et al., 2009). Yet, many studies continue to report qPCR efficiencies directly from the instrument using unrelated plasmid standards, when environmental samples are well-known to potentially contain differing quantities of inhibitors which may not only inhibit the amplification reaction but also interfere with the fluorochemistry (see *Introduction*).

Compounding the effects of unnoticed inhibitors in environmental samples (due to erroneous amplification efficiency calculations) with possibly undetected residual gDNA in RNA samples (due to inappropriate assessment methods), this potentially affects a large number of studies on environmental samples. Depending on which of the above two reasons are at play and in what combination, the dual possibilities of both over- and underestimating the presence of specific taxonomic lineages and/or microbial activity cannot be easily denied. Thus, it is of utmost importance that future studies take careful note of the points highlighted in **Paper I**, to avoid the potential pitfalls discussed in this chapter.

3.2 Abiotic nitrite decomposition vs. biotic nitrite reduction

It has previously been noted that highly acidic soils from Fjaler are able to quickly produce and accumulate relatively large quantities of NO and N_2O regardless of O_2 presence and carbon content (Lim, 2013; Liu et al., 2010; Mørkved et al., 2007). Thus, postulation that these soils suffer from high levels of chemodenitrification were naturally expected (Schreiber et al., 2012). Although NO production (and thus nitrite decomposition) has been suggested to be dominated by chemodenitrification at pH <4.5 (McKenney et al., 1990), we hypothesised that it would be unlikely for microorganisms to fully rely upon chemical processes to control nitrite and NO levels. Instead, one could reasonably expect a selection for regulatory traits that would assist the cells in controlling these potentially harmful compounds. Thus, we set out to ascertain the extent of chemically-decomposed and -evolved nitrite and NO in our soils, respectively. **Paper II** aimed to disentangle abiotic from biotic processes via a series of sterilisation and N-addition experiments, and determine the role played by abiotic and biotic nitrite processes by modelling the respective contributions.

Because sterilisation methods may affect abiotic nitrogen processes (Dail et al., 2001), this made choosing an appropriate sterilisation technique highly important. Otherwise, the compounded effects of sterilisation with abiotic decomposition would make it difficult to determine the exact effects of chemodenitrification under "normal" circumstances. Although previous studies have attempted to elucidate adverse effects of different sterilisation techniques in soils (Labeda et al., 1975; Powlson and Jenkinson, 1976), a "perfect" method does not exist and all techniques come with their own set of problems. As pointed out by Trevors (1996), the sterilisation technique favoured often depends on the type of study, convenience, and financial expense. Since a number of methods were available to us, and high organic content environments are known to be chemically complex especially when interacting with nitrogenous compounds (Thorn and Mikita, 2000), four different sterilisation techniques – chemical (glutaraldehyde immersion), gaseous (chloroform fumigation), heat-based (autoclaving), and radiative (gamma-irradiation) – were tested on our soils. Analysis of nitrite decomposition rates, the gases evolved from treated soils, and residual bioactivity (measured by growth analysis) determined that only gamma-irradiated soils had sufficiently low levels of biological activity not to contribute to nitrite reduction, while maintaining the native chemistry of the soils (**Paper II, Fig. 1**).

Due to the complex nature of soils and its interference with N-oxyanion extraction, we also needed to determine the proportion of nitrite that was able to bind to the soil matter without the

use of KCl as an extractant. Typically used in soil studies to release bound nitrite (to get accurate nitrite measurements), recent research with KCl has proven that the simultaneous release of exchangeable acidity also causes the destruction of the released nitrite when this method is employed to neutral or acidic soils (Homyak et al., 2015). Since our soils range from highly acidic to neutral pH, the application of such traditional methods would cause severe problems in our analysis. Thus, we utilised nitrate (which is chemically stable) to determine the partitioning of ions in each soil by extracting with KCl and water. Since nitrate and nitrite are very similar molecules and would be partitioned in the same way, we were then able to use our calculated "partitioning factor" to correct for water-extracted nitrite measurements (**Paper II, Table 1**).

Using the gamma-irradiated soil and our calculated partitioning factor, we were able to determine the rate of abiotic nitrite decay, and NO and N_2O production in our soils. Nitrite decomposition in our soils strongly reflected first order kinetics, and the decay rate constants were strongly correlated with the proportion of undissociated HNO_2, as predicted by soil pH (**Paper II, Fig. 2**). This indicated that pH was a reliable predictor of such chemical nitrite decomposition in our soils. Additionally, not all added nitrite was recoverable as nitrite or N-gas (NO, N_2O or N_2) in sterilised soils of pH <6, suggesting the abiotic formation of nitrosated soil organic N. Thus, the interplay of enzymatic and chemical nitrite transformations is complex, with some of the added N potentially lost to non-biologically available soil organic N (**Paper II, Fig. 4**).

Taking this into account, we estimated the rates of biological nitrite reduction (V_{NIR}) and chemical nitrite decomposition (V_{ADEC}) for all soils (**Paper II, Fig. 3**). Despite the theoretical nature of these calculations, we are confident that these values likely reflected the processes in the soil because we could account for almost all of the added nitrate-N in all soils tested (**Paper III, Table 2**). The results of the modelling revealed convincing evidence of enzyme-dominated nitrite reduction at the start of the experiment regardless of pH (**Paper II, Fig. 3**). As was expected, chemical decomposition was insignificant at pH >6 throughout the experiment. At pH <6, abiotic and biotic nitrite transformations were equal after enzymatic dominance during the first 15 h, partially supporting the suggestions by Spott et al. (2011) that chemical nitrosation may be indistinguishable from biological reactions under acidic conditions. Most surprisingly, enzymatic nitrite reduction strongly controlled nitrite transformations at pH <4 throughout the experiment, except during a relatively short 15 h window >30 h after the start of the experiment. While we are uncertain what caused this

temporary loss of enzymatic control, we postulate that it may be related to the start of N_2O reduction in that soil. Reports of nitrate use over N_2O are abound in the literature (Burns et al., 1996; Mania et al., 2016), and there has been some suggestion that there is a preferential use of more oxidised electron acceptors over N_2O by denitrification enzymes (Betlach and Tiedje, 1981). This appears to be supported by the gas kinetics of our most acidic soil, since the maximum N_2O accumulated was similar to the total N_2 recovered, suggesting that the more oxidised N-compounds were reduced to N_2O first. Furthermore, there was no change in the total electron flow (V_{e^-}), suggesting that N2OR had successfully competed with NIR for available electrons.

There has been little indication within the literature to suggest that abiotic nitrite transformations in acidic environments (pH ≤5.5) may be further differentiated with increasing acidity (Van Cleemput and Samater, 1996; Spott et al., 2011). In **Paper II**, we have provided strong contrary evidence, and also showed that nitrite control in acidic environments may be biologically-driven during denitrification. Nevertheless, abiotic nitrite decomposition was not insignificant, and played a role in the fate of added nitrate-N (by diverting to soil nitroso-compound formation). Thus, this highlights the importance of considering abiotic nitrite kinetics when determining N redox transformations in acid soils.

3.3 Anaerobic nitrate consumption at acidic and neutral pH

Despite continued interest in the denitrification process in soil environments, relatively little is known about the microbial communities that perform the reduction reactions. Much of what we understand of such environments have come from bacterial isolates extensively studied under laboratory conditions, as well as primer-based analyses that dominate much of the literature. Recent years have introduced a new research aspect, utilising meta-omics and bioinformatics tools to better characterise the reductase genes involved in denitrification (Graf et al., 2014; Orellana et al., 2014). However, much research on denitrification in soils has focused exclusively on the genetic potential (DNA) of these communities, and few attempt to differentiate between the active and inactive parts of the community (Chen et al., 2012; Coyotzi et al., 2017; Hamonts et al., 2013; Orellana et al., 2014; Yu et al., 2014). Moreover, even fewer studies combined the monitoring of the denitrification kinetics and transcription (Brenzinger et al., 2015; Henderson et al., 2010; Palmer et al., 2016), leading to a marked disconnect between our knowledge of denitrification potential, microbial response, and field observations. Thus in **Paper III**, we endeavoured to link all three by carefully monitoring all denitrification-related N-compounds, as well as taking DNA and RNA samples over time for meta-omic sequencing and characterisation.

As aforementioned in the *Introduction*, multiple biological nitrogen processes may take place simultaneously. Knowing this, we restricted concomitant processes by creating a fully anoxic environment, under which only anammox, denitrification, and DNRA may take place – both nitrifier denitrification and nitrification-coupled-denitrification while occurring at low O_2 concentrations, have been observed to be inhibited in the absence of O_2 (Zhu et al., 2013). Previous studies have evidenced the co-occurrence of these three anaerobic processes, and were thus considered alongside denitrification when analysing the MG and MT (Dong et al., 2009; Long et al., 2013; Smith et al., 2015). However, the gas kinetics of both soils (soil 3.8 and soil 6.8, with the numbers referring to the soil pH) revealed complete denitrification to N_2 from nitrate, with no residual nitrite, NO, or N_2O leftover (**Paper II, Fig. 3** and **Paper III, Fig. 1**). Considering that all added nitrate-N was recovered as N_2 gas in soil 6.8 (see **Paper II** and *Section 3.2* for details), there was no evidence of anammox or DNRA in the neutral pH soil. Furthermore, although AOA (Thaumarchaea) are known to dominate in low pH environments (Prosser and Nicol, 2012), 16S rRNA gene analysis revealed the absence of Thaumarchaea in soil 3.8 (**Paper III, Fig. S2**). On the other hand, DNRA is positively correlated with pH and is not regarded to be important under acidic conditions (Hu et al., 2015; Rütting et al., 2011;

Stevens et al., 1998). Thus, there was little evidence that either anammox or DNRA played any significant role in either soils during this experiment.

Prior to the start of the experiment (defined by anaerobiosis), the soils were revitalised by a 72 h pre-incubation with clover. This was done to remove/prevent the effect of sudden C-addition to the system, which is known to falsely increase denitrifier gene and transcript abundances in the short-term (Henderson et al., 2010). Since denitrification in soils is known to be carbon-limited and given that the choice of added substrate is able to strongly affect denitrification, clover was selected as a naturally-occurring source of carbon (Morley et al., 2014; Wang et al., 2015). The steady 16S rRNA gene profile (**Paper III, Fig. 2**) confirmed that any changes in denitrification transcripts we eventually observed were due to transcriptional regulation, rather than growth bursts caused by the removal of carbon limitation from the system.

As suspected in the *Introduction*, the MG and MT revealed the issues caused by primer bias. Whereas in previous studies soil 3.8 was regarded to have no measurable transcriptional response (Liu et al., 2010), it was low but detectable in both the MG and MT (**Paper III, Fig. 3**). Additionally, the previous report of strong *nirS* and negligible *nirK* genetic potential and transcriptional response was also likely caused by insufficiently broad-range targeting primers – current analysis of the MG and MT revealed a strong dominance of *nirK* genes over *nirS*, which persisted throughout the incubation and regardless of soil type (**Paper III, Fig. 3**).

Further comparing the MG and MT data to the phenome, we noted a surprisingly similar denitrification potential despite remarkably different gas kinetics patterns (**Paper III, Fig. 1 and 3**). With the exception of a much higher abundance of *qnor* in soil 3.8 (understandable given the likelihood for NO build-up due to fast chemical nitrite decomposition), almost all other denitrification genes were at similar levels in both soils. Of note, despite performing the same function, all three sets of denitrification enzyme functional homologue genes (*nap*/*nar*, *nirK*/*nirS*, and *cnor*/*qnor*) showed obvious dominance of one over the other. This seems to support one of two theories: (i) Differential expression based on environmental conditions may select for specific genes if said conditions persist (Wang et al., 1999); or (ii) The complexity hypothesis and deletion bias suggests a higher probability of successful horizontal gene transfer and retention of functionally-independent genes that are immediately functional upon acquisition, unlike those requiring accessory genes (Albalat and Cañestro, 2016; Kuo and Ochman, 2009; Mira et al., 2001). Alternatively, both theories may be in play – Nap/Nar are known to be sensitive to nitrate concentrations (*Introduction*), whereas both NirS and cNor

require accessory genes for functionality, unlike their more dominant counterparts. Regardless, the pressures of selection appear to be pH-independent at Fjaler, given the similar gene dominance in both the highly acidic and neutral pH soils.

Although only data from early in the incubation was available for soil 3.8, there were some notable differences in the response of the two soils. Most significant was the prioritisation of *qnor* transcription at pH 3.8 over that of *nirK*, and vice versa at pH 6.8 (**Paper III, Fig. 3**). Since **Paper II** showed that biological reduction (and not chemodenitrification) played the major role in nitrite suppression for the first 30 h in soil 3.8, *nirK* transcription while low was obviously not insignificant. Similarly, although *qnor* transcription was lower in soil 6.8, relatively strong suppression of NO was observed in both soils (**Paper III, Fig. 1**). This possibly reflects the importance of controlling the central signalling molecule, NO, since it is known to regulate all denitrification-related genes via the Crp-Fnr superfamily of transcriptional regulators. The accumulation of nitrite at pH 6.8 in turn indicates high NAR activity, which is also suggested by the high transcription levels of *nar*.

Most revealing in the MT data, is the detection of *nosZ* transcription at pH 3.8 early in the incubation, in spite of the lack of N_2O reduction and N_2 production (**Paper III, Fig. 1 and 3**). This lends strong support to the hypothesis that an essential post-transcriptional modification is lacking at low pH (Liu et al., 2014). The lack of copper in the active site is known to render NosZ catalytically inactive (Dreusch et al., 1997), and NosZ proteins isolated from the model denitrifying organism *Paracoccus denitrificans* at pH 6 were lacking copper in the active site (Lycus et al., unpublished data). However, as seen in **Paper II Fig. 3**, soil 3.8 was fully capable of reducing all accumulated N_2O to N_2, albeit with a delay of ≈40 h, mirroring a recently isolated *Rhodanobacter* sp. from the same soil that showed similar high N_2O accumulation and delayed N_2 (Lycus et al., 2017). Thus, assuming that copper-deficiency in the active site of NosZ is the cause for its non-functionality at low pH, there is a yet unknown reason for the reinstatement of copper that is common to both pure cultures and complex soil environments. Alternatively, the late N_2O reduction may also be caused by delayed *nosZ* transcription (such as that seen in the Acidobacteria and Bacteroidetes of soil 6.8, **Paper III, Fig. 4**), which may in turn be due to some form of transcriptional regulation. Furthermore, a separate factor that may influence both copper insertion and transcriptional activity, is the probable alkalisation of microsites due to denitrification metabolic activity (Brenzinger et al., 2015), leading to hotspots of N2OR activity at more favourable pH.

Irrespective of the reason behind N2OR inactivity, this example of transcript-phenotype inconsistency lends support to the growing evidence for the importance of MG-MT-phenome interlinked studies, and that genetic potential and/or transcriptional activity alone may be insufficient to predict phenotypic observations (Chen et al., 2012; Henderson et al., 2010). An issue which is, unfortunately, not only constrained to denitrification (Rocca et al., 2015).

Further to the importance of linking different types of data, the MG and MT of the soils confirmed that there was a certain degree of modularity of the denitrification process in both soils. While the gas observations (**Paper III, Fig. 1**) were unable to differentiate between the microorganisms performing denitrification, the MG and MT revealed not only differences between the organisms possessing each reductase gene, but also that some groups of organisms were later expressed than others (**Paper III, Fig. 4-5**). Of note, none of the taxonomic profiles of the denitrification genes and transcripts were identical, strongly hinting towards modular denitrification by multiple organisms in our soils. Although the current depth of bioinformatics exploration was insufficient to verify or disprove the presence or activity of complete denitrifiers, the uneven occurrence of some bacterial groups across denitrification genes (such as the phyla Nitrospirae and Verrucomicrobia) further strengthens the hypothesis of modular denitrification in complex environmental samples. Such observations could not have been easily made with either gas kinetics or MG data alone.

Interestingly, the transient accumulation of NO was substantial in both soils (**Paper III, Fig. 1**), suggesting that denitrification in these soils could indeed result in significant NO emissions. In the environment, NO diffusing from denitrifying microenvironments would have to pass through oxic water before reaching the atmosphere, and here the autoxidation of NO would probably scavenge a substantial fraction of the produced NO before reaching air-filled pores. This is because the autoxidation of NO is a third order reaction (Nadeem et al., 2013), and the calculated half-life of NO is hence inversely proportional to its concentration. In fully aerated water, the half-life of 10 nM NO is 14 h, but the half-life of 3 µM NO (which was the highest concentrations measured in our experiments) is only 2.9 min. The potential connection of this short NO half-life to HONO emissions is obvious (since NO_2 is the primary product of autoxidation), but such speculations are beyond the scope of this thesis.

Collectively, **Paper III** provides deep insights into the effects of long-term pH alteration on its community composition, microbial activity, as well as gas production under anaerobic conditions upon the addition of nitrate. Since these soils receive biannual N-fertilisation, their propensity to lose added fertiliser in the form of nitrite, NO, N_2O or N_2 is of great atmospheric

interest (the former two being precursors to HONO, N_2O being a greenhouse gas, and N_2 being chemically inert). Of note, the lack of functional N2OR at pH 3.8 in the presence of (i) comparable levels of N2OR genes in both soils, and (ii) detectable N2OR transcripts, strongly discourages the independent use of genes and transcripts to determine a soil's denitrification potential. The phenotypic context under which molecular data is applied must be taken into consideration as well.

This page intentionally left blank

4 Concluding remarks and future perspectives

As the American physician Dr Martin H. Fischer once said, "All the world is a laboratory to the inquiring mind," and those of us who have chosen to investigate environmental processes have, in a way, taken that quite literally. True enough to Dr Fischer's words, over 150 years have passed since the conception of the idea of a form of global nitrogen cycling (Aulie, 1971) yet we are still busily investigating one of the key series of processes upon which all life on Earth depends. This thesis aimed to elucidate the biological and chemical processes involved in anaerobic nitrogen transformation under highly acidic and neutral-pH conditions, in a high organic content soil environment. Enzymes and processes controlling nitrite, NO, and N_2O concentrations (NIR, NOR and N2OR) were of particular interest due to the potential cytotoxic effects of the former two substrates at uncontrolled levels, as well as the potential adverse atmospheric effects of the latter two.

Collectively, **Papers II** and **III** revealed that although nitrite levels were kept low under highly acidic conditions as expected, enzymatic nitrite reduction played a larger role in nitrite suppression than abiotic nitrite decomposition. Not only have we shown that biological denitrification is not insignificant under acidic conditions, this thesis has provided new insight to differential chemical degradation under highly acidic conditions, which had not previously been considered at pH $\leq$5.5 (Van Cleemput and Samater, 1996; Spott et al., 2011). This strongly highlights the complexity of chemical processes and the importance of taking abiotic N-processes into account in acidic environments, as well as the potential pitfalls of extrapolating existing knowledge (where complex chemistry is concerned).

Perhaps because there was an absence of significant chemical nitrite degradation at neutral pH, relatively high NIR transcription was observed, presumably as an attempt to further reduce nitrite to NO. Nevertheless, large quantities of nitrite was accumulated throughout the experiment, which potentially meant high HONO field emissions. However, our calculations of abiotically-formed undissociated HNO_2 indicated the improbability of high HONO emissions at pH 6.8, barring the existence of enzyme-mediated HONO production, as assumed by Oswald et al. (2013). In contrast, NO accumulated to similar levels in both soils regardless of pH, in spite of differing genetic potential and high NIR:NOR transcription ratios in soil 6.8, suggesting high NO-derived HONO emissions from both soils in the field.

Of special interest, despite detectable *nosZ* transcription at pH 3.8, there was no corresponding N_2O reduction or N_2 production post-anaerobiosis. This strongly supports

previous hypotheses that N2OR at low pH suffers from the failure of a post-transcriptional modification process, resulting in detectable transcript levels but no functional N2OR enzyme (Liu et al., 2014). Additionally, the combination of MG and MT results coupled with phenotypic gas measurements gave strong support for a modular denitrification process that is split across multiple microbial guilds in lieu of complete denitrifiers performing the entire process. These results potentially complicate existing knowledge about the denitrification process, since much comes from model denitrifying organisms performing either complete (to N_2) or incomplete (to N_2O) denitrification, such as *Paracoccus denitrificans*, *Pseudomonas stutzeri*, or *Agrobacterium tumefaciens* (Bakken et al., 2012; Vollack and Zumft, 2001). Together these results emphasise the exquisite need to link genes with transcripts and phenotype to formulate a more complete picture, and strongly underscores the need for more community-based multi-omic studies (MG, MT and phenome).

Although much has been revealed in these studies, the exact fate of the immobilised nitrite in acid soils (pH <6) is still unconfirmed. Based on previous studies, we assumed in **Paper II** that exogenous nitrite was lost to the abiotic nitrosation of organic compounds in the soil (Stevenson et al., 1970; Stevenson and Swaby, 1964; Thorn and Mikita, 2000). Since the soils we used are high in organic matter (humus in particular), the proportion of nitrite lost to abiotic nitrosation could be verified with the use of stable nitrogen isotopes (^{15}N) and analysis of the soil material after completing biological denitrification. The same method could also be used to determine the exact proportion of abiotically- vs. biotically-formed NO and N_2O, especially since chemically-formed N_2O will be revealed as a hybrid N-compound (Phillips et al., 2016; Spott et al., 2011).

Given the current success of extracting, sequencing and analysing the metatranscriptome of soil 3.8, the transcription of denitrification genes over a longer period of time could be analysed to determine if the currently present but non-transcribing *nosZ*-carrying organisms may become active at a later stage. Moreover, although we are certain that there were no major changes to the community composition of soil 6.8 during this short incubation, it remains a possibility that less abundant acid-tolerant *nosZ*-carrying microorganisms (e.g. *Rhodanobacter* spp.) may experience a burst of anaerobic growth, leading to the increased N_2O reduction seen after 48 h in soil 3.8. Thus, it could be of interest to monitor potential changes in the community composition and/or MT of soil 3.8 leading to complete nitrate reduction to N_2.

Furthering this line of investigation, the transcriptional mechanisms and regulation behind the preferential use of nitrate (over other less oxidised electron receptors such as N_2O) could be

elucidated with pure culture studies on denitrifiers that accumulate nitrite vs. those that do not. A potential group of organisms that may shed much light upon this belongs to the genus *Thauera* – organisms of this genus have been observed to handle nitrate differently despite their shared taxonomic lineage (Liu et al., 2013). Although it had been convincingly shown that the presence of nitrate could adversely affect nitrite reduction in some organisms, the transcriptional reasons behind this has not yet been investigated and may prove to be of importance in explaining the phenomena of nitrite-accumulators amongst the denitrifiers.

Pure culture studies would also help to identify nitrite and NO regulatory mechanisms. Previous studies have identified the importance of NO and the transcriptional regulator Crp-Fnr superfamily in controlling denitrification gene expression via specific regulators (Bergaust et al., 2012; Saunders et al., 1999; Vollack and Zumft, 2001), yet there is still much that is unknown in the exact mechanism of control. Targeted mutant studies have the potential to shed light on how exactly NO regulates the Crp-Fnr superfamily, and how these transcription regulators in turn controls denitrification gene operon expression.

Gene-based studies aside, the direct investigation of non-functional N2OR via protein isolation experiments could help to clarify the post-transcriptional phenomena preventing N_2O reduction, as reported in both pure cultures of *Paracoccus denitrificans* (Bergaust et al., 2010) and complex soil communities (Liu et al., 2010, 2014). Recently, copper-deficient N2OR was isolated from *Paracoccus denitrificans* grown at pH 6 by members of our research group (Lycus et al., unpublished data), supporting the hypothesis of copper insertion failure into the active centres of NosZ at below-neutral pH. This could be taken further with a transcriptome-wide analysis of cells with and without copper-inserted NosZ (grown under neutral and acidic conditions, respectively), identifying potential genes and/or regulators responsible for this phenomenon. Current gene candidates include those encoding copper chaperones (e.g. *nosL* of the *nos* operon), or the *nos* operon regulator *nosR*, which is needed to maintain NosZ activity (Wunsch et al., 2003; Wunsch and Zumft, 2005). Alternatively, a study of the periplasmic proteome of these cells, including small proteins such as copper chaperones, could also reveal protein-based reasons for the absence of copper in NosZ.

A tangent possibility for exploration, given the high genetic potential of *nirB* and *nrf* detected in both soils, would be the degree of DNRA microbial activity and N-transformations in these soils. Evidence in the literature suggests that DNRA is favoured over denitrification under high carbon decomposition conditions (Hardison et al., 2015), which is not unlikely under field conditions at Fjaler. However, there are also signs that this is dependent upon nitrate/nitrite

concentrations, and that DNRA may only outcompete denitrification under nitrate-limited conditions (Smith et al., 2015). As such, DNRA may be negatively selected for in these soils during specific seasons because of the high biannual N-addition. Hence, an investigation into anaerobic microbial activity in soils from different seasons may also be potentially enlightening.

5 References

Abed, R. M. M., Lam, P., de Beer, D., and Stief, P. (2013). High rates of denitrification and nitrous oxide emission in arid biological soil crusts from the Sultanate of Oman. *ISME J.* 7, 1862–1875. doi:10.1038/ismej.2013.55.

Abu Al-Soud, W., and Rådström, P. (1998). Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. *Appl. Environ. Microbiol.* 64, 3748–3753. Available at: http://aem.asm.org/content/64/10/3748.short [Accessed October 22, 2015].

Albalat, R., and Cañestro, C. (2016). Evolution by gene loss. *Nat. Rev. Genet.* 17, 379–391. doi:10.1038/nrg.2016.39.

Albers, C. N., Jensen, A., Bælum, J., and Jacobsen, C. S. (2013). Inhibition of DNA Polymerases Used in Q-PCR by Structurally Different Soil-Derived Humic Substances. *Geomicrobiol. J.* 30, 675–681. doi:10.1080/01490451.2012.758193.

Arbeli, Z., and Fuentes, C. L. (2007). Improved purification and PCR amplification of DNA from environmental samples. *FEMS Microbiol. Lett.* 272, 269–75. doi:10.1111/j.1574-6968.2007.00764.x.

Aulie, R. P. (1971). Origin of the Idea of the Nitrogen Cycle. *Am. Biol. Teach.* 33, 461–471. doi:10.2307/4443650.

Bachoon, D. S., Otero, E., and Hodson, R. E. (2001). Effects of humic substances on fluorometric DNA quantification and DNA hybridization. *J. Microbiol. Methods* 47, 73–82. doi:10.1016/S0167-7012(01)00296-2.

Bakken, L. R., Bergaust, L., Liu, B., and Frostegård, Å. (2012). Regulation of denitrification at the cellular level: a clue to the understanding of N2O emissions from soils. *Philos. Trans. R. Soc. London B Biol. Sci.* 367.

Bakken, L. R., and Frostegård, Å. (2006). "Nucleic Acid Extraction from Soil," in *Nucleic Acids and Proteins in Soil*, eds. P. Nannipieri and K. Smalla (Springer Berlin Heidelberg), 49–73. doi:10.1007/3-540-29449-x_3.

Bancroft, K., Grant, I. F., and Alexander, M. (1979). Toxicity of NO2: Effect of Nitrite on Microbial Activity in an Acid Soil. *Appl. Envir. Microbiol.* 38, 940–944. Available at: http://aem.asm.org/content/38/5/940.short [Accessed April 13, 2016].

Barney, B. M., Lee, H.-I., Dos Santos, P. C., Hoffman, B. M., Dean, D. R., and Seefeldt, L. C. (2006). Breaking the N2 triple bond: insights into the nitrogenase mechanism. *Dalt. Trans.*, 2277. doi:10.1039/b517633f.

Beller, H. R., Kane, S. R., Legler, T. C., and Alvarez, P. J. J. (2002). A Real-Time Polymerase Chain Reaction Method for Monitoring Anaerobic, Hydrocarbon-Degrading Bacteria Based on a Catabolic Gene. *Environ. Sci. Technol.* 36, 3977–3984. doi:10.1021/es025556w.

Bergaust, L., Mao, Y., Bakken, L. R., and Frostegård, Å. (2010). Denitrification Response Patterns during the Transition to Anoxic Respiration and Posttranscriptional Effects of Suboptimal pH on Nitrogen Oxide Reductase in Paracoccus denitrificans. *Appl. Environ. Microbiol.* 76, 6387–6396. doi:10.1128/aem.00608-10.

Bergaust, L., Shapleigh, J., Frostegård, Å., and Bakken, L. R. (2008). Transcription and activities of NOx reductases in Agrobacterium tumefaciens: the influence of nitrate, nitrite and oxygen availability. *Environ. Microbiol.* 10, 3070–3081. doi:10.1111/j.1462-2920.2007.01557.x.

Bergaust, L., van Spanning, R. J. M., Frostegård, A., and Bakken, L. R. (2012). Expression of nitrous oxide reductase in Paracoccus denitrificans is regulated by oxygen and nitric oxide through FnrP and NNR. *Microbiology* 158, 826–34. doi:10.1099/mic.0.054148-0.

Betlach, M. R., and Tiedje, J. M. (1981). Kinetic explanation for accumulation of nitrite, nitric oxide, and nitrous oxide during bacterial denitrification. *Appl. Environ. Microbiol.* 42, 1074–84. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=244157&tool=pmcentrez&rendertype=abstract [Accessed January 8, 2014].

Blackmer, A. M., and Bremner, J. M. (1978). Inhibitory effect of nitrate on reduction of N2O to N2 by soil microorganisms. *Soil Biol. Biochem.* 10, 187–191. doi:10.1016/0038-0717(78)90095-0.

Bleakley, B. H., and Tiedje, J. M. (1982). Nitrous Oxide Production by Organisms Other than Nitrifiers or Denitrifiers. *Appl. Environ. Microbiol.* 44, 1342–1348. Available at: http://aem.asm.org/content/44/6/1342.abstract.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–20. doi:10.1093/bioinformatics/btu170.

Bowman, L. A. H., McLean, S., Poole, R. K., and Fukuto, J. M. (2011). "The Diversity of Microbial Responses to Nitric Oxide and Agents of Nitrosative Stress: Close Cousins but Not Identical Twins," in *Advances in Microbial Physiology*, ed. K. P. Robert (Academic Press), 135–219. doi:http://dx.doi.org/10.1016/B978-0-12-387661-4.00006-9.

Bray, N. L., Pimentel, H., Melsted, P., and Pachter, L. (2016). Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* 34, 525–527. doi:10.1038/nbt.3519.

Bremner, J. M. (1997). Sources of nitrous oxide in soils. *Nutr. Cycl. Agroecosystems* 49, 7–16. doi:10.1023/A:1009798022569.

Brenzinger, K., Dörsch, P., and Braker, G. (2015). pH-driven shifts in overall and transcriptionally active denitrifiers control gaseous product stoichiometry in growth experiments with extracted bacteria from soil. *Front. Microbiol.* 6. doi:10.3389/fmicb.2015.00961.

Brochier-Armanet, C., Boussau, B., Gribaldo, S., and Forterre, P. (2008). Mesophilic crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. *Nat. Rev. Microbiol.* 6, 245–252. doi:10.1038/nrmicro1852.

Buchfink, B., Xie, C., and Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12, 59–60. doi:10.1038/nmeth.3176.

Burford, J. R., and Bremner, J. M. (1975). Relationships between the denitrification capacities of soils and total, water-soluble and readily decomposable soil organic matter. *Soil Biol. Biochem.* 7, 389–394. doi:10.1016/0038-0717(75)90055-3.

Burns, L. C., Stevens, R. J., and Laughlin, R. J. (1996). Production of nitrite in soil by simultaneous nitrification and denitrification. *Soil Biol. Biochem.* 28, 609–616. doi:10.1016/0038-0717(95)00175-1.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., et al. (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 55, 611–22. doi:10.1373/clinchem.2008.112797.

Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R., and Zechmeister-Boltenstern, S. (2013). Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Philos. Trans. R. Soc. London B Biol. Sci.* 368. Available at: http://rstb.royalsocietypublishing.org/content/368/1621/20130122 [Accessed April 20, 2017].

Canfield, D. E., Glazer, A. N., and Falkowski, P. G. (2010). The evolution and future of Earth's nitrogen cycle. *Science* 330, 192–6. doi:10.1126/science.1186120.

Cantera, J. J. L., and Stein, L. Y. (2007). Molecular diversity of nitrite reductase genes (nirK) in nitrifying bacteria. *Environ. Microbiol.* 9, 765–776. doi:10.1111/j.1462-2920.2006.01198.x.

Chen, Y., Neufeld, J. D., Dumont, M. G., Friedrich, M. W., and Murrell, J. C. (2010a). "Metagenomic Analysis of Isotopically Enriched DNA," in *Methods in molecular biology (Clifton, N.J.)*, 67–75. doi:10.1007/978-1-60761-823-2_4.

Chen, Z., Liu, J., Wu, M., Xie, X., Wu, J., and Wei, W. (2012). Differentiated response of denitrifying communities to fertilization regime in paddy soil. *Microb. Ecol.* 63, 446–59. doi:10.1007/s00248-011-9909-5.

Chen, Z., Luo, X., Hu, R., Wu, M., Wu, J., and Wei, W. (2010b). Impact of Long-Term Fertilization on the Composition of Denitrifier Communities Based on Nitrite Reductase Analyses in a Paddy Soil. *Microb. Ecol.* 60, 850–861. doi:10.1007/s00248-010-9700-z.

van Cleemput, O. (1998). Subsoils: chemo-and biological denitrification, N2O and N2 emissions. *Nutr. Cycl. Agroecosystems* 52, 187–194. doi:10.1023/A:1009728125678.

Van Cleemput, O., and Samater, A. H. (1996). Nitrite in soils: accumulation and role in the formation of gaseous N compounds. *Fertil. Res.* 45, 81–89. doi:10.1007/BF00749884.

Cole, J. A., and Richardson, D. J. (2008). Respiration of Nitrate and Nitrite. *EcoSal Plus* 3. doi:10.1128/ecosal.3.2.5.

Courtois, S., Frostegard, A., Goransson, P., Depret, G., Jeannin, P., and Simonet, P. (2001). Quantification of bacterial subgroups in soil: comparison of DNA extracted directly from soil or from cells previously released by density gradient centrifugation. *Environ. Microbiol.* 3, 431–439. doi:10.1046/j.1462-2920.2001.00208.x.

Coyne, M. S., Arunakumari, A., Averill, B. A., and Tiedje, J. M. (1989). Immunological identification and distribution of dissimilatory heme cd1 and nonheme copper nitrite reductases in denitrifying bacteria. *Appl. Environ. Microbiol.* 55, 2924–2931. Available at: http://aem.asm.org/content/55/11/2924.abstract.

Coyotzi, S., Doxey, A. C., Clark, I. D., Lapen, D. R., Van Cappellen, P., and Neufeld, J. D. (2017). Agricultural soil denitrifiers possess extensive nitrite reductase gene diversity. *Environ. Microbiol.* doi:10.1111/1462-2920.13643.

Cullen, D. W., and Hirsch, P. R. (1998). Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biol. Biochem.* 30, 983–993. doi:10.1016/S0038-0717(98)00001-7.

Cuypers, H., Viebrock-Sambale, A., and Zumft, W. G. (1992). NosR, a membrane-bound regulatory component necessary for expression of nitrous oxide reductase in denitrifying Pseudomonas stutzeri. *J. Bacteriol.* 174, 5332–9. doi:10.1128/JB.174.16.5332-5339.1992.

Dail, D. B., Davidson, E. A., and Chorover, J. (2001). Rapid abiotic transformation of nitrate in an acid forest soil. *Biogeochemistry* 54, 131–146. doi:10.1023/A:1010627431722.

Daims, H., Lücker, S., and Wagner, M. (2016). A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. *Trends Microbiol.* 24, 699–712. doi:10.1016/j.tim.2016.05.004.

Dalsgaard, T., Stewart, F. J., Thamdrup, B., De Brabandere, L., Revsbech, N. P., Ulloa, O., et al. (2014). Oxygen at nanomolar levels reversibly suppresses process rates and gene expression in anammox and denitrification in the oxygen minimum zone off northern Chile. *MBio* 5, e01966. doi:10.1128/mBio.01966-14.

Decleyre, H., Heylen, K., Bjorn, T., and Willems, A. (2016). Highly diverse nirK genes comprise two major clades that harbour ammonium-producing denitrifiers. *BMC Genomics* 17, 155. doi:10.1186/s12864-016-2465-0.

Dendooven, L., and Anderson, J. M. (1995). Maintenance of denitrification potential in pasture soil following anaerobic events. *Soil Biol. Biochem.* 27, 1251–1260. Available at: http://www.sciencedirect.com/science/article/pii/003807179500067O [Accessed January 9, 2014].

Dong, L. F., Smith, C. J., Papaspyrou, S., Stott, A., Osborn, A. M., and Nedwell, D. B. (2009). Changes in Benthic Denitrification, Nitrate Ammonification, and Anammox Process Rates and Nitrate and Nitrite Reductase Gene Abundances along an Estuarine Nutrient Gradient (the Colne Estuary, United Kingdom). *Appl. Environ. Microbiol.* 75, 3171–3179. doi:10.1128/aem.02511-08.

Dreusch, A., Bürgisser, D. M., Heizmann, C. W., and Zumft, W. G. (1997). Lack of copper insertion into unprocessed cytoplasmic nitrous oxide reductase generated by an R20D substitution in the arginine consensus motif of the signal peptide. *Biochim. Biophys. Acta - Bioenerg.* 1319, 311–318. doi:10.1016/S0005-2728(96)00174-0.

Firestone, M. K., Firestone, R. B., and Tiedje, J. M. (1980). Nitrous Oxide from Soil Denitrification: Factors Controlling Its Biological Production. *Science (80-.).* 208, 749–751. doi:10.1126/science.208.4445.749.

Franzosa, E. A., Hsu, T., Sirota-Madi, A., Shafquat, A., Abu-Ali, G., Morgan, X. C., et al. (2015). Sequencing and beyond: integrating molecular "omics" for microbial community profiling. *Nat. Rev. Microbiol.* 13, 360–372. doi:10.1038/nrmicro3451.

Franzosa, E. A., Morgan, X. C., Segata, N., Waldron, L., Reyes, J., Earl, A. M., et al. (2014). Relating the metatranscriptome and metagenome of the human gut. *Proc. Natl. Acad. Sci.* 111, E2329–E2338. doi:10.1073/pnas.1319284111.

Frostegård, Å., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., et al. (1999). Quantification of Bias Related to the Extraction of DNA Directly from Soils. *Appl. Environ. Microbiol.* 65, 5409–5420. Available at: http://aem.asm.org/content/65/12/5409.abstract.

Gadkar, V. Y., and Filion, M. (2013). Quantitative Real-time Polymerase Chain Reaction for Tracking Microbial Gene Expression in Complex Environmental Matrices. *Curr Issues Mol*

Biol 15, 45–58.

Gamble, T. N., Betlach, M. R., and Tiedje, J. M. (1977). Numerically Dominant Denitrifying Bacteria from World Soils. *Appl. Environ. Microbiol.* 33, 926–939. Available at: http://aem.asm.org/content/33/4/926.abstract.

Gingold, H., and Pilpel, Y. (2011). Determinants of translation efficiency and accuracy. *Mol. Syst. Biol.* 7, 481. doi:10.1038/msb.2011.14.

Glass, C., and Silverstein, J. (1998). Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Res.* 32, 831–839. doi:10.1016/S0043-1354(97)00260-1.

Graf, D. R. H., Jones, C. M., and Hallin, S. (2014). Intergenomic Comparisons Highlight Modularity of the Denitrification Pathway and Underpin the Importance of Community Structure for N2O Emissions. *PLoS One* 9, e114118. doi:10.1371/journal.pone.0114118.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., and Bailey, M. J. (2000). Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA-Based Microbial Community Composition. *Appl. Environ. Microbiol.* 66, 5488–5491. doi:10.1128/aem.66.12.5488-5491.2000.

Guo, J. H., Liu, X. J., Zhang, Y., Shen, J. L., Han, W. X., Zhang, W. F., et al. (2010). Significant acidification in major Chinese croplands. *Science* 327, 1008–10. doi:10.1126/science.1182570.

Hamonts, K., Clough, T. J., Stewart, A., Clinton, P. W., Richardson, A. E., Wakelin, S. A., et al. (2013). Effect of nitrogen and waterlogging on denitrifier gene abundance, community structure and activity in the rhizosphere of wheat. *FEMS Microbiol. Ecol.* 83, 568–584. doi:10.1111/1574-6941.12015.

Hardison, A. K., Algar, C. K., Giblin, A. E., and Rich, J. J. (2015). Influence of organic carbon and nitrate loading on partitioning between dissimilatory nitrate reduction to ammonium (DNRA) and N2 production. *Geochim. Cosmochim. Acta* 164, 146–160. doi:10.1016/j.gca.2015.04.049.

Henderson, S. L., Dandie, C. E., Patten, C. L., Zebarth, B. J., Burton, D. L., Trevors, J. T., et al. (2010). Changes in Denitrifier Abundance, Denitrification Gene mRNA Levels, Nitrous Oxide Emissions, and Denitrification in Anoxic Soil Microcosms Amended with Glucose and Plant Residues. *Appl. Environ. Microbiol.* 76, 2155–2164. doi:10.1128/aem.02993-09.

Herold, M. B., Baggs, E. M., and Daniell, T. J. (2012). Fungal and bacterial denitrification are differently affected by long-term pH amendment and cultivation of arable soil. *Soil Biol. Biochem.* 54, 25–35. doi:10.1016/j.soilbio.2012.04.031.

Van Den Heuvel, R. N., Van Der Biezen, E., Jetten, M. S. M., Hefting, M. M., and Kartal, B. (2010). Denitrification at pH 4 by a soil-derived Rhodanobacter-dominated community. *Environ. Microbiol.* 12, 3264–3271. doi:10.1111/j.1462-2920.2010.02301.x.

Homyak, P. M., Vasquez, K. T., Sickman, J. O., Parker, D. R., and Schimel, J. P. (2015). Improving Nitrite Analysis in Soils: Drawbacks of the Conventional 2 M KCl Extraction. *Soil Sci. Soc. Am. J.* 79, 1237–1242. doi:10.2136/sssaj2015.02.0061n.

Hu, H.-W., Chen, D., and He, J.-Z. (2015). Microbial regulation of terrestrial nitrous oxide

formation: understanding the biological pathways for prediction of emission rates. *FEMS Microbiol. Rev.* 39, 729–749. doi:10.1093/femsre/fuv021.

Huggett, J., Dheda, K., Bustin, S., and Zumla, A. (2005). Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun.* 6, 279–84. doi:10.1038/sj.gene.6364190.

Huggett, J. F., Novak, T., Garson, J. A., Green, C., Morris-Jones, S. D., Miller, R. F., et al. (2008). Differential susceptibility of PCR reactions to inhibitors: an important and unrecognised phenomenon. *BMC Res. Notes* 1, 70. doi:10.1186/1756-0500-1-70.

IGBP-DIS (1998). SoilData(V.0) A program for creating global soil-property databases.

Jacob, D. J. (2000). Heterogeneous chemistry and tropospheric ozone. *Atmos. Environ.* 34, 2131–2159. doi:10.1016/S1352-2310(99)00462-8.

Jetten, M. S. M., Wagner, M., Fuerst, J., van Loosdrecht, M., Kuenen, G., and Strous, M. (2001). Microbiology and application of the anaerobic ammonium oxidation ("anammox") process. *Curr. Opin. Biotechnol.* 12, 283–288. doi:10.1016/s0958-1669(00)00211-1.

Jones, C. M., Graf, D. R. H., Bru, D., Philippot, L., and Hallin, S. (2012). The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME J* 7, 417–426. doi:http://www.nature.com/ismej/journal/v7/n2/suppinfo/ismej2012125s1.html.

Jones, C. M., Spor, A., Brennan, F. P., Breuil, M.-C., Bru, D., Lemanceau, P., et al. (2014). Recently identified microbial guild mediates soil N_2O sink capacity. *Nat. Clim. Chang.* 4, 801–805. doi:10.1038/nclimate2301.

Jones, C. M., Stres, B., Rosenquist, M., and Hallin, S. (2008). Phylogenetic Analysis of Nitrite, Nitric Oxide, and Nitrous Oxide Respiratory Enzymes Reveal a Complex Evolutionary History for Denitrification. *Mol. Biol. Evol.* 25, 1955–1966. doi:10.1093/molbev/msn146.

Jung, J., Choi, S., Jung, H., Scow, K. M., and Park, W. (2012). Primers for amplification of nitrous oxide reductase genes associated with Firmicutes and Bacteroidetes in organic-compound-rich soils. *Microbiology*. doi:10.1099/mic.0.060194-0.

Kaiser, G., and Heber, U. (1983). Photosynthesis of leaf cell protoplasts and permeability of the plasmalemma to some solutes. *Planta* 157, 462–470. doi:10.1007/BF00397204.

Keiblinger, K. M., Fuchs, S., Zechmeister-Boltenstern, S., and Riedel, K. (2016). Soil and leaf litter metaproteomics—a brief guideline from sampling to understanding. *FEMS Microbiol. Ecol.* 92. doi:http://dx.doi.org/10.1093/femsec/fiw180.

Kermekchiev, M. B., Kirilova, L. I., Vail, E. E., and Barnes, W. M. (2009). Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Res.* 37, e40–e40. doi:10.1093/nar/gkn1055.

Kim, J., Kim, M. S., Koh, A. Y., Xie, Y., Zhan, X., Qin, J., et al. (2016). FMAP: Functional Mapping and Analysis Pipeline for metagenomics and metatranscriptomics studies. *BMC Bioinformatics* 17, 420. doi:10.1186/s12859-016-1278-0.

Körner, H., Sofia, H. J., and Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp-Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene programs. *FEMS Microbiol. Rev.* 27, 559–592. doi:10.1016/S0168-6445(03)00066-4.

Kraft, B., Strous, M., and Tegetmeyer, H. E. (2011). Microbial nitrate respiration – Genes, enzymes

and environmental distribution. *J. Biotechnol.* 155, 104–117. doi:10.1016/j.jbiotec.2010.12.025.

Krsek, M., and Wellington, E. M. H. (1999). Comparison of different methods for the isolation and purification of total community DNA from soil. *J. Microbiol. Methods* 39, 1–16. doi:10.1016/S0167-7012(99)00093-7.

Kulmala, M., and Petäjä, T. (2011). Soil Nitrites Influence Atmospheric Chemistry. *Science (80-.).* 333, 1586–1587. doi:10.1126/science.1211872.

Kumon, Y., Sasaki, Y., Kato, I., Takaya, N., Shoun, H., and Beppu, T. (2002). Codenitrification and denitrification are dual metabolic pathways through which dinitrogen evolves from nitrate in Streptomyces antibioticus. *J. Bacteriol.* 184, 2963–8. doi:10.1128/jb.184.11.2963-2968.2002.

Kuo, C.-H., and Ochman, H. (2009). The fate of new bacterial genes. *FEMS Microbiol. Rev.* 33, 38–43. doi:10.1111/j.1574-6976.2008.00140.x.

Labeda, D. P., Balkwill, D. L., and Casida Jr., L. E. (1975). Soil sterilization effects on *in situ* indigenous microbial cells in soil. *Can. J. Microbiol.* 21, 263–269. doi:10.1139/m75-037.

Lam, P., Lavik, G., Jensen, M. M., van de Vossenberg, J., Schmid, M., Woebken, D., et al. (2009). Revising the nitrogen cycle in the Peruvian oxygen minimum zone. *Proc. Natl. Acad. Sci.* 106, 4752–4757. doi:10.1073/pnas.0812444106.

Lashof, D. A., and Ahuja, D. R. (1990). Relative contributions of greenhouse gas emissions to global warming. *Nature* 344, 529–531. doi:10.1038/344529a0.

Laughlin, R. J., and Stevens, R. J. (2002). Evidence for Fungal Dominance of Denitrification and Codenitrification in a Grassland Soil. *Soil Sci. Soc. Am. J.* 66, 1540. doi:10.2136/sssaj2002.1540.

Leininger, S., Urich, T., Schloter, M., Schwark, L., Qi, J., Nicol, G. W., et al. (2006). Archaea predominate among ammonia-oxidizing prokaryotes in soils. *Nature* 442, 806–809. doi:10.1038/nature04983.

Lever, M. A., Torti, A., Eickenbusch, P., Michaud, A. B., Šantl-Temkiv, T., and Jørgensen, B. B. (2015). A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. *Front. Microbiol.* 6, 476. doi:10.3389/fmicb.2015.00476.

Levy-Booth, D. J., Campbell, R. G., Gulden, R. H., Hart, M. M., Powell, J. R., Klironomos, J. N., et al. (2007). Cycling of extracellular DNA in the soil environment. *Soil Biol. Biochem.* 39, 2977–2991. doi:10.1016/j.soilbio.2007.06.020.

Lim, N. Y. N. (2013). Optimisation of nucleic acid extraction methods for a low pH soil, quantification of denitrification gene expression, and the analysis of gas kinetics from agricultural peat soils.

Lim, N. Y. N., Roco, C. A., and Frostegard, A. (2016). Transparent DNA/RNA co-extraction workflow protocol suitable for inhibitor-rich environmental samples that focuses on complete DNA removal for transcriptomic analyses. *Front. Microbiol.* 7, 1588. doi:10.3389/FMICB.2016.01588.

Lindahl, V., and Bakken, L. R. (1995). Evaluation of methods for extraction of bacteria from soil. *FEMS Microbiol. Ecol.* 16, 135–142. doi:10.1111/j.1574-6941.1995.tb00277.x.

Liu, B., Frostegård, Å., and Bakken, L. R. (2014). Impaired reduction of N2O to N2 in acid soils is due to a posttranscriptional interference with the expression of nosZ. *MBio* 5, e01383-14. doi:10.1128/mBio.01383-14.

Liu, B., Mao, Y., Bergaust, L., Bakken, L. R., and Frostegård, Å. (2013). Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes. *Environ. Microbiol.*, n/a-n/a. doi:10.1111/1462-2920.12142.

Liu, B., Mørkved, P. T., Frostegård, Å., and Bakken, L. R. (2010). Denitrification gene pools, transcription and kinetics of NO, N2O and N2 production as affected by soil pH. *FEMS Microbiol. Ecol.* 72, 407–417. doi:10.1111/j.1574-6941.2010.00856.x.

Long, A., Heitman, J., Tobias, C., Philips, R., and Song, B. (2013). Co-occurring anammox, denitrification, and codenitrification in agricultural soils. *Appl. Environ. Microbiol.* 79, 168–76. doi:10.1128/AEM.02520-12.

Lüke, C., Speth, D. R., Kox, M. A. R., Villanueva, L., and Jetten, M. S. M. (2016). Metagenomic analysis of nitrogen and methane cycling in the Arabian Sea oxygen minimum zone. *PeerJ* 4, e1924. doi:10.7717/peerj.1924.

Luque-Almagro, V. M., Lyall, V. J., Ferguson, S. J., Roldán, M. D., Richardson, D. J., and Gates, A. J. (2013). Nitrogen oxyanion-dependent dissociation of a two-component complex that regulates bacterial nitrate assimilation. *J. Biol. Chem.* 288, 29692–702. doi:10.1074/jbc.M113.459032.

Lycus, P., Kjendseth, Å. R., Bergaust, L., Bakken, L. R., Frostegård, Å. (2017). [Impaired N2OR assembly at low pH causes N_2O emissions]. *Unpublished data*.

Lycus, P., Bøthun, K., Bergaust, L., Shapleigh, J., Bakken, L., and Frostegård, Å. (2017). Phenotypic and genotypic richness of denitrifiers revealed by a novel isolation strategy. *ISME J.* In Press.

Maeda, K., Morioka, R., Hanajima, D., and Osada, T. (2010). The Impact of Using Mature Compost on Nitrous Oxide Emission and the Denitrifier Community in the Cattle Manure Composting Process. *Microb. Ecol.* 59, 25–36. doi:10.1007/s00248-009-9547-3.

Magalhães, A. M. T., and Chalk, P. M. (1987). Factors affecting formation of methyl nitrite in soils. *J. Soil Sci.* 38, 701–709. doi:10.1111/j.1365-2389.1987.tb02167.x.

Mahmoudi, N., Slater, G. F., and Fulthorpe, R. R. (2011). Comparison of commercial DNA extraction kits for isolation and purification of bacterial and eukaryotic DNA from PAH-contaminated soils. *Can. J. Microbiol.* 57, 623–628. doi:10.1139/w11-049.

Mahne, I., and Tiedje, J. M. (1995). Criteria and methodology for identifying respiratory denitrifiers. *Appl. Environ. Microbiol.* 61, 1110–1115. Available at: http://aem.asm.org/content/61/3/1110.abstract.

Malm, S., Tiffert, Y., Micklinghoff, J., Schultze, S., Joost, I., Weber, I., et al. (2009). The roles of the nitrate reductase NarGHJI, the nitrite reductase NirBD and the response regulator GlnR in nitrate assimilation of Mycobacterium tuberculosis. *Microbiology* 155, 1332–1339. doi:10.1099/mic.0.023275-0.

Mania, D., Heylen, K., van Spanning, R. J. M., and Frostegård, Å. (2014). The nitrate-ammonifying and nosZ -carrying bacterium Bacillus vireti is a potent source and sink for nitric and nitrous oxide under high nitrate conditions. *Environ. Microbiol.* 16, 3196–3210. doi:10.1111/1462-2920.12478.

Mania, D., Heylen, K., van Spanning, R. J. M., and Frostegård, Å. (2016). Regulation of nitrogen metabolism in the nitrate-ammonifying soil bacterium *Bacillus vireti* and evidence for its ability to grow using N_2O as electron acceptor. *Environ. Microbiol.* 18, 2937–2950. doi:10.1111/1462-2920.13124.

Mardis, E. R. (2017). DNA sequencing technologies: 2006–2016. *Nat. Protoc.* 12, 213–218. doi:10.1038/nprot.2016.182.

Mason, O. U., Hazen, T. C., Borglin, S., Chain, P. S. G., Dubinsky, E. A., Fortney, J. L., et al. (2012). Metagenome, metatranscriptome and single-cell sequencing reveal microbial response to Deepwater Horizon oil spill. *ISME J.* 6, 1715–27. doi:10.1038/ismej.2012.59.

McKenney, D. J., Lazar, C., and Findlay, W. J. (1990). Kinetics of the Nitrite to Nitric Oxide Reaction in Peat. *Soil Sci. Soc. Am. J.* 54, 106. doi:10.2136/sssaj1990.03615995005400010016x.

Medinets, S., Skiba, U., Rennenberg, H., and Butterbach-Bahl, K. (2015). A review of soil NO transformation: Associated processes and possible physiological significance on organisms. *Soil Biol. Biochem.* 80, 92–117. doi:10.1016/j.soilbio.2014.09.025.

Meiklejohn, J. (1940). Aerobic Denitrification. *Ann. Appl. Biol.* 27, 558–573. doi:10.1111/j.1744-7348.1940.tb07526.x.

Mira, A., Ochman, H., and Moran, N. A. (2001). Deletional bias and the evolution of bacterial genomes. *Trends Genet.* 17, 589–596. doi:10.1016/S0168-9525(01)02447-7.

Moran, M. A., Satinsky, B., Gifford, S. M., Luo, H., Rivers, A., Chan, L.-K., et al. (2013). Sizing up metatranscriptomics. *ISME J* 7, 237–243. doi:http://www.nature.com/ismej/journal/v7/n2/suppinfo/ismej201294s1.html.

Moreno-Vivián, C., Cabello, P., Martínez-Luque, M., Blasco, R., and Castillo, F. (1999). Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial nitrate reductases. *J. Bacteriol.* 181, 6573–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10542156 [Accessed February 10, 2017].

Mørkved, P. T., Dörsch, P., and Bakken, L. R. (2007). The N2O product ratio of nitrification and its dependence on long-term changes in soil pH. *Soil Biol. Biochem.* 39, 2048–2057. doi:10.1016/j.soilbio.2007.03.006.

Morley, N. J., Richardson, D. J., Baggs, E. M., Lindow, S., and Senoo, K. (2014). Substrate Induced Denitrification over or under Estimates Shifts in Soil N2/N2O Ratios. *PLoS One* 9, e108144. doi:10.1371/journal.pone.0108144.

Muller, E. B., Stouthamer, A. H., and van Verseveld, H. W. (1995). Simultaneous NH3 oxidation and N2 production at reduced O2 tensions by sewage sludge subcultured with chemolithotrophic medium. *Biodegradation* 6, 339–349. doi:10.1007/BF00695264.

Nadeem, S., Dörsch, P., and Bakken, L. R. (2013). Autoxidation and acetylene-accelerated oxidation of NO in a 2-phase system: Implications for the expression of denitrification in ex

situ experiments. *Soil Biol. Biochem.* 57, 606–614. doi:http://dx.doi.org/10.1016/j.soilbio.2012.10.007.

Narayanasamy, S., Jarosz, Y., Muller, E. E. L., Heintz-Buschart, A., Herold, M., Kaysen, A., et al. (2016). IMP: a pipeline for reproducible reference-independent integrated metagenomic and metatranscriptomic analyses. *Genome Biol.* 17, 260. doi:10.1186/s13059-016-1116-8.

Nelson, D. W., and Bremner, J. M. (1970). Gaseous products of nitrite decomposition in soils. *Soil Biol. Biochem.* 2, 203–IN8. doi:10.1016/0038-0717(70)90008-8.

Nesme, J., Achouak, W., Agathos, S. N., Bailey, M., Baldrian, P., Brunel, D., et al. (2016). Back to the Future of Soil Metagenomics. *Front. Microbiol.* 7, 73. doi:10.3389/fmicb.2016.00073.

Nicolaisen, M. H., Baelum, J., Jacobsen, C. S., and Sørensen, J. (2008). Transcription dynamics of the functional tfdA gene during MCPA herbicide degradation by Cupriavidus necator AEO106 (pRO101) in agricultural soil. *Environ. Microbiol.* 10, 571–9. doi:10.1111/j.1462-2920.2007.01476.x.

Nömmik, H., and Thorin, J. (1972). "Transformations of 15N-labelled nitrite and nitrate in forest raw humus during anaerobic incubation," in *Isotopes and Radiation in Soil Plant Relationships Including Forestry*, ed. IAEA (Vienna, Austria: IAEA-SM-151/58), 369–382.

Orellana, L. H., Rodriguez-R, L. M., Higgins, S., Chee-Sanford, J. C., Sanford, R. A., Ritalahti, K. M., et al. (2014). Detecting nitrous oxide reductase (NosZ) genes in soil metagenomes: method development and implications for the nitrogen cycle. *MBio* 5, e01193-14. doi:10.1128/mBio.01193-14.

Oswald, R., Behrendt, T., Ermel, M., Wu, D., Su, H., Cheng, Y., et al. (2013). HONO Emissions from Soil Bacteria as a Major Source of Atmospheric Reactive Nitrogen. *Science (80-.).* 341, 1233–1235. doi:10.1126/science.1242266.

Palmer, K., and Horn, M. A. (2012). Actinobacterial nitrate reducers and proteobacterial denitrifiers are abundant in N2O-metabolizing palsa peat. *Appl. Environ. Microbiol.* 78, 5584–96. doi:10.1128/AEM.00810-12.

Palmer, K., Köpp, J., Gebauer, G., and Horn, M. A. (2016). Drying-Rewetting and Flooding Impact Denitrifier Activity Rather than Community Structure in a Moderately Acidic Fen. *Front. Microbiol.* 7, 727. doi:10.3389/fmicb.2016.00727.

Palta, M. M., Ehrenfeld, J. G., and Groffman, P. M. (2013). Denitrification and Potential Nitrous Oxide and Carbon Dioxide Production in Brownfield Wetland Soils. *J. Environ. Qual.* 42, 1507. doi:10.2134/jeq2012.0392.

Pauleta, S. R., Dell'Acqua, S., and Moura, I. (2013). Nitrous oxide reductase. *Coord. Chem. Rev.* 257, 332–349. doi:10.1016/j.ccr.2012.05.026.

Paulin, M. M., Nicolaisen, M. H., Jacobsen, C. S., Gimsing, A. L., Sørensen, J., and Bælum, J. (2013). Improving Griffith's protocol for co-extraction of microbial DNA and RNA in adsorptive soils. *Soil Biol. Biochem.* 63, 37–49. doi:10.1016/j.soilbio.2013.02.007.

Penton, C. R., Johnson, T. A., Quensen, J. F., Iwai, S., Cole, J. R., and Tiedje, J. M. (2013). Functional genes to assess nitrogen cycling and aromatic hydrocarbon degradation: primers and processing matter. *Front. Microbiol.* 4. doi:10.3389/fmicb.2013.00279.

Peršoh, D., Theuerl, S., Buscot, F., and Rambold, G. (2008). Towards a universally adaptable method for quantitative extraction of high-purity nucleic acids from soil. *J. Microbiol. Methods* 75, 19–24. doi:http://dx.doi.org/10.1016/j.mimet.2008.04.009.

Phillips, R. L., Song, B., McMillan, A. M. S., Grelet, G., Weir, B. S., Palmada, T., et al. (2016). Chemical formation of hybrid di-nitrogen calls fungal codenitrification into question. *Sci. Rep.* 6, 39077. doi:10.1038/srep39077.

Porter, L. K. (1969). Gaseous Products Produced by Anaerobic Reaction of Sodium Nitrite with Oxime Compounds and Oximes Synthesized from Organic Matter. *Soil Sci. Soc. Am. J.* 33, 696. doi:10.2136/sssaj1969.03615995003300050023x.

Powlson, D. S., and Jenkinson, D. S. (1976). The effects of biocidal treatments on metabolism in soil—II. Gamma irradiation, autoclaving, air-drying and fumigation. *Soil Biol. Biochem.* 8, 179–188. doi:10.1016/0038-0717(76)90002-X.

Prosser, J. I. (2015). Dispersing misconceptions and identifying opportunities for the use of "omics" in soil microbial ecology. *Nat. Rev. Microbiol.* 13, 439–446. doi:10.1038/nrmicro3468.

Prosser, J. I., and Nicol, G. W. (2012). Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. *Trends Microbiol.* 20, 523–531. doi:10.1016/j.tim.2012.08.001.

Purkhold, U., Pommerening-Röser, A., Juretschko, S., Schmid, M. C., Koops, H. P., and Wagner, M. (2000). Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. *Appl. Environ. Microbiol.* 66, 5368–82. doi:10.1128/AEM.66.12.5368-5382.2000.

Qu, Z., Bakken, L. R., Molstad, L., Frostegård, Å., and Bergaust, L. L. (2016). Transcriptional and metabolic regulation of denitrification in *Paracoccus denitrificans* allows low but significant activity of nitrous oxide reductase under oxic conditions. *Environ. Microbiol.* 18, 2951–2963. doi:10.1111/1462-2920.13128.

Qu, Z., Wang, J., Almøy, T., and Bakken, L. R. (2014). Excessive use of nitrogen in Chinese agriculture results in high $N_2O/(N_2O+N_2)$ product ratio of denitrification, primarily due to acidification of the soils. *Glob. Chang. Biol.* 20, 1685–1698. doi:10.1111/gcb.12461.

Quail, M., Smith, M. E., Coupland, P., Otto, T. D., Harris, S. R., Connor, T. R., et al. (2012). A tale of three next generation sequencing platforms: comparison of Ion torrent, pacific biosciences and illumina MiSeq sequencers. *BMC Genomics* 13, 341. doi:10.1186/1471-2164-13-341.

Raut, N., Dörsch, P., Sitaula, B. K., and Bakken, L. R. (2012). Soil acidification by intensified crop production in South Asia results in higher N2O/(N2 + N2O) product ratios of denitrification. *Soil Biol. Biochem.* 55, 104–112. doi:http://dx.doi.org/10.1016/j.soilbio.2012.06.011.

Ravishankara, A. R., Daniel, J. S., and Portmann, R. W. (2009). Nitrous Oxide (N2O): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. *Science (80-.).* 326. Available at: http://science.sciencemag.org/content/326/5949/123.full [Accessed April 4, 2017].

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S., and Taylor, C. J. (2001). Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell. Mol. Life Sci.* 58, 165–178. doi:10.1007/PL00000845.

RNA-Seq Blog (2015). RPKM, FPKM and TPM, clearly explained. Available at: http://www.rna-seqblog.com/rpkm-fpkm-and-tpm-clearly-explained/ [Accessed April 6, 2017].

Rocca, J. D., Hall, E. K., Lennon, J. T., Evans, S. E., Waldrop, M. P., Cotner, J. B., et al. (2015). Relationships between protein-encoding gene abundance and corresponding process are commonly assumed yet rarely observed. *ISME J.* 9, 1693–1699. doi:10.1038/ismej.2014.252.

Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005). Dissimilatory Metabolism of Nitrogen Oxides in Bacteria: Comparative Reconstruction of Transcriptional Networks. *PLoS Comput. Biol.* 1, 0415–0431. doi:10.1371/journal.pcbi.0010055.

Roessner, U., and Bowne, J. (2009). What is metabolomics all about? *www.BioTechniques.com* 46, 363–365. doi:10.2144/000113133.

Ruijter, J. M., Pfaffl, M. W., Zhao, S., Spiess, A. N., Boggy, G., Blom, J., et al. (2013). Evaluation of qPCR curve analysis methods for reliable biomarker discovery: Bias, resolution, precision, and implications. *Methods* 59, 32–46. doi:10.1016/j.ymeth.2012.08.011.

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B., et al. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37, e45. doi:10.1093/nar/gkp045.

Russenes, A. L., Korsaeth, A., Bakken, L. R., and Dörsch, P. (2016). Spatial variation in soil pH controls off-season N2O emission in an agricultural soil. *Soil Biol. Biochem.* 99, 36–46. doi:10.1016/j.soilbio.2016.04.019.

Russow, R., Stange, C. F., and Neue, H.-U. (2009). Role of nitrite and nitric oxide in the processes of nitrification and denitrification in soil: Results from 15N tracer experiments. *Soil Biol. Biochem.* 41, 785–795. doi:10.1016/j.soilbio.2009.01.017.

Rütting, T., Boeckx, P., Müller, C., and Klemedtsson, L. (2011). Assessment of the importance of dissimilatory nitrate reduction to ammonium for the terrestrial nitrogen cycle. *Biogeosciences Discuss.* 8, 1169–1196. doi:10.5194/bgd-8-1169-2011.

Salter, S. J., Cox, M. J., Turek, E. M., Calus, S. T., Cookson, W. O., Moffatt, M. F., et al. (2014). Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.* 12, 87. doi:10.1186/s12915-014-0087-z.

Samouilov, A., Woldman, Y. Y., Zweier, J. L., and Khramtsov, V. V. (2007). Magnetic resonance study of the transmembrane nitrite diffusion. *Nitric Oxide* 16, 362–370. doi:10.1016/j.niox.2006.12.006.

Sánchez, C., Itakura, M., Okubo, T., Matsumoto, T., Yoshikawa, H., Gotoh, A., et al. (2014). The nitrate-sensing NasST system regulates nitrous oxide reductase and periplasmic nitrate reductase in *Bradyrhizobium japonicum*. *Environ. Microbiol.* 16, 3263–3274. doi:10.1111/1462-2920.12546.

Sanford, R. A., Wagner, D. D., Wu, Q., Chee-Sanford, J. C., Thomas, S. H., Cruz-García, C., et al. (2012). Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. *Proc. Natl. Acad. Sci. U. S. A.* 109, 19709–14. doi:10.1073/pnas.1211238109.

Saunders, N. F. W., Houben, E. N. G., Koefoed, S., de Weert, S., Reijnders, W. N. M., Westerhoff, H. V., et al. (1999). Transcription regulation of the nir gene cluster encoding nitrite reductase of Paracoccus denitrificans involves NNR and NirI, a novel type of membrane protein. *Mol.*

Microbiol. 34, 24–36. doi:10.1046/j.1365-2958.1999.01563.x.

Schalk-Otte, S., Seviour, R. J., Kuenen, J. G., and Jetten, M. S. M. (2000). Nitrous oxide (N2O) production by Alcaligenes faecalis during feast and famine regimes. *Water Res.* 34, 2080–2088. doi:http://dx.doi.org/10.1016/S0043-1354(99)00374-7.

Schreiber, F., Wunderlin, P., Udert, K. M., and Wells, G. F. (2012). Nitric oxide and nitrous oxide turnover in natural and engineered microbial communities: biological pathways, chemical reactions, and novel technologies. *Front. Microbiol.* 3, 372. doi:10.3389/fmicb.2012.00372.

Seefeldt, L. C., Hoffman, B. M., and Dean, D. R. (2009). Mechanism of Mo-dependent nitrogenase. *Annu. Rev. Biochem.* 78, 701–22. doi:10.1146/annurev.biochem.78.070907.103812.

Shapleigh, J. P. (2013). "Denitrifying Prokaryotes," in *The Prokaryotes* (Berlin, Heidelberg: Springer Berlin Heidelberg), 405–425. doi:10.1007/978-3-642-30141-4_71.

Shen, Q. ., Ran, W., and Cao, Z. . (2003). Mechanisms of nitrite accumulation occurring in soil nitrification. *Chemosphere* 50, 747–753. doi:10.1016/S0045-6535(02)00215-1.

Sidstedt, M., Jansson, L., Nilsson, E., Noppa, L., Forsman, M., Rådström, P., et al. (2015). Humic substances cause fluorescence inhibition in real-time polymerase chain reaction. *Anal. Biochem.* 487, 30–7. doi:10.1016/j.ab.2015.07.002.

Šimek, M., Jíšová, L., and Hopkins, D. W. (2002). What is the so-called optimum pH for denitrification in soil? *Soil Biol. Biochem.* 34, 1227–1234. doi:10.1016/S0038-0717(02)00059-7.

Smith, C. J., Dong, L. F., Wilson, J., Stott, A., Osborn, A. M., and Nedwell, D. B. (2015). Seasonal variation in denitrification and dissimilatory nitrate reduction to ammonia process rates and corresponding key functional genes along an estuarine nitrate gradient. *Front. Microbiol.* 6, 542. doi:10.3389/fmicb.2015.00542.

Smith, C. J., and Osborn, A. M. (2009). Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol. Ecol.* 67, 6–20. doi:10.1111/j.1574-6941.2008.00629.x.

Spataro, F., and Ianniello, A. (2014). Sources of atmospheric nitrous acid: State of the science, current research needs, and future prospects. *J. Air Waste Manage. Assoc.* 64, 1232–1250. doi:10.1080/10962247.2014.952846.

Spiro, S. (2012). Nitrous oxide production and consumption: regulation of gene expression by gas-sensitive transcription factors. *Philos. Trans. R. Soc. B Biol. Sci.* 367, 1213–1225. doi:10.1098/rstb.2011.0309.

Spott, O., Russow, R., and Stange, C. F. (2011). Formation of hybrid N2O and hybrid N2 due to codenitrification: First review of a barely considered process of microbially mediated N-nitrosation. *Soil Biol. Biochem.* 43, 1995–2011. doi:10.1016/j.soilbio.2011.06.014.

Stevens, R. J., and Laughlin, R. J. (1995). Nitrite Transformations during Soil Extraction with Potassium Chloride. *Soil Sci. Soc. Am. J.* 59, 933. doi:10.2136/sssaj1995.03615995005900030044x.

Stevens, R. J., Laughlin, R. J., and Malone, J. P. (1998). Soil pH affects the processes reducing nitrate to nitrous oxide and di-nitrogen. *Soil Biol. Biochem.* 30, 1119–1126.

doi:10.1016/S0038-0717(97)00227-7.

Stevenson, F. J., Harrison, R. M., Wetselaar, R., and Leeper, R. A. (1970). Nitrosation of Soil Organic Matter: III. Nature of Gases Produced by Reaction of Nitrite with Lignins, Humic Substances, and Phenolic Constituents Under Neutral and Slightly Acidic Conditions. *Soil Sci. Soc. Am. J.* 34, 430–435. doi:10.2136/sssaj1970.03615995003400030024x.

Stevenson, F. J., and Swaby, R. J. (1964). Nitrosation of Soil Organic Matter: I. Nature of Gases Evolved During Nitrous Acid Treatment of Lignins and Humic Substances1. *Soil Sci. Soc. Am. J.* 28, 773–778. doi:10.2136/sssaj1964.03615995002800060027x.

Stieglmeier, M., Mooshammer, M., Kitzler, B., Wanek, W., Zechmeister-Boltenstern, S., Richter, A., et al. (2014). Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *ISME J.* 8, 1135–1146. doi:10.1038/ismej.2013.220.

Stremińska, M. A., Felgate, H., Rowley, G., Richardson, D. J., and Baggs, E. M. (2012). Nitrous oxide production in soil isolates of nitrate-ammonifying bacteria. *Environ. Microbiol. Rep.* 4, 66–71. doi:10.1111/j.1758-2229.2011.00302.x.

Strohm, T. O., Griffin, B., Zumft, W. G., and Schink, B. (2007). Growth Yields in Bacterial Denitrification and Nitrate Ammonification. *Appl. Environ. Microbiol.* 73, 1420–1424. doi:10.1128/aem.02508-06.

Su, H., Cheng, Y., Oswald, R., Behrendt, T., Trebs, I., Meixner, F. X., et al. (2011). Soil nitrite as a source of atmospheric HONO and OH radicals. *Science* 333, 1616–8. doi:10.1126/science.1207687.

Suharti, Hendrik A. Heering, and, and Vries*, S. de (2004). NO Reductase from Bacillus azotoformans Is a Bifunctional Enzyme Accepting Electrons from Menaquinol and a Specific Endogenous Membrane-Bound Cytochrome c551†. doi:10.1021/BI0488101.

Sutlovic, D., Gamulin, S., Definis-Gojanovic, M., Gugic, D., and Andjelinovic, S. (2008). Interaction of humic acids with human DNA: proposed mechanisms and kinetics. *Electrophoresis* 29, 1467–72. doi:10.1002/elps.200700699.

Tan, S. C., and Yiap, B. C. (2009). DNA, RNA, and protein extraction: the past and the present. *J. Biomed. Biotechnol.* 2009, 574398. doi:10.1155/2009/574398.

Taxis, T. M., Wolff, S., Gregg, S. J., Minton, N. O., Zhang, C., Dai, J., et al. (2015). The players may change but the game remains: network analyses of ruminal microbiomes suggest taxonomic differences mask functional similarity. *Nucleic Acids Res.* 43, gkv973. doi:10.1093/nar/gkv973.

Tebbe, C. C., and Vahjen, W. (1993). Interference of Humic Acids and DNA Extracted Directly fromSoil in Detection and Transformation of RecombinantDNA from Bacteria and a Yeast. *Appl. Environ. Microbiol.* 59, 2657–2665. Available at: http://aem.asm.org/content/59/8/2657.abstract [Accessed November 6, 2014].

Thomson, A. J., Giannopoulos, G., Pretty, J., Baggs, E. M., and Richardson, D. J. (2012). Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 367, 1157–68. doi:10.1098/rstb.2011.0415.

Thorn, K. A., and Mikita, M. A. (2000). Nitrite fixation by humic substances: Nitrogen-15 nuclear magnetic resonance evidence for potential intermediates in chemodenitrification. *Soil Sci. Soc.*

Am. J. 64, 568–582. Available at: https://pubs.er.usgs.gov/publication/70022880 [Accessed February 22, 2017].

Throbäck, I. N., Enwall, K., Jarvis, Å., and Hallin, S. (2004). Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol. Ecol.* 49, 401–417. doi:10.1016/j.femsec.2004.04.011.

Töwe, S., Kleineidam, K., and Schloter, M. (2010). Differences in amplification efficiency of standard curves in quantitative real-time PCR assays and consequences for gene quantification in environmental samples. *J. Microbiol. Methods* 82, 338–341. doi:http://dx.doi.org/10.1016/j.mimet.2010.07.005.

Trevors, J. T. (1996). Sterilization and inhibition of microbial activity in soil. *J. Microbiol. Methods* 26, 53–59. doi:10.1016/0167-7012(96)00843-3.

Tveit, A., Schwacke, R., Svenning, M. M., and Urich, T. (2013). Organic carbon transformations in high-Arctic peat soils: key functions and microorganisms. *ISME J.* 7, 299–311. doi:10.1038/ismej.2012.99.

Tveit, A. T., Urich, T., Frenzel, P., and Svenning, M. M. (2015). Metabolic and trophic interactions modulate methane production by Arctic peat microbiota in response to warming. *Proc. Natl. Acad. Sci.* 112, E2507–E2516. doi:10.1073/pnas.1420797112.

Twin, J., Bradshaw, C. S., Garland, S. M., Fairley, C. K., Fethers, K., and Tabrizi, S. N. (2013). The Potential of Metatranscriptomics for Identifying Screening Targets for Bacterial Vaginosis. *PLoS One* 8. doi:10.1371/journal.pone.0076892.

Větrovský, T., and Baldrian, P. (2013). The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLoS One* 8, e57923. doi:10.1371/journal.pone.0057923.

Vishnivetskaya, T. A., Layton, A. C., Lau, M. C. Y., Chauhan, A., Cheng, K. R., Meyers, A. J., et al. (2014). Commercial DNA extraction kits impact observed microbial community composition in permafrost samples. *FEMS Microbiol. Ecol.* 87, 217–30. doi:10.1111/1574-6941.12219.

Vollack, K. U., and Zumft, W. G. (2001). Nitric oxide signaling and transcriptional control of denitrification genes in Pseudomonas stutzeri. *J. Bacteriol.* 183, 2516–26. doi:10.1128/JB.183.8.2516-2526.2001.

Wang, H., and Gunsalus, R. P. (2000). The nrfA and nirB nitrite reductase operons in Escherichia coli are expressed differently in response to nitrate than to nitrite. *J. Bacteriol.* 182, 5813–22. doi:10.1128/JB.182.20.5813-5822.2000.

Wang, H., Tseng, C. P., and Gunsalus, R. P. (1999). The napF and narG nitrate reductase operons in Escherichia coli are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite. *J. Bacteriol.* 181, 5303–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10464201 [Accessed March 15, 2017].

Wang, L., Tian, J., and Li, Y. (2015). Nitrite accumulation and nitrous oxide emission during denitrification processes with quinoline or indole as the sole carbon source. *J. Chem. Technol. Biotechnol.* 90, 1317–1328. doi:10.1002/jctb.4439.

Wang, S., Radny, D., Huang, S., Zhuang, L., Zhao, S., Berg, M., et al. (2017). Nitrogen loss by

anaerobic ammonium oxidation in unconfined aquifer soils. *Sci. Rep.* 7, 40173. doi:10.1038/srep40173.

White, R. A., Bottos, E. M., Roy Chowdhury, T., Zucker, J. D., Brislawn, C. J., Nicora, C. D., et al. (2016). Moleculo Long-Read Sequencing Facilitates Assembly and Genomic Binning from Complex Soil Metagenomes. *mSystems* 1. doi:10.1128/mSystems.00045-16.

Wijler, J., and Delwiche, C. C. (1954). Investigations on the denitrifying process in soil. *Plant Soil* 5, 155–169. doi:10.1007/BF01343848.

Wood, D. E., and Salzberg, S. L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* 15, R46. doi:10.1186/gb-2014-15-3-r46.

Wrage, N., Velthof, G. ., van Beusichem, M. ., and Oenema, O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol. Biochem.* 33, 1723–1732. doi:10.1016/S0038-0717(01)00096-7.

Yu, Y., Zhang, J., Chen, W., Zhong, W., Zhu, T., and Cai, Z. (2014). Effect of land use on the denitrification, abundance of denitrifiers, and total nitrogen gas production in the subtropical region of China. *Biol. Fertil. Soils* 50, 105–113. doi:10.1007/s00374-013-0839-x.

Zhu, X., Burger, M., Doane, T. A., and Horwath, W. R. (2013). Ammonia oxidation pathways and nitrifier denitrification are significant sources of N2O and NO under low oxygen availability. *Proc. Natl. Acad. Sci. U. S. A.* 110, 6328–33. doi:10.1073/pnas.1219993110.

Zipper, H., Buta, C., Lämmle, K., Brunner, H., Bernhagen, J., and Vitzthum, F. (2003). Mechanisms underlying the impact of humic acids on DNA quantification by SYBR Green I and consequences for the analysis of soils and aquatic sediments. *Nucleic Acids Res.* 31, e39. doi:10.1093/nar/gng039.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* 61, 533–616. Available at: http://mmbr.asm.org/content/61/4/533.abstract.

Zumft, W. G. (2005). Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme–copper oxidase type. *J. Inorg. Biochem.* 99, 194–215. doi:http://dx.doi.org/10.1016/j.jinorgbio.2004.09.024.

Paper I

Transparent DNA/RNA co-extraction workflow protocol suitable for inhibitor-rich environmental samples that focuses on complete DNA removal for transcriptomic analyses.

Natalie YN Lim, Constance A Roco, and Åsa Frostegård

Frontiers in Microbiology (2016), 7:1588.

This page intentionally left blank

frontiers in Microbiology

METHODS
published: 18 October 2016
doi: 10.3389/fmicb.2016.01588

Transparent DNA/RNA Co-extraction Workflow Protocol Suitable for Inhibitor-Rich Environmental Samples That Focuses on Complete DNA Removal for Transcriptomic Analyses

Natalie Y. N. Lim[1], Constance A. Roco[1,2] and Åsa Frostegård[1]*

[1] *Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Aas, Norway,*
[2] *Department of Microbiology, Cornell University, Ithaca, NY, USA*

OPEN ACCESS

Edited by:
Graeme W. Nicol,
Université de Lyon, France

Reviewed by:
Hinsby Cadillo-Quiroz,
Arizona State University, USA
Kuk-Jeong Chin,
Georgia State University, USA

****Correspondence:***
Natalie Y. N. Lim
natalie.lim@nmbu.no

Specialty section:
This article was submitted to Terrestrial Microbiology, a section of the journal Frontiers in Microbiology

Received: *10 June 2016*
Accepted: *22 September 2016*
Published: *18 October 2016*

Citation:
Lim NYN, Roco CA and Frostegård Å (2016) Transparent DNA/RNA Co-extraction Workflow Protocol Suitable for Inhibitor-Rich Environmental Samples That Focuses on Complete DNA Removal for Transcriptomic Analyses. Front. Microbiol. 7:1588. doi: 10.3389/fmicb.2016.01588

Adequate comparisons of DNA and cDNA libraries from complex environments require methods for co-extraction of DNA and RNA due to the inherent heterogeneity of such samples, or risk bias caused by variations in lysis and extraction efficiencies. Still, there are few methods and kits allowing simultaneous extraction of DNA and RNA from the same sample, and the existing ones generally require optimization. The proprietary nature of kit components, however, makes modifications of individual steps in the manufacturer's recommended procedure difficult. Surprisingly, enzymatic treatments are often performed before purification procedures are complete, which we have identified here as a major problem when seeking efficient genomic DNA removal from RNA extracts. Here, we tested several DNA/RNA co-extraction commercial kits on inhibitor-rich soils, and compared them to a commonly used phenol-chloroform co-extraction method. Since none of the kits/methods co-extracted high-quality nucleic acid material, we optimized the extraction workflow by introducing small but important improvements. In particular, we illustrate the need for extensive purification prior to all enzymatic procedures, with special focus on the DNase digestion step in RNA extraction. These adjustments led to the removal of enzymatic inhibition in RNA extracts and made it possible to reduce genomic DNA to below detectable levels as determined by quantitative PCR. Notably, we confirmed that DNase digestion may not be uniform in replicate extraction reactions, thus the analysis of "representative samples" is insufficient. The modular nature of our workflow protocol allows optimization of individual steps. It also increases focus on additional purification procedures prior to enzymatic processes, in particular DNases, yielding genomic DNA-free RNA extracts suitable for metatranscriptomic analysis.

Keywords: RNA extraction, purification, genomic DNA removal, enzyme inhibition, environmental sample

INTRODUCTION

With the advent of the meta-omics era, it has become increasingly commonplace to aim for metagenomic/metatranscriptomic analyses of environmental samples. Despite advances in the sequencing front, upstream methods required to obtain the high quality DNA and RNA needed for these analyses have fallen behind and there is often a need to optimize existing methods when applying them to a new sample type. The choice of extraction method affects the ensuing purity and yield of nucleic acid material, which in turn affects subsequent downstream processes. This calls for rapid and simple extraction and/or purification methods that yield high quality and quantities of nucleic acids. However, this is but a pipe dream in many cases, due to the presence of "inhibitory compounds." These well-known, yet poorly understood compounds are ubiquitous to most environments. They are abundant in most soils and are often classified under the blanket term of "humic and fulvic compounds, and/or polyphenolic compounds" (Tebbe and Vahjen, 1993; Krsek and Wellington, 1999; Hirsch et al., 2010; Mettel et al., 2010), yet there is little certainty that this is an accurate enough description of all enzyme-influencing compounds present in soil. Additionally, although it is known that inhibitors affect many DNA-transforming processes including hybridization, quantification and amplification (Tebbe and Vahjen, 1993; Bachoon et al., 2001; Zipper et al., 2003; Wang et al., 2012), many studies focus primarily on their effect on DNA polymerases (Abu Al-Soud and Rådström, 1998; Kermekchiev et al., 2009; Baar et al., 2011), disregarding the effect these same inhibitors may have on other enzymes performing other processes. Another complicating factor is that enzymes show various degrees of resistance to different inhibitors (Tebbe and Vahjen, 1993; Abu Al-Soud and Rådström, 1998; Baar et al., 2011). Thus, along with the development of new and efficient enzymes, there is a strong need for improved purification strategies.

Presently available methods can be divided into two: those that co-extract both DNA and RNA from single reactions, and those that extract DNA and RNA from separate reactions. While extracting nucleic acids separately is markedly simpler, with a wider variety of highly optimized kits and methods available, single reaction DNA/RNA co-extractions offer the benefit of more comparable data, especially from highly heterogeneous samples such as soils. This has spawned a multitude of novel methods and kits from independent researchers (Purdy et al., 1996; Griffiths et al., 2000; Peršoh et al., 2008; Mettel et al., 2010; Lever et al., 2015) and large multinational companies alike, as well as many comparisons of such methods and kits (Krsek and Wellington, 1999; LaMontagne et al., 2002; Dineen et al., 2010; Mahmoudi et al., 2011; Vishnivetskaya et al., 2014). Despite extensive testing of both kit and non-kit based methods, no single method has been found to work for all environment types (Frostegård et al., 1999; Krsek and Wellington, 1999; LaMontagne et al., 2002; Vishnivetskaya et al., 2014), and the "best" method is often difficult to determine, where one kit or reagent may provide, for example, better replication or quantity, but at the detriment of quality (Krsek and Wellington, 1999; Mahmoudi et al., 2011; Cruaud et al., 2014; Vishnivetskaya et al., 2014). Furthermore, there are fewer studies based on metatranscriptomics compared to metagenomics, resulting in a disproportionate focus on DNA-based methods over RNA ones.

Metatranscriptomic analyses require high quality RNA that is free of inhibitors and genomic DNA (gDNA). The presence of inhibitors greatly affects RNA high throughput sequencing due to the relatively large quantities of RNA required. Unlike DNA-based analyses, where "diluting out the inhibitor effect" is always an option, metatranscriptomic analyses often require concentrating samples in order to achieve sufficient material for the sequencing process, thus further exacerbating the inhibitory effect. Even if we ignored any effect the inhibitory compounds may have on the RNA extraction and DNA removal process, this need to concentrate samples makes inhibitor removal an extremely important step in RNA analysis. Thus, there is a consistent necessity to optimize existing methods and/or kits to suit one's needs. Although commercial kits have the potential to yield high quality nucleic acids, the proprietary nature of kit components make it difficult for optimization or up-scaling. Such changes to the extraction procedure or increased sample volumes may be necessary for samples with low biomass and/or activity, containing little mRNA, when metatranscriptomic analysis is sought after.

The present study aimed to identify and overcome key problematic steps during the co-extraction of high quality DNA and RNA from inhibitor-rich soil samples for the purposes of meta-omic analyses. The efficacy of commercially available nucleic acid extraction kits were tested, and the nucleic acid extracts' yield and purity were compared to the extracts obtained using the method by Nicolaisen et al. (2008) that was used in a previously published paper investigating the same soils (Liu et al., 2010). Finding little benefit in using the extraction kits, we took lessons learnt from a different modular extraction method (Lever et al., 2015), and further optimized Nicolaisen et al. (2008) method in an iterative manner, starting with the types of beads used for cell lysis and the nucleic acid precipitant. Different purification kits were also compared by examining the efficiencies of nucleic acid targeting enzymes (polymerases, DNases and reverse transcriptases) used on crude total nucleic acids (TNA) extracted by the aforementioned optimized method. Special attention was paid to the removal of gDNA from RNA samples. This step is often incorrectly assessed, despite being a potential source of major bias in downstream mRNA analyses. The proposed protocol, which is an optimization of existing phenol-chloroform based procedures, with additional purification at critical points, proved to yield nucleic acids suitable for metagenomic and metatranscriptomic analyses when tested on soils with high levels of inhibitors. The new method and workflow are transparent, which allows optimizations (as necessary) at various steps in the procedure.

MATERIALS AND METHODS

Soils

Three agricultural soils, chosen because of their extraction difficulty with commercial kits and non-kit methods (Liu et al., 2010), were used to determine the quality of DNA and RNA from co-extraction reactions. Soils FL (pH 3.65) and FH (pH 7.39) are high organic content peat soils (40–45% soil organic C, 2% organic N) (Liu et al., 2010) from a long-term field experimental site in Fjaler in western Norway (61°17′42″, 5°03′03″). FL is the original un-limed soil, and FH was limed in 1978 with 800 m^3 of shell sand per hectare of soil (Sognnes et al., 2006). Soil Å (pH 5.5) is a high clay-content soil (39% sand, 40% silt, 21% clay, 3% soil organic C, 0.22% organic N) from a grassland site in Ås in southeast Norway (59°39′44″, 10°45′50″). All soils were immediately transported to the laboratory, sieved (4.5 mm) upon arrival, then stored in sealed plastic bags at 4°C. All pH values were measured in 0.01 M $CaCl_2$ (1:5 (ww to volume) soil to $CaCl_2$ solution) immediately prior to using the soil. Soils FH and FL were used in the testing of all kits and methods, and soil Å was only used as a comparison for kits/methods that showed at least some success with the other two soils.

Soil Treatment

In the present study we targeted denitrification gene transcripts to evaluate methods for DNA/mRNA isolation. Several successive experiments were performed where different extraction kits/methods were tested (see below). Using field-fresh soil for each of these would introduce undesired variation, due to seasonal differences in the soil. Instead, to achieve the best possible comparison of extraction methods, all soils used in this study were sampled at the same time and kept at 4°C until use (2–6 months after arrival).

A small amount of a natural C source was added, to standardize the conditions and to secure that the organisms would have enough energy to induce transcription of the targeted denitrification genes (Liu et al., 2010). Soils FH and FL were revitalized from cold storage by addition of 5 mg dried, powdered clover g^{-1} soil wet weight (ww), amended with 8–11 mM nitrate (in soil moisture), then incubated at 15°C for 72 h. Soil Å was used in a separate experiment (C. A. Roco, unpublished data) and was exposed to different lengths of oxic and anoxic periods over 4 weeks in glass vials incubated at 15°C. During this incubation, clover (1 mg g^{-1} soil, dry weight (dw)) and nitrate (0.065–0.65 μmol g^{-1} soil, dw) was added every 2–5 days (for a total of 11 times) to maintain microbial activity.

At the end of the 72 h (FH and FL) or 4 weeks (Å) incubation, the soils were transferred to air-tight glass vials and sealed with butyl-rubber septa and aluminum crimps, then made anoxic by six cycles of gas evacuation and helium filling (Liu et al., 2010). These vials were incubated anoxically to stimulate the production of denitrification gene transcripts. Gases (CO_2, O_2, NO, N_2O, and N_2) produced in the headspace were measured every 3 h with a GC and NO analyzer (Molstad et al., 2007), and used to guide soil sampling for denitrification genes – reduction of N_2O gas to N_2 gas was taken as an indicator for nitrous oxide reductase gene (*nosZ*) transcription. For each sample, one vial was opened and the soil within was snap frozen in liquid nitrogen then stored at −80°C until nucleic acid extraction.

Kit and Non-kit Nucleic Acid Extraction

Figure 1 presents a schematic diagram of the different key steps examined to obtain an optimized protocol for co-extraction of DNA and RNA from soil. Our criteria for the successful application of a kit or method was the ability to obtain high quality DNA and RNA (both rRNA and mRNA) from our samples. Quality was assessed as follows: (1) DNA extracts should be amplifiable with little or no inhibition, as judged by successful PCR amplification and comparable qPCR efficiency to plasmid standards; and (2) RNA extracts must be free of gDNA (as determined by qPCR, see below), and should yield positive results when reverse transcribed and assessed with qPCR. Three DNA- and three TNA extraction kits were tested for their ability to extract nucleic acids that are suitable for downstream processes, according to manufacturer's instructions (**Table 1**). In the present paper, the RNA PowerSoil kits are considered one kit because the DNA Accessory Kit (AK) cannot be used separately. Where applicable, lysis was achieved by bead-beating as described below.

The PowerLyzer DNA (PL), FastDNA SPIN (FDS), and ZR Soil (SM) kits were used as benchmark DNA extractions because of their previous success in our laboratory with soil FH and in the literature in extracting DNA from soil and other environmental

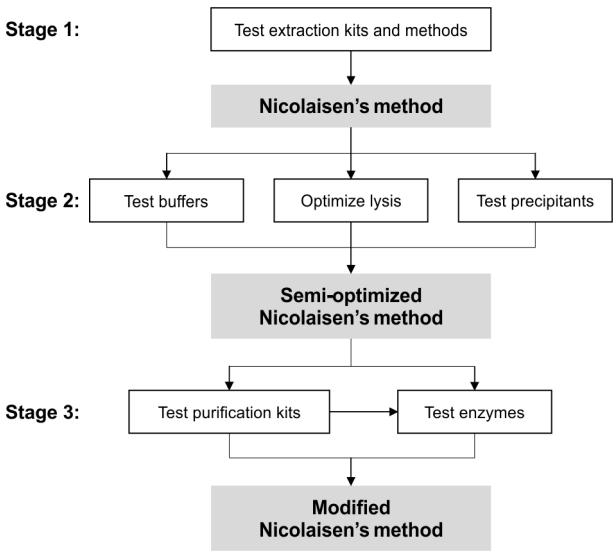


FIGURE 1 | Schematic diagram of the optimization process. In Stage 1 of the process, various extraction kits and Nicolaisen's method (as listed in **Table 1**) was tested on soils FH and FL (see text for soil descriptions). In Stage 2, various extraction buffers, lysis conditions, and nucleic acid precipitants were tested using Nicolaisen's method as the base, creating a new "semi-optimized Nicolaisen's method." In the final Stage 3, DNases/reverse transcriptases and purification kits were tested concurrently for their ability to completely remove genomic DNA, and was briefly tested in combination. The end result is the "Modified Nicolaisen's method," which is based on the workflow as outlined in **Figure 2**.

TABLE 1 | List of extraction and purification kits tested in this study[a].

Use	Target	Kit name	Abbreviation	Company
Extraction	DNA	PowerLyzer PowerSoil DNA Isolation Kit	PL	MO BIO Laboratories
Extraction	DNA	FastDNA SPIN Kit for Soil	FDS	MP Biomedicals
Extraction	DNA	ZR Soil Microbe DNA MiniPrep	SM	Zymo Research
Extraction	DNA/RNA	MasterPure RNA Purification Kit[b]	MP	Epicentre Biotechnologies
Extraction	DNA/RNA	PowerMicrobiome RNA Isolation Kit	PM	MO BIO Laboratories
Extraction	RNA	RNA PowerSoil Total RNA Isolation Kit	PS	MO BIO Laboratories
Extraction	DNA	RNA PowerSoil DNA Elution Accessory Kit (used in conjunction with the above RNA kit)	AK	MO BIO Laboratories
Purification	DNA	E.Z.N.A. Cycle Pure Kit	CP	Omega Bio-Tek
Purification	DNA	MinElute Reaction Cleanup Kit	MRC	QIAGEN
Purification	DNA	Genomic DNA Clean & Concentrator	gDCC	Zymo Research
Purification	RNA	RNeasy Mini Kit	RM	QIAGEN
Purification	RNA	RNA Clean & Concentrator – 5	RCC	Zymo Research
Purification	DNA/RNA	*OneStep* PCR Inhibitor Removal Kit	OPIR	Zymo Research

[a] *The purification kits were tested in combination with the modified method described in this paper.*
[b] *The lysate was obtained using the phenol-chloroform extraction as detailed previously (Nicolaisen et al., 2008).*

samples (Mahmoudi et al., 2011; Vishnivetskaya et al., 2014; Wesolowska-Andersen et al., 2014; Young et al., 2014). The rest of the kits were selected according to the manufacturer's claim that they are able to co-extract DNA and RNA fractions from the same soil sample. The kits were compared to the phenol-chloroform extraction method as modified by Nicolaisen et al. (2008), referred to here as the Nicolaisen's method, which is based on the extraction procedure by Griffiths et al. (2000).

The lysis step of Nicolaisen's method was optimized by testing different lysis options (FastPrep-24 Instrument vs. vortex), lysis beads type (garnet vs. glass), one size (garnet: 0.15 mm; glass: 0.10-0.11 mm) vs. multiple bead sizes (garnet beads: 0.15 and 0.7 mm; glass beads: 0.10–0.11, 1.0, and 2.5–3.5 mm), and the number of cycles of lysis (once, twice, or thrice). Different buffers for the lysis of bacteria were also tested: CTAB (hexadecyltrimethylammonium bromide) buffer (pH 5.7 and 8.0, and 120 mM or 250 mM ionic strength) with 1% (w/v) polyvinylpolypyrrolidone (PVPP); GES (guanidinium thiocyanate-EDTA-sarcosyl) buffer (pH 4); and phenol (pH 4 or 8) (Supplementary Table S1). Additionally, we tested the effectiveness of 30% polyethylene glycol (PEG) 6000 (following Nicolaisen's method) and isopropanol as nucleic acid precipitants. The results are described in Supplementary Material, pp. 1–2 and Supplementary Figures S1–S5.

Purification Kits

In the following, the term "primary" when used to describe nucleic acids refers to the resuspended or eluted nucleic acids obtained from the extraction procedure or kit, and is equivalent to "Extract I" in **Figure 2**. In addition to the purification steps already included in the above extraction methods and kits to obtain the primary extract, purification kits (listed in **Table 1**) were tested in various combinations on the primary extracts: MinElute Reaction Cleanup Kit (MRC), RNeasy Mini Kit (RM) (both from QIAGEN), E.Z.N.A. Cycle Pure Kit (CP) (Omega Bio-Tek), Genomic DNA Clean & Concentrator (gDCC), RNA Clean & Concentrator-5 (RCC) and *OneStep* PCR Inhibitor Removal Kit (OPIR) (all from Zymo Research).

DNase Digestion of Total RNA

Based on our previous experience (Liu et al., 2010), residual gDNA is often leftover after DNase treatment of RNA fractions, making this step a major bottleneck, especially for inhibitor-rich soil samples. The following DNases were tested for their ability to remove amplifiable DNA from TNA samples: DNase I (Sigma), RNase-Free DNase Set (QIAGEN), RNase-Free DNase I (Epicentre Biotechnologies) and TURBO DNA-*free* DNase Kit (Ambion, Life Technologies). All DNases were used according to manufacturers' instructions, with the exception of incubation time, which we varied from 15 min to 2 h. The efficiency of each DNase treatment was determined by comparing the purified DNA fractions (Extract III in **Figure 2**) with the non-reverse transcribed RNA (Extract V in **Figure 2**), via quantitative PCR (qPCR) amplification of the 16S rRNA or the *nosZ* genes (details below).

Reverse Transcriptases

Several reverse transcriptases were compared using RNA extracts obtained from soils FL and FH during the iterative method optimization. The purpose was to ensure successful cDNA synthesis in extraction replicates from inhibitor-rich soils. Because trials with RNA extracts from Nicolaisen's method and the extraction kits were not able to yield cDNA (see Comparison of Methods for Nucleic Acid Extraction, Supplementary Data section "The Effectiveness of Dedicated Nucleic Acid Extraction Kits," and an earlier study Liu et al., 2010), the assessment focused on the presence (but not quantity) of detectable *nosZ* cDNA in the absence of gDNA. Reverse transcriptase efficiency was not assessed in this study. The following reverse transcriptases were tested according to manufacturers' instructions: High Capacity RNA-to-cDNA Master Mix (Applied Biosystems), SuperScript VILO MasterMix (Invitrogen), PrimeScript RT Reagent Kit (Takara Bio), and

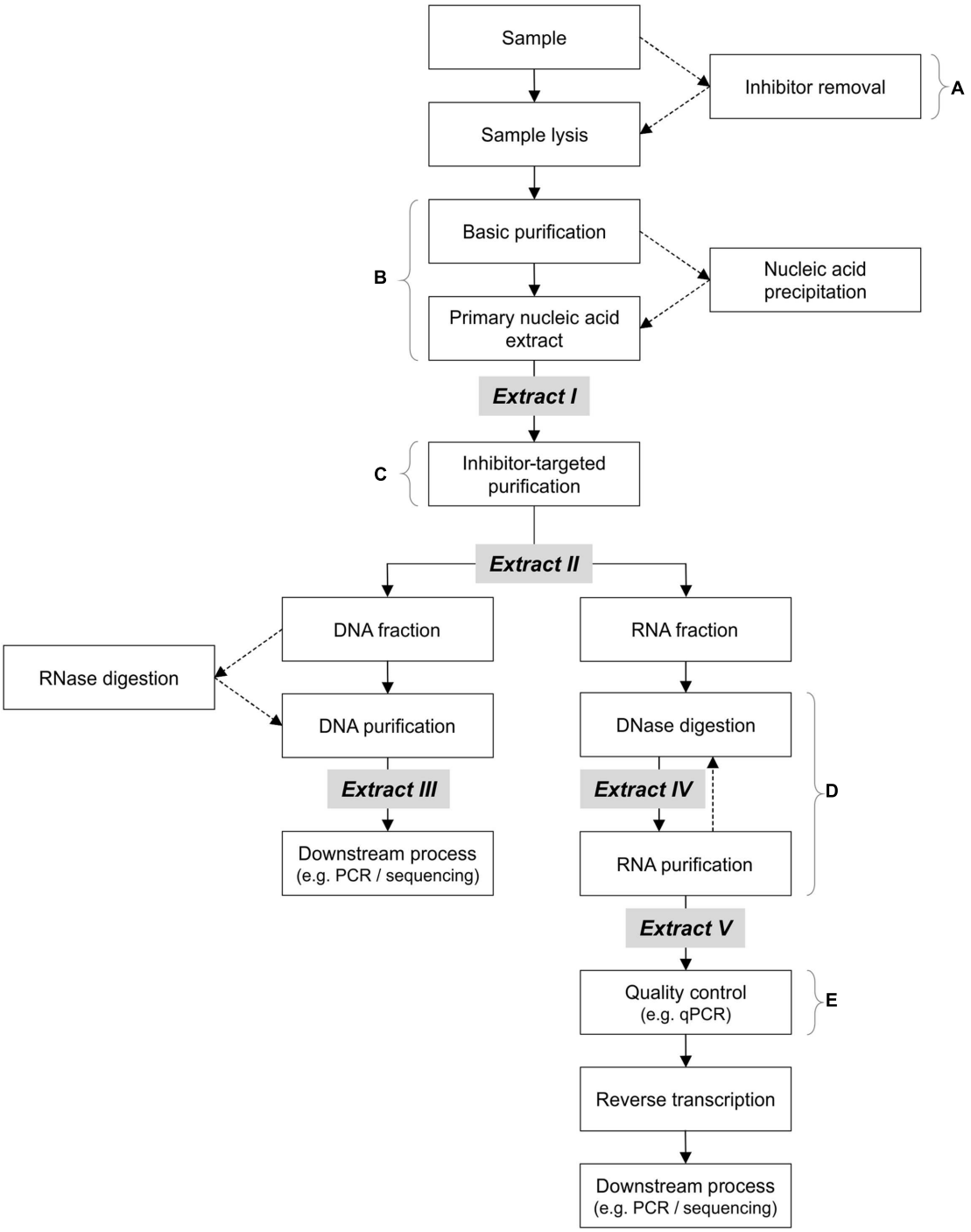


FIGURE 2 | Suggested DNA/RNA co-extraction workflow for environmental samples, with stronger emphasis on thorough purification prior to all enzymatic steps (including DNase digestion). Optional steps are indicated by dotted arrows. Note that RNase digestion (between Extracts II and III) may be necessary for better results downstream, but may be omitted as a separate step (in the current study, RNase is present in the qPCR mix). **(A)** Pre-lysis inhibitor removal is only advisable if quick methods are used, or if mRNA is not the target molecule (lengthy inhibitor removal procedures compromise RNA integrity). **(B)** Various methods may be used, such as phenol/chloroform procedures or nucleic acid precipitation. **(C)** This purification step should target the removal of enzymatic-inhibitors (e.g., humic/fulvic acids and polyphenolics). **(D)** Purification of partially digested RNA extracts with residual genomic DNA aids in the removal of enduring inhibitors, prior to further digestion. **(E)** Stringent and well-documented quality control via rigorous and sensitive detection (preferably quantitative methods) is necessary to detect residual amplifiable gDNA **prior** to reverse transcription.

Maxima Reverse Transcriptase (Thermo Scientific). Random hexamer primers and dNTPs (provided by the respective manufacturers, either bought separately or provided in the kit) were used with all reverse transcriptases. To improve the rate of successful *nosZ* transcript reverse transcription (present in low quantities in the samples compared to 16S rRNA), the maximum volume of RNA template (8–10 μL, corresponding to 150–200 ng RNA) was used in each reaction. Due to the comparatively low quantities of RNA in the extracts (compared to pure culture RNA extractions), the quantity of RNA in these volumes never exceeded the manufacturers' recommended maximum quantity of RNA template (ranging from 500 ng to 5 μg total RNA). Additionally, the differing template quantities/volumes used in this study did not affect the failure or success of cDNA synthesis, as determined by the absence or presence of amplifiable *nosZ* cDNA (see Test of DNases and Reverse Transcriptases).

Optimized Non-kit Extraction Method That Mitigates Inhibitor Effect

Based on the results from the above tests (as described in Supplementary Material, pp. 1–2 and Supplementary Figures S1–S5), some additions and modifications were made based on several widely used phenol-chloroform extraction methods, including Nicolaisen's method (Griffiths et al., 2000; Nicolaisen et al., 2008; Mettel et al., 2010). **Figure 2** depicts our suggested workflow protocol, and is the basis for our method. Briefly describing the method, 0.2–0.25 g of soil was lysed by bead-beating in 2 mL screw-capped microcentrifuge tubes containing glass beads, CTAB extraction buffer (with 1% w/v PVPP), and phenol-chloroform-isoamyl alcohol (25:24:1), and the nucleic acids were washed with ethanol then precipitated. The following are the differences to Nicolaisen's method: (i) Three sizes of glass beads were used for lysis (0.10–0.11, 1.0, and 2.5–3.5 mm); (ii) the samples were lysed in a FastPrep-24 Instrument by two cycles at 6.0 m s^{-1} for 45 s, with intermittent cooling between each cycle to prevent overheating of the samples and instrument; (iii) after removing residual phenol with chloroform, up to 500 μL of the aqueous phase was transferred; (iv) the nucleic acids (both DNA and RNA) were precipitated with 0.2 volumes of 3 M sodium acetate (buffered to pH 5.2 with glacial acetic acid) and an equal volume of isopropanol, then continuously inverted for 2 min at room temperature; and (v) the ethanol-washed TNA pellet was dried in a SpeedVac Concentrator then resuspended in DEPC-treated nuclease-free water.

After this primary extraction, and before any further enzymatic downstream treatment, the resuspended TNA (Extract I in **Figure 2**) was purified with the OPIR kit, according to manufacturer's instructions. Extract II (**Figure 2**) was then divided in two fractions, one for DNA and one for RNA. To ensure maximum removal of inhibitory compounds, the DNA fraction was further purified with the gDCC kit. For the RNA fraction, gDNA was removed with the TURBO DNase kit, before purification with the RCC kit. If residual gDNA was detected in the eluate (via qPCR using primers targeting the 16S rRNA or *nosZ* genes), a second round of DNase digestion and purification with the RCC kit was performed (but without the OPIR kit prior to digestion). Additional use of OPIR prior to the second digestion did not improve RNA purity, but instead resulted in the loss of material (data not shown). The qPCR-certified gDNA-free RNA was then reverse transcribed to cDNA with random hexamers using the Maxima Reverse Transcriptase, both according to manufacturer's instructions. All resulting nucleic acids (DNA, non-reverse transcribed RNA, and cDNA) were quantified after extraction and/or purification (see below), then stored at −80°C until use. This procedure of 'purification before enzymatic processes' was also used on primary extracts from the most effective extraction kit, RNA Powersoil kit (PS), to ensure high quality RNA for sequencing (see Results section).

Analysis of Nucleic Acid Quality and Quantity

Extracts II, III, IV, and V (the primary TNA, purified DNA, the DNase-treated RNA, and purified RNA fractions, respectively; see **Figure 2**) were quantified by spectrofluorometry using the Qubit dsDNA BR Assay Kit and Qubit RNA BR Assay Kit (Qubit Fluorometer, Invitrogen, Life Technologies). Spectrophotometric analysis (NanoDrop Spectrophotometer, NanoDrop Technologies, Thermo Fisher Scientific) was used for preliminary evaluation of nucleic acid quality, via the assessment of the absorbance ratios $A_{260/230}$ and $A_{260/280}$. As is common practice, $A_{260/230}$ absorbance ratios nearing 2.0 were regarded as contaminated with humic substances, whereas ratios below 1.5 were regarded as failure to extract nucleic acids (Cullen and Hirsch, 1998; Krsek and Wellington, 1999; LaMontagne et al., 2002; Peršoh et al., 2008; Mahmoudi et al., 2011). However, due to the high quantities of humic compounds present in soils FL and FH, we only regarded it as failed nucleic acid extraction if the ratio remained under 1.5 after additional clean-up with dedicated purification kits. Protein contamination was indicated by the $A_{260/280}$ ratio, where samples with ratios between 1.7 and 2.0 were considered usable, while purified extraction reactions with ratios < 1.7 were discarded. Estimation of humic content by color (Dineen et al., 2010) was not used in this study, since low amounts of humic substances may be undetectable visually (Bachoon et al., 2001). Additionally, where applicable, gel visualization was used to quickly assess the extent of DNA shearing and/or the presence of rRNA (note that rRNA presence/absence was always further confirmed by PCR/qPCR following reverse transcription). For reasons of simplicity, in this paper the term "usable nucleic acids" refers to nucleic acids of sufficient enough quality to be used in further experiments, i.e., downstream processes such as qPCR were not inhibited or inversely affected by co-extracted inhibitory compounds.

Verification of Inhibitor and gDNA Absence

To confirm amplifiability of extracted DNA and synthesized cDNA, and the complete digestion of gDNA in RNA samples, the presence of the 16S rRNA, *narG* and *nosZ* genes were assessed via PCR and qPCR. For both PCR and qPCR, DNA samples were diluted to between 1:10 and 1:50 of the original extract, which translated to 1–10 ng of DNA per reaction. All cDNA and RNA

samples (DNase-digested) were used without dilution. For PCR, each 25 μL amplification reaction contained 1 μL of template, 0.4 μM of each primer, 0.125 U of *TaKaRa* Taq (Takara Bio), 400 μM of each dNTP and 2.5 μL of 10X PCR Buffer. The primers used were: 27F and 518R for the 16S rRNA gene (Weisburg et al., 1991; Muyzer et al., 1993), 1960f and 2650r for the *narG* gene (Philippot et al., 2002), and Z-F and 1622R for the *nosZ* gene (Kloos et al., 2001; Throbäck et al., 2004). The optimized thermal cycling conditions were 95°C for 5 min, 30–35 cycles of 95°C for 30 s, x for 45 s, 72°C for 30 s, and a final extension of 72°C for 7 min, where $x = 54$°C (16S rRNA gene), or 60°C (*narG* and *nosZ* gene). For qPCR the StepOnePlus Real-Time PCR System (Applied Biosystems) was used. All samples were amplified in simultaneous reactions to compare the DNase digestion and reverse transcription efficiency. Each 20 μL reaction contained SYBR *Premix Ex Taq* II (Tli RNaseH Plus; Takara Bio) used according to manufacturer's instructions, and included 0.4 μM of each primer and 2 μL of template. The qPCR cycling conditions for all primer sets were the same as above, with the following exceptions: an additional 20 s at 82°C at the end of each cycle to measure the fluorescent signal, thereby reducing background signals from primer dimers and unspecific PCR products; the extension time for the primers targeting the *nosZ* gene was prolonged to 60 s; a final melting curve analysis from 60 to 95°C was performed to determine the specificity of amplicons, in lieu of the final extension step; and the amplification reactions were performed for 40 cycles. The detection limit of each qPCR run was five copies per microliter of reaction, which ranged from 4×10^2 to 4×10^5 copies g^{-1} soil (ww).

The raw qPCR fluorescence data was imported into the LinRegPCR program (Ruijter et al., 2009). Unlike commonly reported efficiencies that are calculated by employing the use of serial diluted standards and the construction of calibration plots, LinRegPCR uses the exponential portion of the fluorescence signal curve of each well to determine individual well efficiencies by calculating the deviation from a perfect "one copy to two copies" amplification after each cycle. Efficiencies calculated with standard curves assume equal amplification efficiencies in all calibration and biological samples, and cannot be used objectively to determine the degree of amplification inhibition in biological samples. To overcome this, qPCR curve analysis methods such as LinRegPCR, as used above, have to be used (Ruijter et al., 2013). This allows for more reliable qPCR efficiency determinations that are independent of potential standard-sample variations, including differences in inhibitor content. Moreover, humic substances have been found to inhibit commonly used double-stranded DNA (dsDNA) binding fluorescence dyes, making it doubly important to check individual sample amplification efficiencies (Sidstedt et al., 2015).

Additional Nucleic Acid Quality Control and Sequencing

Multiple samples of DNA and RNA extracted from all three soils using our revised extraction method, and PS kit-extracted (and further purified as described in the simplified extraction method) soil Å RNA extracts, were sent for metagenomic and metatranscriptomic sequencing at The Roy J. Carver Biotechnology Center (CBC)/W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign, using HiSeq 2500 technology. Prior to shipping on liquid nitrogen vapor (Cryoport), we confirmed that all nucleic acids were of high quality (DNA or gDNA-free RNA as verified by qPCR). Independent verification of the RNA quality, including confirmation of the absence of gDNA, was also performed at the CBC. A sample of the sequenced reads from soil FH and FL were trimmed for adaptors and quality using Trimmomatic (MINLEN: 70, TRAILING: 15) (Bolger et al., 2014). The trimmed sequences were uploaded to MG-RAST and annotated (Meyer et al., 2008). Annotated FH and FL soil sequences are available online on the MG-RAST database (project ID 14446, project name "Fjaler_HiSeq").

RESULTS

Comparison of Methods for Nucleic Acid Extraction

No single dedicated nucleic acid extraction kit was applicable to all soils. The kits that managed to obtain both DNA and RNA (kits MP, PM and PS+AK) are compared to the unmodified Nicolaisen's method in **Table 2**. For a comparison of all kits tested, see Supplementary Table S2 and explanatory text in Supplementary Material, p. 1. As seen, PS was the most successful kit, obtaining gDNA-free RNA in two of the three soils. The PS kit utilizes nucleic acid-specific elution buffers to preferentially elute DNA or RNA from the nucleic acid binding column. However, as per manufacturer's strict instructions, neither centrifugal (positive) nor vacuum (negative) pressure could be applied to the columns (supplied in the kit), and the gravitational drip process took over 4 h (and up to 8 h) per sample to complete for FL and Å soils, due to clogging of the column. Despite the long procedure at room temperature, preliminary trials with the PS kit (without the AK kit) produced promising results, yielding 6.71 ± 1.01 μg RNA g^{-1} soil (ww) and amplifiable cDNA (16S rRNA) in the absence of amplifiable gDNA. The long extraction time required at room temperature may potentially compromise the quality and quantity of extracted mRNA, which puts any absence or low mRNA copy numbers in doubt. The only available option provided by the manufacturer was the application of positive pressure to the top of the column. Unfortunately, the outcome varied between soil types Å and FL: High quality rRNA and mRNA was obtained from soil Å, although a supplementary two rounds of 'purification-digestion-purification' was required (i.e., RNA purification was performed after each digestion). In contrast, for soil FL, positive pressure application co-extracted such large quantities of inhibitory compounds that both the extracted DNA (eluted with the AK kit) and RNA remained brown (suggesting a high content of organic compounds) and was unusable in downstream processes in spite of attempted clean-up with additional purification kits. Moreover, the extracts were not reliably quantifiable prior to further purification (NanoDrop and Qubit readings returned "error" and "out of range" messages, respectively). NanoDrop quality assessments

TABLE 2 | Comparison of DNA and RNA co-extraction methods and kits, tested on soils FH (high pH peat, pH 7.39), FL (low pH peat, pH 3.65), and Å (low pH clay soil, pH 5.5).

Method/Kit	Nicolaisen's method[a]			MP[b]		PM[b]		PS + AK[b]			Optimized method		
TNA purification prior to digest[c]	−			+		−		+			+		
Soils tested	FH	FL	Å	FH	FL	FH	FL	FH	FL	Å	FH	FL	Å
Amplifiable DNA[d]	+	+	+	+	+	+	+	+	±[f]	+	+	+	+
Complete removal of DNA after 1st digestion[d,e]	+	−	−	+	−	+	−	+	−	−	+	+	−
Complete removal of DNA after 2nd digestion[d,e]	+	−	−	+	±[f]	+	−	+	−	+	+	+	+
cDNA synthesis	+	NT	NT	+	±[f]	+	NT	+	NT	+	+	+	+

[a] *Method from Nicolaisen et al. (2008).*
[b] *See **Table 1** for list of kit abbreviations.*
[c] *TNA purification with the OPIR kit.*
[d] *See text for details on DNA amplification and removal assessment.*
[e] *DNA was digested with TURBO DNase, and RCC kit was used for purification after each digestion.*
[f] *Results from replicates varied, likely due to the presence of inhibitory compounds.*
gDNA, genomic DNA; NT, not tested because of residual gDNA.

revealed highly variable $A_{260/280}$ ratio ranges that failed to improve with additional purification: 1.41–1.58 for the DNA eluate and 1.34–1.79 for the RNA eluate (see also Supplementary Table S2). Tellingly, the DNA and reverse transcribed RNA could not be amplified (fluorescence signal did not pass threshold after $\geq$35 cycles in the qPCR using primers targeting the 16S rRNA gene). The PS kit therefore did not provide sufficient quality of nucleic acids from soil FL because of the long extraction time required at room temperature and the inability to speed up the process with positive pressure application.

Purification Kits and Enzymatic Inhibition

In the final stage of optimization (**Figure 1**), various purification kits (listed in **Table 1**) were tested on FH and FL extracts from the best extraction kits (listed in **Table 2**) and our optimized version of Nicolaisen's method (utilizing the most optimally tested buffer and precipitant as stated in the Supplementary Material, pp. 1–2). Regardless of method or kit used for the extraction, the DNA yielded from both FH and FL in Extract I (**Figure 2**) was amplifiable, but the results were variable in consistency and strength (strong and consistent amplification was defined by the presence of equally bright amplicons on agarose gels, see Supplementary Figure S1). Due to the inhibitor-rich nature of the soils tested, we found that nucleic acid purification kits were always necessary to secure high quality, fully uninhibited material for downstream processes such as PCR amplification.

These further purification steps, regardless of the purification kit used, greatly improved the purity of DNA extracts. For example, purification of FL extracts with gDCC improved the $A_{260/280}$ ratio from 1.59 $\pm$ 0.05 to 1.81 $\pm$ 0.09, and the $A_{260/230}$ ratio from 1.17 $\pm$ 0.07 to 1.65 $\pm$ 0.04. Eluates from these DNA purification kits were always amplifiable: Amplification of these purified DNA extracts resulted in brighter and more consistent amplicon bands (on agarose gel) when the same quantity of pre-purification DNA was used, independent of primers used (Supplementary Figure S1). This indicated that the inhibitory compounds interfering with the PCR amplification of the TNA (Extract I, **Figure 2**) were removed by purification with DNA clean-up kits (note that step C in **Figure 2** had not yet been included during this early purification kit testing).

For RNA, on the other hand, the quality of the extracts varied, as seen from differences in residual gDNA for soils FH and FL below. We were able to obtain gDNA-free RNA from soil FH (gDNA undetectable via qPCR analysis after 35 cycles), although DNase digestion was always required to remove the residual gDNA, regardless of kit or method used (including the PS kit, despite its preferential eluent system). These RNA extracts from soil FH were successfully reverse transcribed, as judged from the amplification of the resulting cDNA using qPCR (detected after $\leq$35 cycles). In contrast, RNA extracts from soil FL often contained qPCR-amplifiable gDNA (detected after $\leq$35 cycles) that was not removable even after repeated rounds of extended DNase digestion (1–2 h) and RNA clean-up kit purification (regardless of purification kit used). There was often residual gDNA in these primary extracts even after a second digestion or, in cases where gDNA was completely digested (in the qPCR), the RNA in the sample was no longer detectable (undetectable after $\geq$35 cycles, after reverse transcription followed by qPCR).

During the first two stages of optimization (**Figure 1**), we observed that enzymatic issues in the RNA fraction (e.g., incomplete DNase digestion as described above) coincided with Taq polymerase inhibition in the DNA fraction (polymerase inhibition is described above and in Supplementary Figures S1 and S4), suggesting that the same inhibitors associated with Taq polymerase activity could be the main reason behind the interference with other enzymes (i.e., DNase and reverse transcriptase). Thus in Stage 3 of optimization (**Figure 1**), we used the OPIR kit, a TNA purification kit that specializes in inhibitor removal, on the primary TNA Extract I (**Figure 2**) prior to any enzymatic process (including DNase digestion). In addition to improved DNA quality, we observed little loss of nucleic acid material. For example, purification of 3–4 μg of DNA g^{-1} soil (ww) resulted in 2.5–3.5 μg using OPIR (compared to 2–2.3 μg using gDCC), and the Extract II (**Figure 2**) DNA was as equally amplifiable as Extract III (**Figure 2**) DNA purified with dedicated DNA purification kits, confirming the removal of

Taq polymerase inhibitors. The improved TNA quality was also observed by enhanced DNase digestion. A single, non-extended digestion using the TURBO DNase kit (see below), performed according to manufacturer's instructions, reduced the quantity of residual gDNA in the digested RNA Extracts V (**Figure 2**) from FH and FL soils to below the limit of PCR and qPCR detection (conservatively estimated to 2 copies μL^{-1} reaction; in this case corresponding to 1.6×10^4 16S rRNA gene copies g^{-1} soil, ww).

Thus, we concluded that using the OPIR kit prior to a DNA or RNA purification kit was the best option for obtaining high quality DNA or RNA extracts, respectively. With the addition of the OPIR kit, we did not observe any difference in the quality of DNA or RNA yielded by any of the purification kits tested, so the choice of DNA and RNA purification kit used in subsequent extractions was decided by load capacity and cost per reaction. For our purposes, the OPIR, gDCC, and RCC kits satisfied these criteria and were used on the DNA and RNA extracts sent for metagenomic and metatranscriptomic analysis, respectively.

Test of DNases and Reverse Transcriptases

In the second part of Stage 3 optimization (**Figure 1**), OPIR kit purified, inhibitor-free extracts from all three soil types were used to test different DNases (Extract II) and reverse transcriptases (Extract V). Of the DNases tested, TURBO DNase was the most active at 2 Units μL^{-1} (as described in the respective product information sheets), and was also the most efficient at removing gDNA from samples even in the presence of low quantities of inhibitors (residual gDNA was undetectable with qPCR after $\geq$35 cycles when using TURBO DNase, compared to $\leq$35 cycles using the other DNases). Coupling this DNase with the OPIR kit made a potent combination for alleviating the inhibitory effect, thus digesting more gDNA in the TNA extracts.

To investigate the reproducibility of gDNA removal, we quantified the *nosZ* gene in TNA that was extracted from 45 soil Å samples and digested in two consecutive rounds (**Figure 3**). The soil had been exposed to different oxygen regimes, and incubated anoxically for different time periods (see Materials and Methods), but these treatments did not affect the copy numbers of *nosZ* in the gDNA content of the samples (**Figure 3A**). Although residual gDNA persisted in some samples from soil Å after the first DNase digestion (Extract IV), purification with an RNA purification kit (e.g., RCC) followed by a second DNase digestion often completely removed the remaining gDNA in Extract V (**Figure 3**). The first digestion ensured that any RNA clean-up kit used (in this case, RCC) did not become overloaded by the large quantities of extracted gDNA, which would result in the loss of RNA. Using qPCR on these RNA extracts, we showed that two rounds of DNase digestion reduced the number of *nosZ* gene copies to below the qPCR detection limit (conservatively estimated to 2 copies μL^{-1} reaction; in this case corresponding to 400 copies g^{-1} soil (ww)) for all samples (**Figure 3**). This is compared to a single DNase digestion, where only 6 of 45 samples had undetectable quantities of *nosZ* DNA, and the residual gDNA in the remaining samples was $0.002 \pm 0.002\%$ of the original. Although these percentage numbers are small,

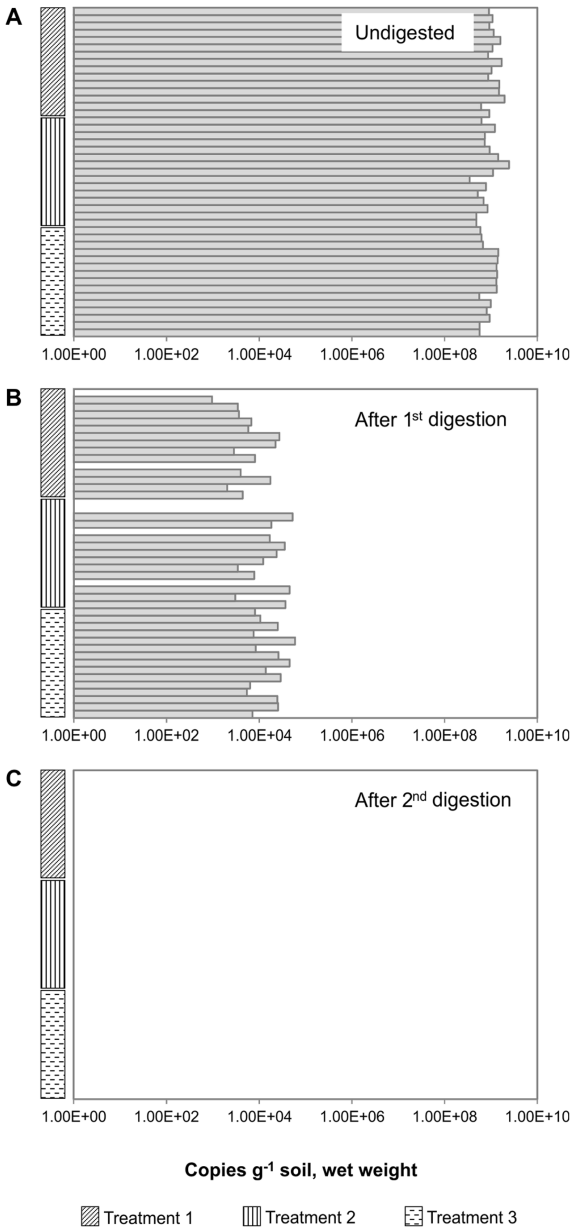


FIGURE 3 | Removal of gDNA by consecutive DNase digestions of total nucleic acids (TNA) extracted from 45 Å soil samples. The soil had been exposed to different oxygen regimes (here called Treatments 1, 2, and 3), for details see section "Materials and Methods." The soils were incubated anoxically to stimulate denitrification gene expression, and samples were taken at time intervals. TNA was extracted using the optimized and simplified method, and the *nosZ* was quantified by qPCR. **(A)** After extraction via the optimized method, all samples were tested for the presence of DNA. Neither the different oxygen regimes nor the stimulation of gene expression affected the number of *nosZ* genes in the gDNA from the different samples. **(B)** The first digest removed most amplifiable genomic DNA (gDNA) present. **(C)** The second DNase treatment removed amplifiable gDNA in all samples. There was no relationship between the starting DNA quantity and the success of complete gDNA removal ($R^2 = 0.0189$). This highlights the importance of checking all RNA samples and not only representative samples, as there may be high variability among samples from the same source and extraction procedure.

they translate to a residual gDNA of between 900 and 60 000 copies of *nosZ* genes g^{-1} soil (ww). Notably, the soil samples retained different quantities of residual gDNA in RNA fractions despite identical extraction procedures, as indicated by qPCR (**Figure 3**). This differed from the DNA fractions that contained equally amplifiable and relatively similar quantities of gDNA in replicate extractions (**Figure 3A**).

Using these high quality gDNA-free RNA extracts for reverse transcription, there was no observable difference in the cDNA synthesis success rate between the reverse transcriptases tested – *nosZ* cDNA was always undetectable in partially purified RNA, and consistently detectable in high-quality RNA, regardless of the reverse transcriptase used. In this study, Maxima Reverse Transcriptase was chosen for use with the optimized method because it had the highest capacity and was thus the least likely to be overloaded by the total RNA in each sample (5 μg total RNA). Thus, for the optimized method, we used a combination of the OPIR and RCC purification kits and TURBO DNase to obtain high-quality RNA extracts prior to cDNA synthesis with the Maxima Reverse Transcriptase.

Optimized and Transparent Method for Non-kit Based Extraction

Using the results from the optimization of the lysis and precipitation steps of Nicolaisen's method (see Supplementary Material, pp. 1–2 and Supplementary Figures S1–S5), we revised the method as described in the section "Materials and Methods." We compared the revised method with the different extraction kits and the original Nicolaisen's method, and observed no advantage to using extraction kits over our revised extraction method. In addition to the shorter average extraction time and quick precipitation, the quality and quantity of nucleic acids extracted using our revised method was equal, if not better, than all the other kits and methods tested. Using the above described combination of purification kits and DNase enzyme, we were able to obtain gDNA-free RNA fractions (Extract IV) in the FL and FH soils after only a 30-min DNase digestion. This is compared to persistent incomplete DNA digestion in soil FL despite extended DNase digestion times of up to 2 h using the unamended Nicolaisen's method, proving that low digestion efficiencies are likely caused by the failure to remove inhibitory compounds. Using our optimized method, the average $A_{260/280}$ and $A_{260/230}$ ratios before purification (Extract I) were 1.84 and 1.66, respectively, and the crude extracted quantities were 50–150 μg DNA g^{-1} soil (ww) and 15–18 μg RNA g^{-1} soil (ww). Analysis by agarose gel electrophoresis revealed reproducible TNA extraction, with large quantities of extracted rRNA that was clearly visible on the gel (Supplementary Figure S2). After a 10- or 20-fold dilution (to attain the desired 1–10 ng of DNA per reaction, as specified in Materials and Methods), Extract I from all soils (FH, FL, and Å) was always at least weakly amplifiable with primers targeting the 16S rRNA gene (as visualized on agarose gels). Additional purification using the OPIR kit, followed by the gDCC and RCC kit for DNA and RNA, respectively, yielded nucleic acids that were always usable in downstream processes.

Using qPCR analysis and primers targeting the 16S rRNA and *nosZ* genes, we confirmed that the purified RNA fraction (Extract V) contained no detectable copies of gDNA. Average 16S rRNA copies were reduced from $1.08 \times 10^{11} \pm 3.32 \times 10^{10}$ (soil FH) and $3.15 \times 10^{10} \pm 1.19 \times 10^{10}$ (soil FL) copies g^{-1} soil (ww) to below the detection limit of qPCR (1.6×10^4 copies g^{-1} soil, ww) in RNA extracts. The RNA extracts were also successfully reverse transcribed to cDNA, and qPCR-amplifiable with primers targeting the *nosZ* gene (3×10^6 and 1×10^5 copies g^{-1} soil, ww in soils FH and FL, respectively).

Analysis of the raw qPCR fluorescence data using LinRegPCR revealed similar efficiencies for both the samples and the purified plasmid standards (**Table 3**), confirming the absence of amplification or dsDNA-binding dye inhibitors in all our amplification reactions. Although these individual amplification efficiencies appear to be low, similar efficiencies seen in the standards indicate that the lower-than-expected efficiencies are likely an effect of poor primer-template matches or the formation of primer dimers affecting the amplification reaction, rather than the presence of inhibitory compounds. For comparison to other studies, the calibration plot-based method of efficiency calculation yields amplification efficiencies of 95.1 and 99.1% for the 16S rRNA and *nosZ* genes, respectively.

Quality Assessment and Reproducibility of DNA and RNA Extracts

DNA and RNA (Extracts III and V) yielded by our simplified TNA extraction method (soils Å, FL, and FH) and RNA (Extract V) from the PS kit (soil Å) (all purified with OPIR/gDCC/RCC kits as described previously), were sent for Illumina HiSeq sequencing at the CBC. All samples were independently verified to be of high quality: RNA extracts were confirmed to be free of gDNA, and both DNA and RNA were successfully sequenced with HiSeq 2500 technology. The resulting sequences were annotated using MG-RAST, and a summary of the annotated data has been included in the Supplementary Table S3. Total Sequence and Clusters of Orthologous Groups (COG) breakdown profiles generated using MG-RAST were highly similar between replicate extractions for both soil FH and FL, indicating good co-extraction replication (Supplementary Figures S6 and S7). Further analysis of the sequences (normalized to Reads per Million, RPM) using bacterial housekeeping genes as a reference of comparison revealed good reproducibility of DNA and RNA extraction replicates (examples of data shown in **Table 4**). There was minor variation for some genes in the RNA duplicates (e.g., *fusA* in R5 and R6), but the reproducibility for the other genes points toward variability in *fusA* gene expression

TABLE 3 | Individual qPCR efficiencies based on LinRegPCR analysis of nucleic acids extracted from soils FH (high pH peat, pH 7.39) and FL (low pH peat, pH 3.65).

Target	Plasmid standard	FH	FL
16S rRNA gene	77.9 ± 3.44%	81.3 ± 3.18%	82.0 ± 3.49%
nosZ DNA	84.2 ± 5.05%	85.4 ± 3.97%	84.2 ± 3.36%
nosZ cDNA	Same as above	80.7 ± 2.51%	81.0 ± 2.86%

TABLE 4 | Example of DNA and RNA meta-ome sequencing reproducibility, based on Reads per Million (RPM) values from MG-RAST annotation of bacterial housekeeping genes, obtained from soils FH (high pH peat, pH 7.39) and FL (low pH peat, pH 3.65).

Gene	FH					FL				
	DNA			RNA		DNA			RNA	
	D1	D2	D3	R5	R6	D4	D5	D6	R11	R12
recA	212.4	208.5	208.3	114.7	164.4	221.4	221.7	221.7	23.4	18.2
gyrB	383.1	392.1	385.6	209.5	277.1	374.8	385.7	383.8	40.6	35.5
fusA	788.4	800.3	794.6	434.9	594.1	764.9	782.9	774.7	201.2	183.6
rpoB	686.0	700.5	702.3	456.7	525.3	693.3	717.9	710.7	205.6	187.2
infB	356.8	359.2	359.3	229.5	298.0	345.6	376.5	368.0	63.0	50.4
atpD	297.5	296.5	298.0	222.9	263.9	340.7	347.9	339.5	57.3	48.1

Samples were sequenced using Illumnia HiSeq 2500 technology, and all values were normalized for total read counts to Reads per Million (RPM). DNA samples were sequenced in triplicate (D1–D3, and D4–D6), and RNA samples were sequenced in duplicate (R5–R6, and R11–R12). The genes were identified in MG-RAST using the following annotations: recA (RecA protein), gyrB (DNA gyrase subunit B), fusA (Translation elongation factor G), rpoB (DNA-directed RNA polymerase beta subunit), infB (Translation initiation factor 2), and atpD (ATP synthase beta chain).

due to incubation conditions, rather than an extraction bias. Together, the sequenced metagenomes and metatranscriptomes give evidence to the reproducibility of DNA and RNA co-extraction using the optimized method.

DISCUSSION

Standardized Workflow vs. Specific Methods

In our search to identify and overcome key problematic steps when extracting DNA/RNA from inhibitor-rich soil samples, we found that commercially available nucleic acid extraction/purification kits are not always better than non-kit methods (e.g., Nicolaisen's method). While the DNA extraction kits fared well, none of the RNA extraction kits tested worked for all our soil samples. Even the best kit tested, the PS kit, only worked for soil Å and FH, but not for soil FL (**Table 2**). Although the PS kit was able to yield usable nucleic acids, varying quantities were extracted from equal starting amounts of a single soil type (**Figure 3A**). Considering the inherent variations in the soil, methods yielding poor replication will only further complicate matters and lead to erroneous conclusions and hypotheses. Previous studies comparing multiple methods have also concluded that extraction methods may substantially affect any downstream data (Inceoglu et al., 2010; Töwe et al., 2010). As such, we once again highlight the importance of determining suitable extraction methods based on the environment of interest. This emphasizes the need for transparent, modular methods such as the one described by Lever et al. (2015), where each step can be optimized to meet the needs for a specific sample type. Similar to their conclusions, we have found that the ease to add and adjust extraction and purification procedures as required has resulted in higher DNA and RNA yields, as well as an improved quality.

We took the study by Lever et al. (2015) further, and were able to pinpoint the important steps in nucleic acid extraction for better quality and quantity of DNA and RNA yields via our systematic testing of extraction methods. Our proposed workflow (**Figure 2**) aims to remove the problems upstream, thereby circumventing downstream problems and avoiding the struggle with persistent residual gDNA or otherwise poor quality nucleic acids. In the current study, we have chosen relative ease and speed over cost, and have opted to use commercial purification kits for each purification step. But, as suggested in our data and indicated in **Figure 2**, it is not the purification kit that determines the usability of the material downstream, but the point during extraction at which the purification step takes place – as early as possible and before enzymatic processes, but without compromising RNA stability. As such, the use of similar purification kits or methods (e.g., gradient centrifugation, Sephadex columns or chromatography) would achieve the same effect, and at a reduced cost. Similarly, the core of our suggested workflow is designed for gene expression analyses, and the restriction of total sample processing time (due to short mRNA half-lives) played a big role in the creation of our proposed workflow (**Figure 2**). Thus, our workflow reflects time-limited sample processing that is incompatible with early purification procedures that require pre-optimization, such as the addition of $Al_2(SO_4)_3$ to remove inhibitors prior to soil disruption (Peršoh et al., 2008).

Effectiveness of the Optimized Nucleic Acid Extraction Workflow

Although there are a large number of published modular DNA and RNA co-extraction methods, many are based on the same fundamentals of (1) sample lysis, (2) phenol-chloroform purification, and (3) nucleic acid precipitation (Griffiths et al., 2000; Arbeli and Fuentes, 2007; Nicolaisen et al., 2008; Kotiaho et al., 2010; Mettel et al., 2010; Paulin et al., 2013; Lever et al., 2015). These papers mostly focused on the buffers/materials used (e.g., composition, concentration, incubation time, etc.) and generally follow the same structure. Here, we instead aimed to characterize and detail the key order of essential steps in the workflow. In particular, additional pre-DNase digestion purification steps were added to aid in better gDNA removal and higher RNA quality. In this study, our modular method changes were grounded on Nicolaisen's et al. (2008) method

because of previous work published on the same soils (Liu et al., 2010). In that study where Nicolaisen's method was used, both the quantity and quality was unsuitable for meta-ome sequencing, and mRNA transcripts extracted from FL soils were undetectable by qPCR, despite similar incubation conditions to those in this study (Liu et al., 2010). Using the optimized method detailed in this paper, at least double the amount of DNA and RNA was co-extracted from the same soils – Liu et al. (2010) only managed to obtain 16.1–26.4 μg DNA g^{-1} soil (ww) and 2.3–7.2 μg RNA g^{-1} soil (ww). Additionally, *nosZ* transcripts that were previously only quantifiable in soil FH (3-6 × 10^5 copies g^{-1} soil, ww) but completely undetectable in soil FL (Liu et al., 2010), were now detectable in both soil FH and FL (see Optimized and Transparent Method for Non-kit Based Extraction).

One plausible reason behind this novel detection of *nosZ* transcripts in soil FL, could be that the higher extraction efficiency of the optimized method provided a "deeper" transcript profile. The nucleic acid yield of the optimized method presented here was ≈ 10 times that of the unmodified Nicolaisen's method (Liu et al., 2010), and corresponded with a nearly 10-fold increase in *nosZ* transcript detection in soil FH. However, when the transcript numbers in soil FL yielded by the optimized method (1 × 10^5 copies g^{-1} soil, ww) are adjusted to correspond with a 10 times lower efficiency (thus 1 × 10^4 copies g^{-1} soil, ww), it is still well above than the detection limit of 8.4 × 10^3 copies g^{-1} soil (ww) of Liu et al. (2010). Since sub-optimal extraction procedures are known to result in unusable downstream products due to persistent inhibition even after additional downstream purification processes (Cullen and Hirsch, 1998; LaMontagne et al., 2002), it is thus more likely that the quality of the isolated mRNA has improved sufficiently for *nosZ* transcript detection in soil FL. Furthermore, while the quality and quantity of RNA from soil FL yielded by Nicolaisen's method was previously too poor for sequencing (Liu et al., 2010), the RNA yielded by the optimized method in this study from both soils were successfully sequenced and annotated (see Results **Table 4**, and Supplementary Table S3; Supplementary Figures S6 and S7). This marked improvement from undetectable mRNA, to the now successful sequencing of both metagenome and metatranscriptome using the same soils, shows that the optimized workflow greatly increased nucleic acid extraction quantity and quality.

Enzymes, Inhibitors, and Purification

As of now, there is no existing method that can accurately determine and quantify the presence of all co-extracted enzyme inhibitors, partly due to the unknown composition of inhibitors. Their presence is instead seen through their interference with enzyme activity, affecting nucleic acid transforming processes including amplification, DNase digestion and reverse transcription. A common solution when faced with co-extracted inhibitors is to dilute the sample, reducing the degree of inhibition (Paulin et al., 2013). However, while a partially inhibited DNA amplification reaction (PCR or qPCR) may still yield usable data, using partially DNase digested RNA extracts with residual gDNA would render any RNA analysis biased and useless. Thus, since it is impossible to calculate the inhibitor-tolerance limit of all enzymatic processes (and enzyme types), it is safer and more effective to focus on purifying nucleic acids than to hope that dilution would reduce the inhibitor effect.

During our purification kit trials, we found that the sequence of steps during nucleic acid extraction is more important than the type of kit or enzyme used. We performed extensive trials using different purification kits at different stages of the extraction procedure, using only the extracts from our revised Nicolaisen's method (commercial extraction kits had rigid procedural structures and the reagents involved were of unknown nature). We hypothesized that many commercial extraction kits failed to yield gDNA-free RNA from the inhibitor-rich soil FL, because DNase is often applied to the primary TNA extract (Extract I) before purification. The aforementioned use of the OPIR kit to purify primary TNA extracts prior to all enzymatic processes was the major breakthrough in the optimization and simplification of the extraction process. By using a specialized method to remove inhibitory compounds prior to DNase digestion, digestion efficiencies were greatly improved and the procedure was shortened significantly. In contrast, the relatively common practice of attempting to remove gDNA without purification via prolonged incubations at non-ideal RNA preservation temperatures potentially compromised the extracted RNA. Thus, it is our recommendation to purify samples prior to the digestion of gDNA to ensure maximal efficiency and speed.

If commercial kits are used for purification prior to DNase digestion, two important factors must be considered: (1) Whether or not the purification kit is RNase-free, and (2) The maximum nucleic acid holding capacity of the kit, especially for column-based purification kits. Unfortunately, DNA purification kits have higher load capacities but are not always RNase-free (e.g., gDCC), and the load capacities of the RNA purification kit columns tested were too low to capture all extracted nucleic acids (e.g., RCC). Using these potentially RNase-contaminated DNA purification kits could result in RNA digestion, whereas the RNA kits would be severely overloaded by DNA from the TNA sample. On the other hand, our kit trials revealed that the dedicated RNA purification kits are more capable of removing inhibitors than the TNA purification kit, and their use to remove residual inhibitors prior to reverse transcription was irreplaceable. Hence, while it is critical for TNA extracts (Extract II) to be purified prior to digestion, it is also essential to purify the digested extracts (Extract IV) with dedicated RNA kits to obtain high quality RNA extracts.

Assessing DNase Digestion for RNA Purification

Using our optimized extraction and purification method, both DNA and RNA fractions were used as templates in qPCR reactions with primers targeting the 16S rRNA gene to determine the quantity and amplifiability of gDNA (**Figure 3**). There was no correlation between the quantity of residual

gDNA and the starting gDNA quantities ($R^2 = 0.0189$). The reason behind this is unclear, but uneven spread of inhibitors creates non-uniform DNase digestion of otherwise identical samples. The presence of samples with residual gDNA alongside those with no amplifiable gDNA highlights the importance of checking all samples for the presence of DNA and not only "representative samples." Such use of "representative samples" to extrapolate the lack of contaminating residual gDNA in all RNA samples may potentially introduce severe biases with respect to the quantification and sequencing of mRNA.

A quick search of the literature using the PubMed search engine and the keywords "RNA," "qPCR or PCR" and "transcript*" revealed a surprisingly large proportion of publications that failed to indicate or demonstrate that their RNA extracts are DNA-free. Our criteria for clear demonstration is, ideally, the use of quantification methods such as qPCR. However, we accepted the use of non-quantitative amplification analysis as a minimum indication. The analysis of unamplified nucleic acid material by electrophoresis (agarose or digital gels) or Nanodrop/Qubit quantification, was not considered sufficient evidence of samples free of amplifiable gDNA because neither is sufficiently sensitive to detect trace quantities of gDNA. Among papers published in Applied and Environmental Microbiology in 2012, 2013, and 2014, only 36, 31, and 13% clearly indicated the lack of gDNA in their RNA extracts according to our definition. This problem is not isolated to one journal, as papers published in 2014 in ISME Journal showed a similar trend, with only 37% of papers clearly addressing the residual gDNA question in RNA extracts. While more papers published in 2015 in Applied and Environmental Microbiology (47%) clearly indicated DNA-free RNA samples, the rest still either provided insufficient evidence, or failed to report that the samples had been quality-controlled prior to further downstream analysis.

While on the surface such quick assessments of gDNA removal appear beneficial, allowing a rapid analysis of the integrity of different nucleic acid fractions (as seen in Supplementary Figures S2 and S3), this creates a false impression of quality control. Low quantities of residual gDNA can still be quantifiable using qPCR in RNA samples, but may not be detectable on an agarose gel as a genomic smear even when using sensitive nucleic acid stains such as GelRed (Biotium) or peqGREEN (Peqlab; data not shown). Our qPCR analysis revealed the presence of substantial quantities of gDNA (**Figure 3**), even though gel visualization (not shown) failed to reveal the presence of gDNA in the purified RNA fraction. Additionally, using either spectrofluoro- or spectrophotometric methods to quantify residual gDNA relies heavily on exceeding minimum detection limits, as well as the assumption that the fluorophores have not been otherwise inhibited (Bachoon et al., 2001; Zipper et al., 2003; Sidstedt et al., 2015), neither of which can be easily presumed where environmental samples are concerned. Thus, we strongly recommend the use of quantitative methods such as qPCR (or amplification procedures at the very least, to amplify the signal from trace gDNA molecules) to definitively determine the efficiency of DNase digestion reactions to avoid overestimations of active microbial communities in soil due to the presence of contaminating gDNA.

CONCLUDING REMARKS

As is known from other studies and indicated in **Table 2**, kits and methods that work well for one soil may not perform similarly for another soil type. Our results highlight how soil types with different properties can affect the quality of nucleic acids extracted via identical methods. This disparity likely arises from the unique inhibitor profiles of each soil type, which in turn interfere with the various nucleic acid transforming enzymes to different extents. As such, it is important to thoroughly purify nucleic acids as much as possible prior to any enzymatic process, including but not restricted to DNase digestion, reverse transcription and amplification. Such purification results in more efficient and effective DNase digestion, reducing incubation times and consequently reducing RNA placement at non-optimal temperatures. However, even with multiple purification techniques, DNase digestion is not always a uniform process (especially with inhibitor-rich soil extracts), and the residual gDNA may vary between samples and replicates. Thus, we strongly recommend the examination of all samples for residual gDNA and not only "representative samples." Furthermore, we propose the use of the more sensitive qPCR method as an indicator of residual gDNA, rather than less sensitive methods such as electrophoretic analysis of unamplified nucleic acid extracts.

AUTHOR CONTRIBUTIONS

All authors contributed to the planning of the work and the revision of the manuscript. In addition, NL and CR performed the experimental work detailed in this paper. NL performed data analysis and the drafting of the manuscript.

ACKNOWLEDGMENTS

The authors thank Jim Shapleigh (Cornell University) for his assistance with bioinformatics analysis, Bioforsk Vest Fureneset and Peter Dörsch (IMV, NMBU) for sampling and providing the Fjaler soils, and Trygve Fredriksen (IMV, NMBU) for assistance in sampling Ås soils. Financial support for CR came from the National Science Foundation (NSF) (DEB-1311335), and partial funding support from Cornell University Programs in Biogeochemistry and Environmental Biocomplexity and Cross-Scale Biogeochemistry and Climate, both of which are supported by NSF and Cornell University's Atkinson Center for a Sustainable Future.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01588

REFERENCES

Abu Al-Soud, W., and Rådström, P. (1998). Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. *Appl. Environ. Microbiol.* 64, 3748–3753.

Arbeli, Z., and Fuentes, C. L. (2007). Improved purification and PCR amplification of DNA from environmental samples. *FEMS Microbiol. Lett.* 272, 269–275. doi: 10.1111/j.1574-6968.2007.00764.x

Baar, C., d'Abbadie, M., Vaisman, A., Arana, M. E., Hofreiter, M., Woodgate, R., et al. (2011). Molecular breeding of polymerases for resistance to environmental inhibitors. *Nucleic Acids Res.* 39:e51. doi: 10.1093/nar/gkq1360

Bachoon, D. S., Otero, E., and Hodson, R. E. (2001). Effects of humic substances on fluorometric DNA quantification and DNA hybridization. *J. Microbiol. Methods* 47, 73–82. doi: 10.1016/S0167-7012(01)00296-2

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Cruaud, P., Vigneron, A., Lucchetti-Miganeh, C., Ciron, P. E., Godfroy, A., and Cambon-Bonavita, M.-A. (2014). Influence of DNA extraction method, 16S rRNA targeted hypervariable regions, and sample origin on microbial diversity detected by 454 pyrosequencing in marine chemosynthetic ecosystems. *Appl. Environ. Microbiol.* 80, 4626–4639. doi: 10.1128/AEM.00592-14

Cullen, D. W., and Hirsch, P. R. (1998). Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biol. Biochem.* 30, 983–993. doi: 10.1016/S0038-0717(98)00001-7

Dineen, S. M., Aranda, R., Dietz, M. E., Anders, D. L., and Robertson, J. M. (2010). Evaluation of commercial RNA extraction kits for the isolation of viral MS2 RNA from soil. *J. Virol. Methods* 168, 44–50. doi: 10.1016/j.jviromet.2010.04.014

Frostegård, Å., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., et al. (1999). Quantification of bias related to the extraction of DNA directly from soils. *Appl. Environ. Microbiol.* 65, 5409–5420.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., and Bailey, M. J. (2000). Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl. Environ. Microbiol.* 66, 5488–5491. doi: 10.1128/aem.66.12.5488-5491.2000

Hirsch, P. R., Mauchline, T. H., and Clark, I. M. (2010). Culture-independent molecular techniques for soil microbial ecology. *Soil Biol. Biochem.* 42, 878–887. doi: 10.1016/j.soilbio.2010.02.019

Inceoglu, O., Hoogwout, E. F., Hill, P., and van Elsas, J. D. (2010). Effect of DNA extraction method on the apparent microbial diversity of soil. *Appl. Environ. Microbiol.* 76, 3378–3382. doi: 10.1128/AEM.02715-09

Kermekchiev, M. B., Kirilova, L. I., Vail, E. E., and Barnes, W. M. (2009). Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. *Nucleic Acids Res.* 37:e40. doi: 10.1093/nar/gkn1055

Kloos, K., Mergel, A., Rösch, C., and Bothe, H. (2001). Denitrification within the genus *Azospirillum* and other associative bacteria. *Funct. Plant Biol.* 28, 991–998. doi: 10.1071/PP01071

Kotiaho, M., Fritze, H., Merilä, P., Juottonen, H., Leppälä, M., Laine, J., et al. (2010). Methanogen activity in relation to water table level in two boreal fens. *Biol. Fertil. Soils* 46, 567–575. doi: 10.1007/s00374-010-0461-0

Krsek, M., and Wellington, E. M. H. (1999). Comparison of different methods for the isolation and purification of total community DNA from soil. *J. Microbiol. Methods* 39, 1–16. doi: 10.1016/S0167-7012(99)00093-7

LaMontagne, M. G., Michel, F. C., Holden, P. A., and Reddy, C. A. (2002). Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *J. Microbiol. Methods* 49, 255–264. doi: 10.1016/S0167-7012(01)00377-3

Lever, M. A., Torti, A., Eickenbusch, P., Michaud, A. B., Šantl-Temkiv, T., and Jørgensen, B. B. (2015). A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. *Front. Microbiol.* 6:476. doi: 10.3389/fmicb.2015.00476

Liu, B., Mørkved, P. T., Frostegård, Å., and Bakken, L. R. (2010). Denitrification gene pools, transcription and kinetics of NO, N2O and N2 production as affected by soil pH. *FEMS Microbiol. Ecol.* 72, 407–417. doi: 10.1111/j.1574-6941.2010.00856.x

Mahmoudi, N., Slater, G. F., and Fulthorpe, R. R. (2011). Comparison of commercial DNA extraction kits for isolation and purification of bacterial and eukaryotic DNA from PAH-contaminated soils. *Can. J. Microbiol.* 57, 623–628. doi: 10.1139/w11-049

Mettel, C., Kim, Y., Shrestha, P. M., and Liesack, W. (2010). Extraction of mRNA from Soil. *Appl. Environ. Microbiol.* 76, 5995–6000. doi: 10.1128/aem.03047-09

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., et al. (2008). The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 9:386. doi: 10.1186/1471-2105-9-386

Molstad, L., Dörsch, P., and Bakken, L. R. (2007). Robotized incubation system for monitoring gases (O2, NO, N2O N2) in denitrifying cultures. *J. Microbiol. Methods* 71, 202–211. doi: 10.1016/j.mimet.2007.08.011

Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700.

Nicolaisen, M. H., Baelum, J., Jacobsen, C. S., and Sørensen, J. (2008). Transcription dynamics of the functional tfdA gene during MCPA herbicide degradation by *Cupriavidus necator* AEO106 (pRO101) in agricultural soil. *Environ. Microbiol.* 10, 571–579. doi: 10.1111/j.1462-2920.2007.01476.x

Paulin, M. M., Nicolaisen, M. H., Jacobsen, C. S., Gimsing, A. L., Sørensen, J., and Bælum, J. (2013). Improving Griffith's protocol for co-extraction of microbial DNA and RNA in adsorptive soils. *Soil Biol. Biochem.* 63, 37–49. doi: 10.1016/j.soilbio.2013.02.007

Peršoh, D., Theuerl, S., Buscot, F., and Rambold, G. (2008). Towards a universally adaptable method for quantitative extraction of high-purity nucleic acids from soil. *J. Microbiol. Methods* 75, 19–24. doi: 10.1016/j.mimet.2008.04.009

Philippot, L., Piutti, S., Martin-Laurent, F., Hallet, S., and Germon, J. C. (2002). Molecular analysis of the nitrate-reducing community from unplanted and maize-planted soils. *Appl. Environ. Microbiol.* 68, 6121–6128. doi: 10.1128/aem.68.12.6121-6128.2002

Purdy, K. J., Embley, T. M., Takii, S., and Nedwell, D. B. (1996). Rapid extraction of DNA and rRNA from sediments by a novel hydroxyapatite spin-column method. *Appl. Environ. Microbiol.* 62, 3905–3907.

Ruijter, J. M., Pfaffl, M. W., Zhao, S., Spiess, A. N., Boggy, G., Blom, J., et al. (2013). Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications. *Methods* 59, 32–46. doi: 10.1016/j.ymeth.2012.08.011

Ruijter, J. M., Ramakers, C., Hoogaars, W. M. H., Karlen, Y., Bakker, O., van den Hoff, M. J. B., et al. (2009). Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 37:e45. doi: 10.1093/nar/gkp045

Sidstedt, M., Jansson, L., Nilsson, E., Noppa, L., Forsman, M., Rådström, P., et al. (2015). Humic substances cause fluorescence inhibition in real-time polymerase chain reaction. *Anal. Biochem.* 487, 30–37. doi: 10.1016/j.ab.2015.07.002

Sognnes, L. S., Fystro, G., Øpstad, S. L., Arstein, A., and Børresen, T. (2006). Effects of adding moraine soil or shell sand into peat soil on physical properties and grass yield in western Norway. *Acta Agric. Scand. Sect. B Soil Plant Sci.* 56, 161–170. doi: 10.1080/09064710500218845

Tebbe, C. C., and Vahjen, W. (1993). Interference of humic acids and DNA extracted directly fromSoil in detection and transformation of recombinantdna from bacteria and a yeast. *Appl. Environ. Microbiol.* 59, 2657–2665.

Throbäck, I. N., Enwall, K., Jarvis, Å., and Hallin, S. (2004). Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol. Ecol.* 49, 401–417. doi: 10.1016/j.femsec.2004.04.011

Töwe, S., Kleineidam, K., and Schloter, M. (2010). Differences in amplification efficiency of standard curves in quantitative real-time PCR assays and consequences for gene quantification in environmental samples. *J. Microbiol. Methods* 82, 338–341. doi: 10.1016/j.mimet.2010.07.005

Vishnivetskaya, T. A., Layton, A. C., Lau, M. C. Y., Chauhan, A., Cheng, K. R., Meyers, A. J., et al. (2014). Commercial DNA extraction kits impact observed microbial community composition in permafrost samples. *FEMS Microbiol. Ecol.* 87, 217–230. doi: 10.1111/1574-6941.12219

Wang, Y., Hayatsu, M., and Fujii, T. (2012). Extraction of bacterial RNA from soil: challenges and solutions. *Microbes Environ.* 27, 111–121. doi: 10.1264/jsme2.ME11304

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703.

Wesolowska-Andersen, A., Bahl, M. I., Carvalho, V., Kristiansen, K., Sicheritz-Pontén, T., Gupta, R., et al. (2014). Choice of bacterial DNA extraction method from fecal material influences community structure as evaluated by metagenomic analysis. *Microbiome* 2:19. doi: 10.1186/2049-2618-2-19

Young, J. M., Rawlence, N. J., Weyrich, L. S., and Cooper, A. (2014). Limitations and recommendations for successful DNA extraction from forensic soil samples: a review. *Sci. Justice* 54, 238–244. doi: 10.1016/j.scijus.2014.02.006

Zipper, H., Buta, C., Lämmle, K., Brunner, H., Bernhagen, J., and Vitzthum, F. (2003). Mechanisms underlying the impact of humic acids on DNA quantification by SYBR green i and consequences for the analysis of soils and aquatic sediments. *Nucleic Acids Res.* 31:e39. doi: 10.1093/nar/gng039

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Material

Transparent DNA/RNA co-extraction workflow protocol suitable for inhibitor-rich environmental samples that focuses on complete DNA removal for transcriptomic analyses

Natalie Y.N. Lim*, Constance A. Roco & Åsa Frostegård

*** Correspondence:** Natalie Y.N. Lim: natalie.lim@nmbu.no

1 Supplementary Data

1.1 The effectiveness of dedicated nucleic acid extraction kits

To create a baseline comparison with other studies and the commercial standard, nucleic acids were extracted from the high (FH) and low (FL) pH peat soils using several kits. As stated in the Materials and Methods, all nucleic acid quantities were measured with Qubit, whereas quality assessment was performed with NanoDrop (numerical values from absorbance ratios) and/or agarose gel visualization of the degree of shearing. None of the extraction kits tested was able to provide both usable DNA and mRNA from both soil types. Additionally, the kits varied in the overall quality and quantity of extracted DNA and RNA, for both dedicated DNA extraction kits and kits that co-extracted DNA and RNA (see Table S2). Aside from the already unacceptable $A_{260/280}$ ratios, the kits also yielded very poor $A_{260/230}$ ratios: ranging from 0.26-1.66 before additional purification, and improving to 1.77-2.14 after purification (primary extracts under 1.0 were considered failures and were not purified).

The gDNA extracted from these kits also differed in the degree of shearing (ranging from approximately 2 to 8 kb and represented by the degree of smearing when run on an agarose gel; which may be potentially important for metagenomic studies, see Figure S3). Notably, despite its intention to extract only RNA by keeping DNA bound to the column, a high molecular weight band on the gel was still clearly visible in the RNA extract from the PS kit, indicating the co-elution of DNA with RNA (see Figure S3).

All the extraction kits performed relatively well with FH soil – some required further purification with a purification kit to achieve stronger amplification of the 16S rRNA or *nosZ* genes, but some amplification was generally achieved even without (see Figure S4). However, when tested on FL, most extraction kits could not yield amplifiable DNA without the aid of further purification (see Figure S4). Interestingly, some kits performed so poorly that the obtained extract from FL remained unusable after subsequent purification with one or more of the purification kits. This is likely due to high quantities of co-extracted inhibitors, and is in line with other studies showing the failure of downstream purification techniques to tackle extremely high levels of humic acid contamination (Cullen and Hirsch, 1998; LaMontagne et al., 2002; Young et al., 2014).

1.2 Optimized lysis and precipitation

Since none of the extraction kits yielded usable nucleic acids from all three soils, we chose to modify a commonly used non-kit method because it gave us more freedom to optimize the individual procedures involved in the extraction process. Nucleic acids were extracted from

high (FH) and low (FL) pH peat soils using Nicolaisen's method, but different lysis procedures, buffers and precipitants were tested. The best lysis was achieved with the three sizes of glass beads, two cycles of lysis (45 s each) using the FastPrep-24 Instrument, and using CTAB extraction buffer and phenol (both buffered to pH 8.0). There was no significant difference when garnet (14.89 ± 3.57 µg DNA g^{-1} soil) or glass (16.04 ± 3.75 µg DNA g^{-1} soil) beads were used ($p > 0.1$), but the quantity extracted increased when three sizes of beads was used (4.27 µg DNA g^{-1} soil) instead of one size (3.17 µg DNA g^{-1} soil). Although there was no significant difference in nucleic acid quantity when using the FastPrep-24 Instrument compared to the vortex ($p > 0.1$), the FastPrep-24 Instrument was used in further experiments for reasons of comparability with existing literature, due to its widespread use (Griffiths et al., 2000; Kotiaho et al., 2010; Mettel et al., 2010; Nicolaisen et al., 2008). The extent of gDNA shearing varied directly with the number of lysis cycles, where one lysis cycle yielded the largest fragments of gDNA (see Figure S5). However, there was no such correlation with the amplifiability (see Figure S5) or quantity of nucleic acid material where bead beating twice yielded the most nucleic acid material (1×, 2× and 3× bead beating generated 4.26 ± 1.67 µg DNA g^{-1} soil, 9.51 ± 1.88 µg DNA g^{-1} soil, and 2.93 ± 2.45 µg DNA g^{-1} soil, respectively). Thus, two cycles of lysis was chosen because it yielded the highest quantity of nucleic acid material obtained, and the gDNA was not badly sheared. For lysis buffers, aside from the pH 8.0 buffered CTAB and phenol, all combinations involving acidic CTAB, other buffer agents, GES buffer, acidic phenol or increased buffer ionic strength either failed to extract RNA (detection limit 0.01 µg RNA g^{-1} soil) or co-extracted large quantities of inhibitors, effectively preventing all downstream processes.

In this study, we used isopropanol as a precipitant to further reduce incubation times. Not only did it require a shorter precipitation time than PEG (2 minutes versus 2 hours), precipitation with isopropanol yielded up to threefold increase of DNA over PEG. Isopropanol consistently yielded higher quantities of both DNA and RNA than PEG 6000, with little cost to nucleic acid purity – precipitation with PEG 6000 yielded 50-75 µg DNA g^{-1} soil (ww), whereas isopropanol precipitation yielded 50-150 µg DNA g^{-1} soil (ww). Thus, where applicable, isopropanol was used as the precipitant in all subsequent extractions. Although there is some contrasting opinion on the role isopropanol may play in co-precipitating or removing inhibitory compounds (Arbeli and Fuentes, 2007; Cullen and Hirsch, 1998; Hänni et al., 1995; Krsek and Wellington, 1999; LaMontagne et al., 2002), early trials in this study comparing the use of isopropanol and PEG had indicated little disadvantage in using isopropanol. Additionally, alcohols are known to provide better yields (Krsek and Wellington, 1999), and isopropanol has also previously been recommended as the precipitant of choice for its potential ability to remove polysaccharides from soil (Cullen and Hirsch, 1998).

2 Supplementary Tables

TABLE S1 | Buffer-phenol combinations tested in the present study

Buffer	Components	Buffer used	Buffer pH	Buffer ionic strength	Phenol pH
Normal [a]	5 % w/v CTAB 0.35 M NaCl 1 % w/v PVPP	Phosphate buffer	8.0	120 mM	8.0
Strong phosphate	10 % w/v CTAB 0.35 M NaCl 1 % w/v PVPP	Phosphate buffer	8.0	250 mM	8.0
Strong Tris	10 % w/v CTAB 0.35 M NaCl 1 % w/v PVPP	Tris buffer	8.0	250 mM	8.0
Acidic phenol only	10 % w/v CTAB 0.35 M NaCl 1 % w/v PVPP	Phosphate buffer	8.0	120 mM	4.0
Acidic buffer and phenol	10 % w/v CTAB 0.35 M NaCl 1 % w/v PVPP	Phosphate buffer	5.7	120 mM	4.0
GES buffer [b]	5 M guanidinium thiocyanate 100 mM EDTA 0.5 % sarcosyl	Acetate buffer	4.0	25 mM	4.0

[a] A modified phenol-chloroform extraction method as published previously by Nicolaisen and colleagues (Nicolaisen et al., 2008).

[b] GES: Guanidinium thiocyanate-EDTA-sarcosyl

TABLE S2 | Comparison of DNA/RNA extraction kits, tested on soils FH (high pH peat, pH 7.39) and FL (low pH peat, pH 3.65).

Kit		FH				FL			
		DNA		RNA		DNA		RNA	
		μg g^{-1} soil (ww)	$A_{260/280}$	μg g^{-1} soil (ww)	$A_{260/280}$	μg g^{-1} soil (ww)	$A_{260/280}$	μg g^{-1} soil (ww)	$A_{260/280}$
PowerLyzer PowerSoil DNA Isolation Kit	PL	24.5	1.79	-	-	4.02	1.56	-	-
FastDNA SPIN Kit for Soil	FDS	16.0	1.71	-	-	4.09	1.71	-	-
ZR Soil Microbe DNA MiniPrep	SM	15.4	1.76	-	-	4.82	1.68	-	-
MasterPure RNA Purification Kit	MP	88.9 (14.7)*	1.53 (1.84)*	5.54	1.71	85.1 (16.0)*	1.78 (1.84)*	3.20	1.67
PowerMicrobiome RNA Isolation	PM	19.3 (15.3)*	1.64 (1.87)*	23.4 (15.6)*	1.64 (2.57)*	15.5 (9.51)*	1.64 (1.85)*	16.45 (9.09)*	1.60 (2.75)*
RNA PowerSoil Total RNA Isolation Kit	PS	-	-	-	-	-	-	6.71	1.92
RNA PowerSoil Total RNA Isolation Kit	PS + AK	45.5	1.50	19.5 (14.6)*	1.56 (1.57)*	37.9	1.53	18.6 (13.96)*	1.61 (1.58)*

All values listed are averages of triplicate extractions.

* Kits that yielded colored extracts (a sign of very large quantities of inhibitory compounds) were further purified. The values within the parentheses are post-purification with the Genomic DNA Clean & Concentrator or RNA Clean & Concentrator – 5

TABLE S3 | Summary of MG-RAST annotated meta-omics data

Soil type	Sample type	ID	Total reads	Passed QC	Average length (bp)
FH	DNA	D1	28 674 145	97.4 %	155
FH	DNA	D2	31 420 570	97.5 %	155
FH	DNA	D3	29 448 386	97.2 %	155
FL	DNA	D4	29 142 448	97.3 %	155
FL	DNA	D5	30 690 762	97.3 %	155
FL	DNA	D6	25 949 776	96.9 %	155
FH	RNA	R1	17 902 594	81.9 %	128
FH	RNA	R2	24 855 082	97.7 %	129
FH	RNA	R3	17 767 603	98.7 %	121
FH	RNA	R4	16 441 508	97.4 %	128
FH	RNA	R5	17 993 765	99.5 %	116
FH	RNA	R6	27 809 492	98.8 %	127
FH	RNA	R7	42 039 146	95.1 %	134
FH	RNA	R8	4 030 430	95.6 %	133
FH	RNA	R9	22 104 868	96.5 %	110
FH	RNA	R10	17 735 486	98.2 %	129
FL	RNA	R11	8 466 353	97.3 %	119
FL	RNA	R12	17 111 152	97.5 %	121
FL	RNA	R13	21 605 163	98.8 %	125
FL	RNA	R14	19 321 965	97.6 %	120

Samples were sequenced with Illumina HiSeq 2500 technology. The samples were trimmed for adaptors and quality controlled to remove short sequences (< 80 bp), then submitted to MG-RAST for annotation. The annotated FH and FL soil sequences are available online in the MG-RAST database (project ID 14446, project name “Fjaler_HiSeq”).

3 Supplementary Figures

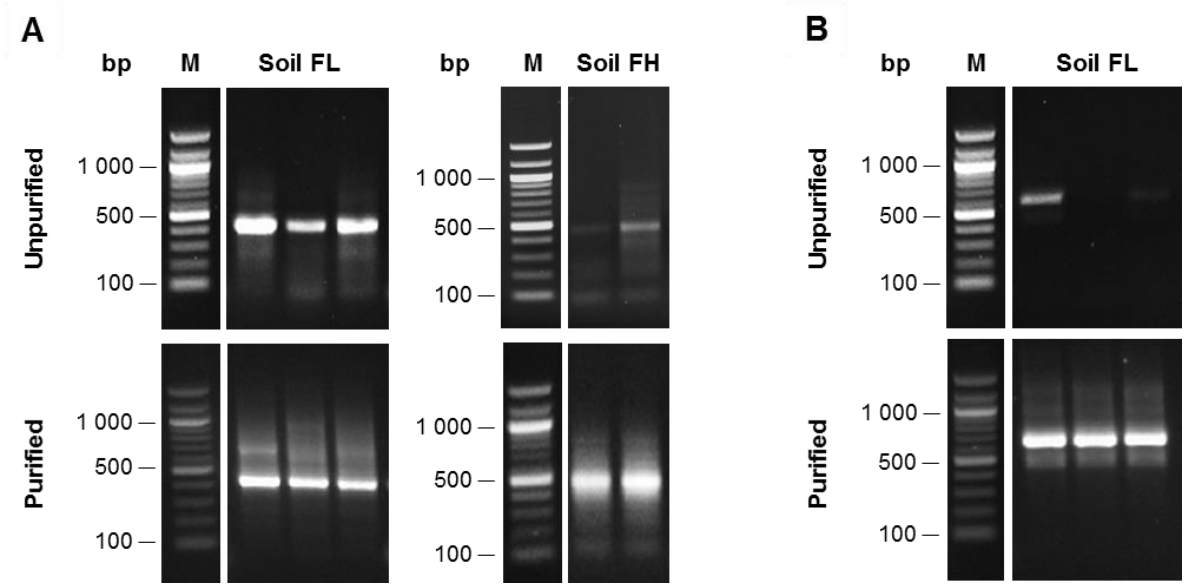


FIGURE S1 | Purifying crude DNA extracts prior to PCR gave stronger and more consistent amplification, regardless of primer used. Triplicate and duplicate samples were extracted from soils FL and FH respectively, using the unmodified Nicolaisen's method. Purified (using the Genomic DNA Clean & Concentrator kit) and unpurified DNA extracts were used in amplification reactions with primers targeting the A) *nosZ* gene (Z-F/1622R, expected amplicon size ~453 bp); or B) *narG* gene (1960f/2650r, expected amplicon size ~650 bp), and equal quantities of product were loaded onto the gels. The intensity of bands were compared by using the marker (M: 100 bp DNA ladder) as a standard of comparison across gels. The 'dimmer' marker bands in the "purified" gels reflect the intensity of the amplicons of interest, which required a shorter gel exposure period for the photograph.

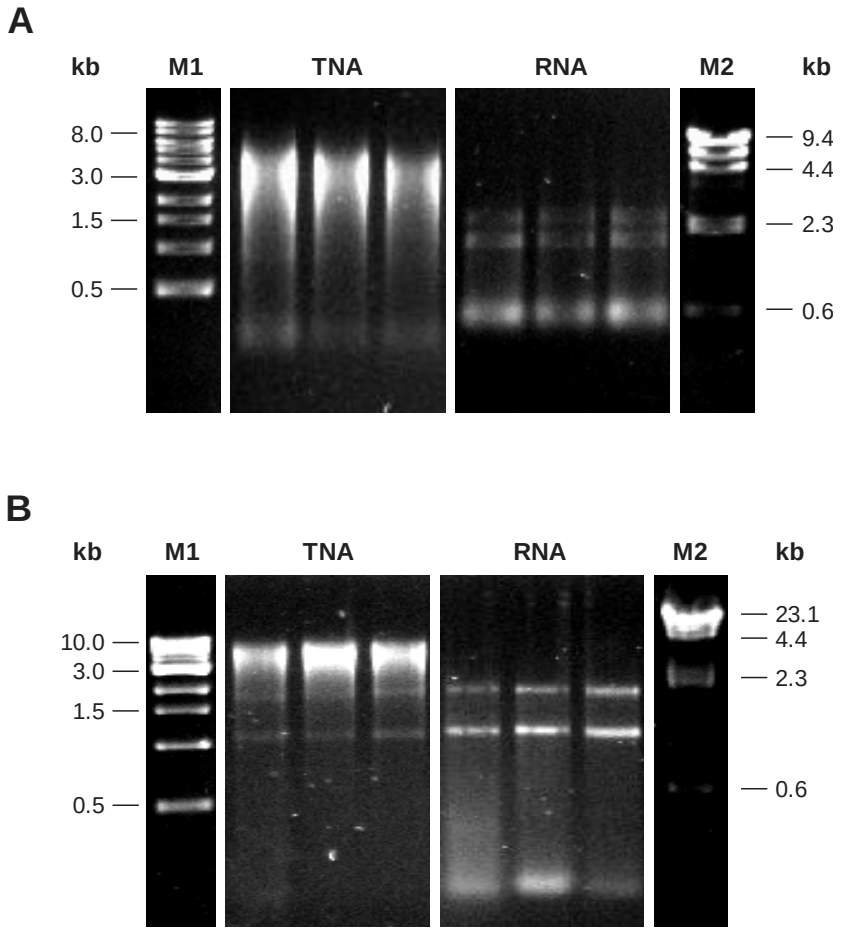


FIGURE S2 | Our simplified extraction method is capable of yielding RNA with little or no residual genomic DNA (gDNA) in the RNA fraction. Triplicate unamplified crude TNA extracts and DNase-digested RNA from soil FL using our simplified extraction method were analyzed on agarose gels to quickly assess the integrity of the nucleic acids. The gDNA smear (between 3 and 8 kb) is easily differentiated from the rRNA bands (the smaller bands under the gDNA smear) by gel electrophoresis. (A) The first gel was used to differentiate higher molecular weight fragments, to analyze the size of the gDNA smear. (B) The second gel was used to clearly separate the two smaller bands (presumed to be 23S and 16S rRNA) from the gDNA smear. The optimized purification of RNA prior to digestion retained most of the 16S and 23S rRNA despite complete digestion of gDNA (further confirmed with qPCR). Equivalent quantities of nucleic acid material (based on g^{-1} soil wet weight) was loaded into each well. Note that the gel has been spliced to remove unrelated samples, and the size of the RNA bands cannot be compared to the DNA markers used. M1: 1 kb DNA ladder. M2: λ DNA-HindIII marker.

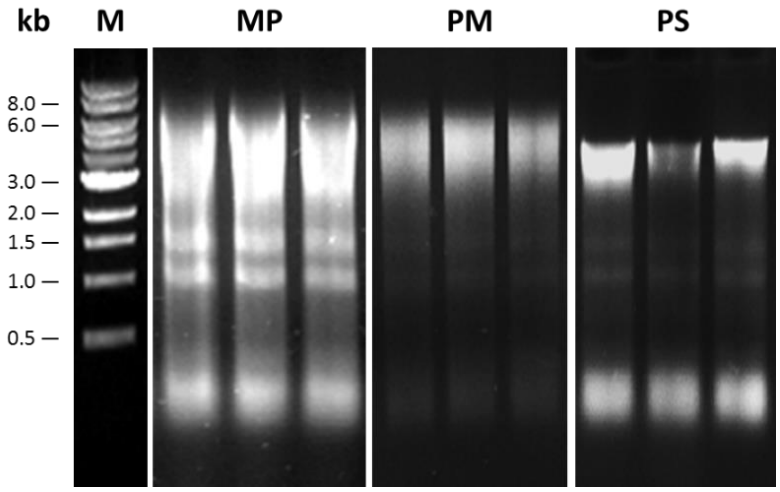


FIGURE S3 | Triplicate crude total nucleic acid (TNA) extracts was analyzed on a 1 % agarose gel to assess the integrity of DNA and RNA extracted from soil FL. The kits were used to extract TNA from three replicate soil samples. The gDNA smear (between 3 and 8 kb) is easily differentiated from the rRNA bands (the smaller bands under the gDNA smear) by gel electrophoresis. Equivalent quantities of extract (based on g^{-1} soil wet weight) were loaded into each well, showing clearly that some kits yield very dilute nucleic acid material. The PowerSoil RNA kit was supposed to elute only RNA, but there is obvious evidence of gDNA in all replicates, and very weak RNA bands are present, representative of the low amounts of RNA that is extracted. MP: MasterPure RNA Purification Kit (Epicentre Biotechnologies), PM: PowerMicrobiome RNA Isolation Kit, PS: RNA PowerSoil Total RNA Isolation Kit (both from MO BIO Laboratories). Note that the gels have been spliced for purposes of comparison. M: 1 kb DNA ladder.

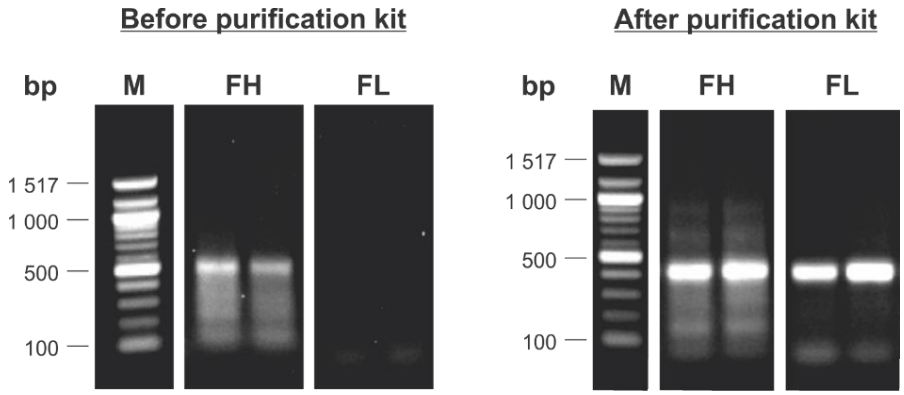


FIGURE S4 | Duplicate primary DNA extracts (Extract I, as seen in FIGURE 2) obtained with extraction kits from soil FH were amplifiable, but not from soil FL. Purification with the Genomic DNA Clean & Concentrator kit (gDCC) resulted in successful DNA amplification of the FL extract, and brighter amplicon bands from the FH extract (as seen by brighter non-specific amplicons greater than the expected size). The example above shows DNA extracts from soils FH and FL obtained using the PowerLyzer PowerSoil DNA Isolation Kit (PL), amplified with primers Z-F and 1622R targeting the *nosZ* gene, with an expected amplicon size of approximately 450 bp. Equal quantities of product were loaded onto the gels. The same trend was observed with other extraction kits. M: 100 bp DNA ladder.

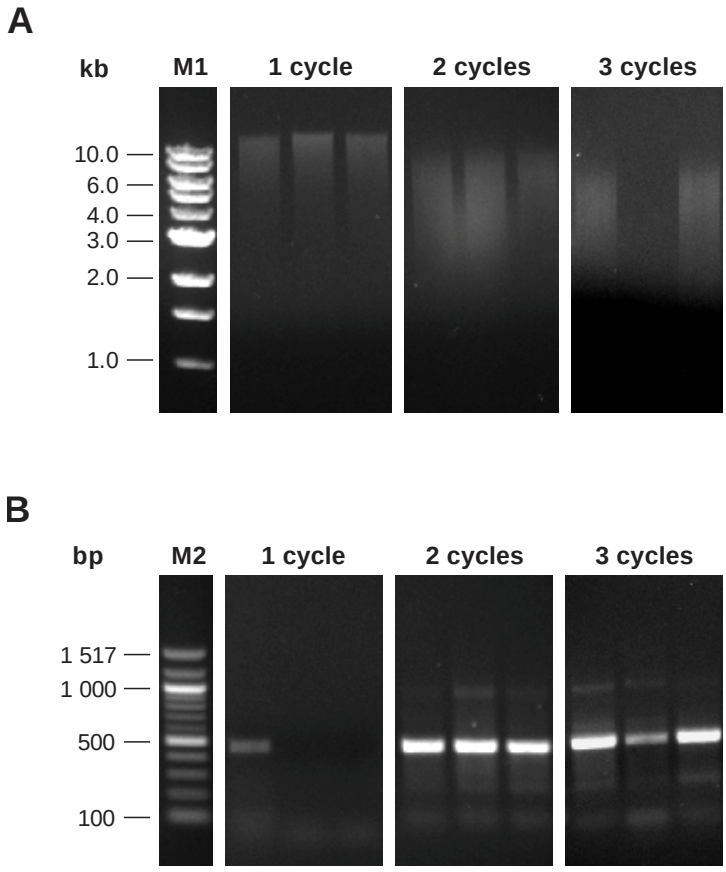


FIGURE S5 | The number of mechanical lysis cycles (via bead beating) affects both genomic DNA (gDNA) shear and amplifiability. Triplicate samples were extracted using the otherwise unmodified Nicolaisen's method and resulting nucleic acids were purified with the Genomic DNA Clean & Concentrator kit (gDCC). (A) The gDNA smear size decreased when samples were put through more than one cycle of bead beating, but there is no visible difference between two or three cycles of bead beating. The faint genomic smear is caused by low extraction efficiencies of a non-optimized method. (B) One cycle of bead beating did not yield amplifiable DNA, and three cycles of bead beating yielded more unspecific amplicons (as judged by stronger bands of the wrong fragment size). PCR was performed with primers Z-F and 1622R targeting the *nosZ* gene, with an expected amplicon size of approximately 450 bp. Equal quantities of product were loaded onto the gels. M1: 1 kb DNA ladder. M2: 100 bp DNA ladder.

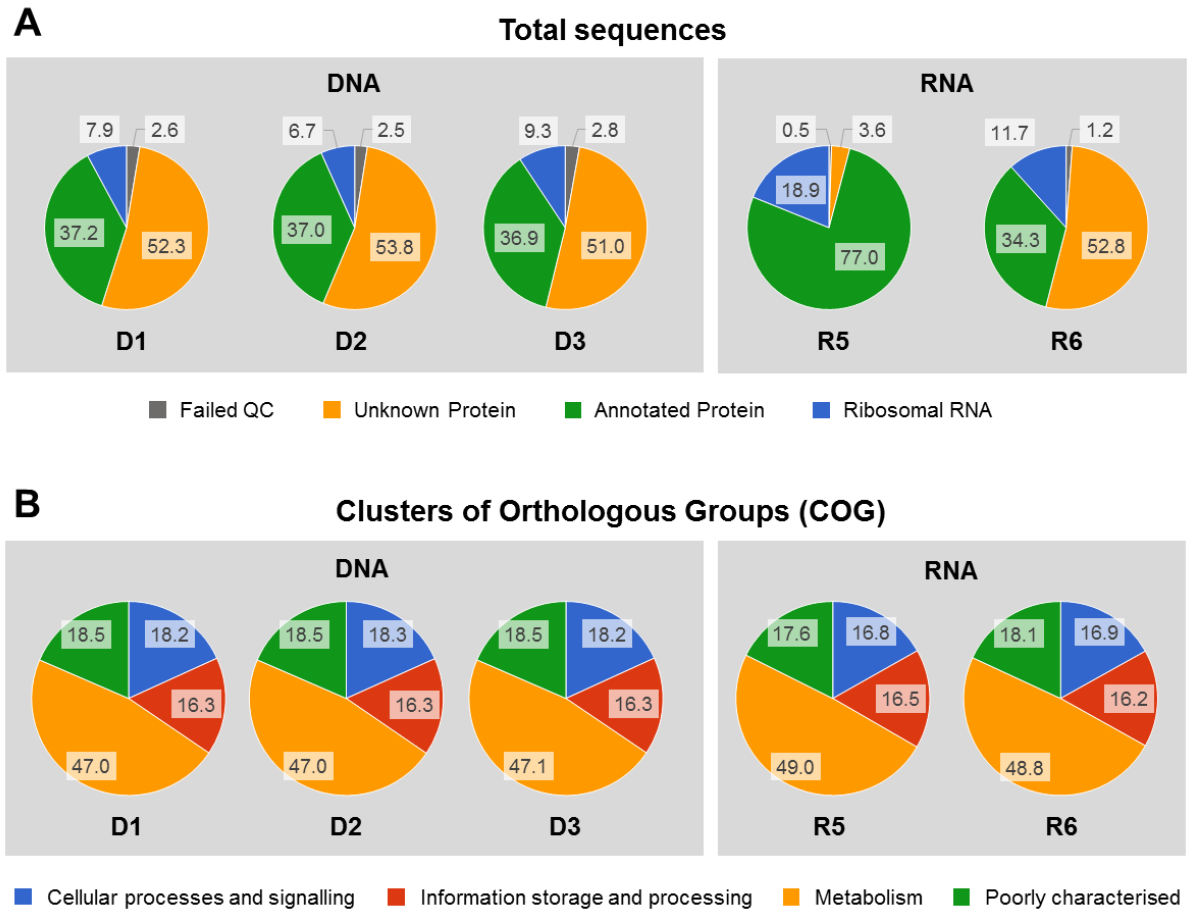
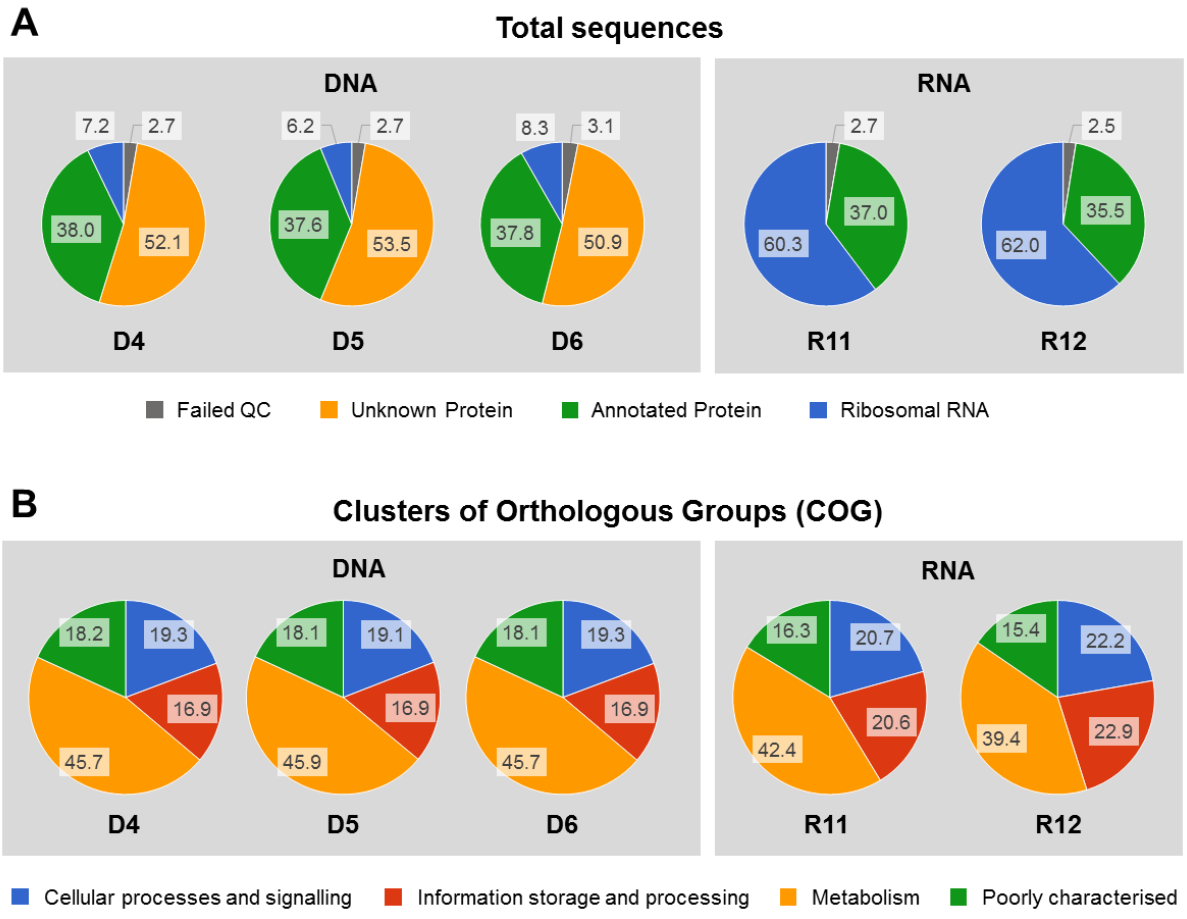


FIGURE S6 | Graphical breakdown of sequenced DNA and RNA samples extracted from soil FH (pH 6.80). DNA and RNA samples were sequenced in triplicate (D1, D2 and D3) and duplicate (R5 and R6), respectively. MG-RAST-annotated profiles were generated using A) Total sequences, and B) Clusters of Orthologous Groups (COG). The COG profile was generated from the green "Annotated Protein" segment of Total Sequences (A). Despite a higher proportion of "unknown protein" sequences in R6, the COG profile indicates good co-extraction replication for both DNA and RNA samples using the optimized method.



178

179

180 **FIGURE S7 | Graphical breakdown of sequenced DNA and RNA samples extracted from**
181 **soil FL (pH 3.80).** DNA and RNA samples were sequenced in triplicate (D4, D5 and D6) and
182 duplicate (R11 and R12), respectively. MG-RAST-annotated profiles were generated using
183 A) Total sequences, and B) Clusters of Orthologous Groups (COG). The COG profile was
184 generated from the green "Annotated Protein" segment of Total Sequences (A). Both Total
185 Sequence and COG profiles indicate good co-extraction replication for both DNA and RNA
186 samples using the optimized method.

187

4 References

Arbeli, Z., and Fuentes, C. L. (2007). Improved purification and PCR amplification of DNA from environmental samples. *FEMS Microbiol. Lett.* 272, 269–75. doi:10.1111/j.1574-6968.2007.00764.x.

Cullen, D. W., and Hirsch, P. R. (1998). Simple and rapid method for direct extraction of microbial DNA from soil for PCR. *Soil Biol. Biochem.* 30, 983–993. doi:10.1016/S0038-0717(98)00001-7.

Griffiths, R. I., Whiteley, A. S., O'Donnell, A. G., and Bailey, M. J. (2000). Rapid Method for Coextraction of DNA and RNA from Natural Environments for Analysis of Ribosomal DNA- and rRNA-Based Microbial Community Composition. *Appl. Environ. Microbiol.* 66, 5488–5491. doi:10.1128/aem.66.12.5488-5491.2000.

Hänni, C., Brousseau, T., Laudet, V., and Stehelin, D. (1995). Isopropanol precipitation removes PCR inhibitors from ancient bone extracts. *Nucleic Acids Res.* 23, 881–882. doi:10.1093/nar/23.5.881.

Kotiaho, M., Fritze, H., Merilä, P., Juottonen, H., Leppälä, M., Laine, J., et al. (2010). Methanogen activity in relation to water table level in two boreal fens. *Biol. Fertil. Soils* 46, 567–575. doi:10.1007/s00374-010-0461-0.

Krsek, M., and Wellington, E. M. H. (1999). Comparison of different methods for the isolation and purification of total community DNA from soil. *J. Microbiol. Methods* 39, 1–16. doi:10.1016/S0167-7012(99)00093-7.

LaMontagne, M. G., Michel, F. C., Holden, P. A., and Reddy, C. A. (2002). Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for microbial community analysis. *J. Microbiol. Methods* 49, 255–264. doi:10.1016/S0167-7012(01)00377-3.

Mettel, C., Kim, Y., Shrestha, P. M., and Liesack, W. (2010). Extraction of mRNA from Soil. *Appl. Environ. Microbiol.* 76, 5995–6000. doi:10.1128/aem.03047-09.

Nicolaisen, M. H., Baelum, J., Jacobsen, C. S., and Sørensen, J. (2008). Transcription dynamics of the functional tfdA gene during MCPA herbicide degradation by Cupriavidus necator AEO106 (pRO101) in agricultural soil. *Environ. Microbiol.* 10, 571–9. doi:10.1111/j.1462-2920.2007.01476.x.

Young, J. M., Rawlence, N. J., Weyrich, L. S., and Cooper, A. (2014). Limitations and recommendations for successful DNA extraction from forensic soil samples: a review. *Sci. Justice* 54, 238–44. doi:10.1016/j.scijus.2014.02.006.

Paper II

Soil pH dependent nitrite kinetics during anoxia; the role of abiotic reactions versus microbial reduction.

Natalie YN Lim, Åsa Frostegård, and Lars R Bakken

Under review in *Soil Biology and Biochemistry*

This page intentionally left blank

Soil pH dependent nitrite kinetics during anoxia; the role of abiotic reactions versus microbial reduction.

Natalie YN Lim [a], Åsa Frostegård [a] and Lars R Bakken [b]

[a] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1432 Ås, Norway

[b] Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, 1432 Ås, Norway

Corresponding author: Natalie YN Lim (Natalie.lim@nmbu.no)

Highlights

- Enzymatic reduction was the primary reason for nitrite suppression at pH <4
- Abiotic and biotic nitrite control was equal at pH≈5
- Abiotic nitrite decomposition mass balance show 50% conversion to nitroso-compounds
- Denitrification in acid soil: 10-20% of nitrate-N converted to nitroso-compounds

Keywords

Nitrite kinetics; chemodenitrification; acidic soil; denitrification;

Abstract

Nitrite concentrations in soils are normally low, but may increase transiently in response to high inputs of ammonia and anoxic spells. This could have cascade effects because nitrite is a signal molecule in the regulation of denitrification, a primary substrate for fungal denitrification, DNRA (dissimilatory nitrate reduction to ammonium) and anammox (anaerobic ammonium oxidation), and causes chemodenitrification. There is evidence that acidic soils accumulate much less nitrite than neutral soils. This could either be due to fast abiotic decomposition at low pH, or that the microbial community keeps nitrite concentrations low by high nitrite reductase activity (relative to nitrate reductase). To explore this, we monitored the kinetics of NO_2^-, NO, N_2O and N_2 during anoxic incubations of organic soils with pH_{CaCl2} ranging from 3.4 to 7.2, taken from a long-term liming experiment. In parallel, we determined the abiotic decomposition rates and its gas products by incubating gamma-irradiated soils amended with nitrite. The acidic soil (pH 3.4) kept nitrite concentrations at 20-50 µM during denitrification, except for a short spike reaching 160 µM. In contrast, the soils with higher pH (4.9 and 7.2) reached nitrite concentrations of >4 mM during denitrification. The analyses of the nitrite kinetics demonstrate that abiotic nitrite decay was significant in the pH 3.4 soil, yet the primary reason for the low nitrite in this soil was a high activity of nitrite reductase. The rates of abiotic nitrite decomposition largely equalled the rates of enzymatic nitrite reduction in soil at pH 4.9, but was insignificant in the pH 7.2 soil. Thus, microbial regulation of denitrification, rather than abiotic decomposition, accounted for the miniscule nitrite accumulation in these acidic soils during anoxic spells. Less than 100% of the nitrite was recovered as N-gas for the soils with pH 3.4 and 4.9, but N-mass balance was restored by taking abiotic nitrosation into account. These findings have profound implications for understanding the fate of nitrate/nitrite in acidic soils.

1 Introduction

Considering both its physiological and ecological importance, the kinetics of nitrite in soils while studied in the past, has not garnered the attention it deserves. Nitrite is a free intermediate in a number of reactions within the nitrogen cycle, including nitrification, denitrification, dissimilatory nitrate reduction to ammonium [DNRA, also known as respiratory ammonification (Mania et al., 2014; Yoon et al., 2015)]. It is also an important component of the regulatory networks of these metabolic pathways, performing the dual roles of being a mandatory reaction intermediate, and a signal molecule involved in controlling these alternative reductive pathways (Mania et al., 2014; Saunders et al., 1999).

Nitrite is chemically unstable, depending on pH and the presence of metals as well as organic compounds, decomposing to nitric oxide (NO), nitrous oxide (N_2O) or dinitrogen (N_2) by dismutation, reactions with metals (Zhu-Barker et al., 2015), or nitrosation [resulting in "hybrid N_2O and N_2" (Spott et al., 2011)]. Nitrite may also form stable covalent bonds with organic matter, especially in acid soils (Thorn and Mikita, 2000). Finally, nitrite in soils may escape to the atmosphere as gaseous nitrous acid (HONO), and this emission plays an important role in OH formation and tropospheric chemistry (Jacob, 2000; Kulmala and Petäjä, 2011; Su et al., 2011).

pH appears to be a key variable determining the biological effects and the fate of nitrite in the environment. Although nitrite is relatively stable and only moderately toxic at high pH, nitrite reactions, decomposition, and toxicity increase with decreasing pH. This reflects that undissociated nitrous acid (HNO_2) is more reactive than NO_2^- (the pK_a of $NO_2^- + H^+ \leftrightarrow HNO_2$ is 3.3), and that cell membranes are permeable to HNO_2 but not to NO_2^- (Kaiser and Heber, 1983; Samouilov et al., 2007). This in turn explains the antimicrobial effects of nitrite addition in acidic environments long observed by soil scientists (Bancroft et al., 1979).Transient accumulation of nitrite in soils typically occurs in response to fertilisation with reduced N (urea or ammonium), due to faster oxidation of ammonia to nitrite than the oxidation of nitrite to nitrate, as demonstrated by Shen et al. (2003). Such transient nitrite accumulation during enhanced nitrification depends on soil pH, and this is ascribed to nitrite oxidisers being sensitive to NH_3: the relative concentration of NH_3 over NH_4^+ increases exponentially with increasing pH (pK_a = 9.2) (Van Cleemput and Samater, 1996). Nitrite has also been observed to accumulate transiently in soil during denitrification (Glass and Silverstein, 1998; Stevens et al., 1998), and peak concentrations appear to increase with soil pH, though the reasons for this are unclear (Shen et al., 2003). It could either be due to fast abiotic nitrite decomposition at low

pH, or early and high expression of nitrite reductase (NIR) genes compared to NO reductase (NOR) genes in acid soils, plausibly caused by transcriptional regulation.

To investigate this, we monitored nitrite and denitrification kinetics during anaerobic incubations of soils of different pH. We found the expected pH-dependency of nitrite accumulation: transient nitrite accumulation decreased with pH. To assess the role of abiotic decomposition, we determined the concentration dependent rates of abiotic nitrite decomposition (and the fraction emitted as NO and N_2O) by incubating sterilised soils amended with nitrite. The first order decay kinetics, and the partitioning to N-gases (NO, N_2O and N_2) was used to assess the abiotic versus enzymatic reduction of N species observed in the live soil. This exercise demonstrated that the nitrite kinetics at neutral pH was entirely controlled by the biological regulation of the different steps in denitrification. In the most acidic soil, the enzymatic reduction of nitrite still dominated during the first 30 h of anoxia, but chemical decomposition gained momentum: this happened when the organisms finally managed to express N_2O reductase (N2OR), hinting at a competition for electrons between the two reductases.

2 Materials & Methods

2.1 Soils

Organic soils were collected from a long-term experimental field site in Fjaler, western Norway (61°17'42"N, 5°03'03"E) (Liu et al., 2010). The site is divided into 24 plots and limed with shell sand, 0-800 m^3 per hectare (1977) creating a pH range from pH 3.1 to pH 7.8 (Sognnes et al., 2006). In this paper, soils from three lime treatments pH were used: soil **L** (un-limed soil, pH 3.16-3.80), soil **M** (medium lime; 200 m^3 shell sand per hectare, pH 5.79-5.89), and soil **H** (high lime; 800 m^3 shell sand per hectare, pH 6.77-6.80). Two replicate plots were sampled treatments L and H; and one plot from treatment M. The soil from each plot was analysed separately. Only one plot was sampled from M because shell sand was unevenly distributed in the replicate plot, resulting in a pH that was too close to soil L for our purposes (the pH at the time of sampling was 4.34). All pH values were measured in 0.01 M $CaCl_2$ [1:5 w/w, soil fresh weight (fw) to 0.01 M $CaCl_2$] prior to using the soil. The soil organic C contents were 49, 45 and 40 % of dry weight (dw) in soil L, M and H, respectively. The declining C content with increasing pH was primarily due to the increasing amounts of shell sand added in 1977.

The soils were nearly water saturated when sampled (taken during the rainy season), and were immediately dried to reach a moisture level that allowed sieving (8 mm, followed by 4 mm). Large roots and plant residues were removed during the drying process, and the soils were frequently mixed by hand to avoid edge effects. The sieved soils were stored moist [61, 59 and 46 % moisture (w/w) in soil L, M and H, respectively] at 4 °C until use. The water holding capacity (WHC) of each soil was determined by flooding and free drainage in filter funnels; WHC was 82, 78 and 68 % moisture (% of fw) for soil L, M and H, respectively.

2.2 Soil sterilisation

Removing all bioactivity from the soils is necessary to determine the kinetics of abiotic decomposition of N-oxyanions (NO_3^- and NO_2^-). To determine the most suitable way to sterilise the soils with minimal effects on the soil chemistry, four commonly used sterilisation methods were tested on soils L and H. The methods were chosen based on their historical and/or frequent use in the literature (Labeda et al., 1975; Silva Aquino, 2012; Trevors, 1996; Tuominen et al., 1994).

Autoclaving: Soil (10 g fw) was measured into pre-weighed 120 mL serum vials, covered with aluminium foil, then autoclaved for 15 min at 121 °C and 15 psi. The extra moisture in the

vials post-autoclaving (condensation water) was removed by drying in a 50 °C oven until the vials reached the original weight. The aluminium foil covers were removed and the vials were sealed with pre-sterilised air-tight rubber septa and aluminium crimps in a class II biosafety cabinet.

Chloroform fumigation: Soil was transferred to disposable aluminium specimen containers, and kept to less than 5 cm in depth to ensure effective transport of chloroform into the soil matrix. The chloroform was water-washed to remove ethanol (the stabilising agent in chloroform), and transferred to a large glass evaporation dish with glass beads and boiling chips, then placed in the lower compartment of a chemical-resistant glass vacuum desiccator. The soil samples were placed on the perforated porcelain plate in the desiccator, which was then evacuated until the chloroform boiled, then kept under vacuum for 1 min before venting to laboratory air. This evacuation procedure was repeated three times, then the chamber was left sealed with a chloroform atmosphere for 24 h. The chloroform was then removed from the desiccator, and the soil was rinsed by evacuation and venting the chamber to laboratory air 15 times. The samples were left to laboratory air for 24 h before repeating the chloroform fumigation again. This "fumigation and air-exposed" procedure was repeated thrice. During the final air-exposure process, the samples were left on a laminar-air flow bench for 1.5 h to evaporate any residual chloroform left in the soil prior to transferring to glass vials and sealed with septa and crimps.

Gamma irradiation: Soil samples were given a dose of 27.8 kGy (^{60}Co) (at the Institute of Energy Technology, Kjeller, Norway). The gamma-irradiated soil was stored for 3 months at 4 °C before use, to deplete free radicals generated by radiolysis.

Glutaraldehyde immersion: Due to the similarity of modes of action of formaldehyde and glutaraldehyde, glutaraldehyde was used as a safer chemical equivalent to the more commonly used formaldehyde. Glutaraldehyde solution (2 %) was adjusted with HCl and NaOH to the pH of the two soils to be sterilised, and used to flood soil samples. The soils were transferred to Buchner funnels with a filter paper insert, and the glutaraldehyde solution was applied to the soil, left flooded for 15 min, before draining with vacuum addition. This "flood-drain" procedure was repeated four times (total exposure time ≈60 min). The soil was then left on vacuum for a further 30 min to remove excess liquid. The glutaraldehyde-treated soil was transferred to glass vials and sealed with septa and crimps in a biosafety cabinet.

2.3 Determining residual biological activity

The success of each sterilisation method was tested by incubating soils with filter-sterilised $NaNO_2$ (0.5 μmol g^{-1} soil fw), with and without glutamate (2.5 μmol g^{-1} soil fw), to aid in the detection of metabolic activity. The sterilised soils (10 g fw) were placed in 120 mL serum vials, the air replaced with He (to enable the detection of denitrification products) or He+1 vol% O_2 (for measuring O_2 consumption and CO_2 production). The O_2 consumption, CO_2 production, denitrification and/or chemodenitrification rates were monitored for 5 days. A water bath and thermostat kept the samples at 15 °C. The evolution and consumption of gases were monitored using a robotised auto-sampling and incubation system (Molstad et al., 2007). Headspace gases were sampled and measured automatically every 3-5 h by the system using a gas chromatograph and NO analyser: CO_2 and O_2 were monitored for respiratory activity, whereas NO, N_2O and N_2 gases were used to determine denitrification activity and abiotic NO_2^- decomposition to NO and N_2O. The amounts of NO and N_2O are either reported as measured (mol $vial^{-1}$), or as cumulated production, which is the measured amounts corrected for the losses by sampling (see Molstad et al., 2007).

Immediately following the oxic incubation, the numbers of viable organisms in the sterilised soils were determined by dilution plating on one-tenth (10 %) strength tryptic soy agar (TSA, Difco) with cycloheximide (100 μg/mL), and on malt agar (MA, Sigma-Aldrich) with streptomycin (100 μg/mL), to enumerate bacteria and fungi, respectively. The soils were dispersed in sterile water (1:4, w/w) by vigorous shaking and allowed to settle for ≈5 min before the supernatant was diluted and plated on agar, using both pour- and spread-plate techniques. The plates were incubated 15 °C for 4 days, and colony numbers were recorded daily.

2.4 Nitrite measurements

To monitor the fast degradation of nitrite in the acidic soils, a quick method for measuring nitrate and nitrite was developed. Briefly, 0.2-0.5 g of soil (fw) was transferred to pre-weighed microcentrifuge tubes for nitrite measurement, and sterile MilliQ water (1:2 w/w, soil fw to water) was added to extract the nitrite from the soil matrix. The soil slurry was agitated with a vortex for 5-10 s, then the soil solids were pelleted by centrifugation (17 600 x *g* for 2 min). Following, 10 μL of the supernatant was immediately injected into a purging device where nitrite or nitrate+nitrite (depending on reducing agent and temperature) was instantaneously reduced to NO which was transported (by a stream of N_2) through a Sievers Nitric Oxide Analyzer 280i system (NOA, GE Analytical Instruments). The integrated NO peaks were used

to estimate nitrite and nitrite+nitrate in the injected sample (calibrated by injecting standards). The reducing agents and temperatures were 1 M HCl with ≈50 mM VCl_3 (95 ^oC) to reduce nitrite+nitrate, and 1 % w/v NaI in 50 % acetic acid (room temperature) to reduce only nitrite. This chemiluminescence nitrate and nitrite measurement is capable of detecting picomole quantities in the injected liquid (Braman and Hendrix, 1989; Cox, 1980).

We suspected that the fast extraction with water could be affected by anion exchange, and tested this by comparing our water extraction procedure with the standard extraction in 2 mM KCl. This comparison was done for nitrate, rather than nitrite, since KCl is suspected to cause degradation of nitrite under acidic and neutral pH conditions (Homyak et al., 2015). The amount of nitrate extracted in water was 50-60 % of that extracted by 2 mM KCl (Supplementary Table S1), thus confirming a significant anion exchange capacity of the soils, leading to the recovery of only 50-60 % of the nitrite when using our rapid water extraction procedure.

To determine the kinetics of anion exchange, we measured the recovery of nitrite added to gamma-irradiated soils in short term experiments: microcentrifuge tubes containing 0.2 g soil fw (≈30 % dw) were given a dose of 100 nmol NO_2^- (10 μL of 10 mM KNO_2), and extracted at different times within the first 10 min. The measured concentrations showed a rapid decline during the first 5 min in all soils, approaching apparent equilibrium levels (50-60 % recovered) after 8-10 min (Supplementary material, Fig. S1). The concentration dependency of this anion partitioning (sorbed/free anions) was tested by adding a range of nitrite concentrations (50-1000 nmol per vial containing 0.2 g soil fw) which was extracted after 10 min. The fraction of nitrite recovered in the water extract (F) was practically constant over the entire concentration range for the two soils tested, F=0.49 and 0.65 for L and H, respectively (Supplementary material Fig. S2). These values were used for correcting the nitrite concentrations as measured in subsequent experiments (assuming an intermediate F value of 0.57 for soil M).

2.5 Kinetics of nitrite decomposition and gas production in gamma-irradiated soils

Gamma-irradiated soils were used to determine the kinetics of abiotic nitrite decay and the gas products. A first approach to determine nitrite decay under aerobic conditions was a 5 h experiment in microcentrifuge tubes: nitrite was added (10 μL of 10 mM NO_2^- = 100 nmol NO_2^- $vial^{-1}$) to a series of microcentrifuge tubes containing 0.2 g fw soil (≈0.1 g dw), and residual nitrite was measured at intervals using the rapid water extraction procedure described above. The length of the experiment proved too short to determine the decay rate in soil M and H,

hence a longer term experiment was conducted with these soils: gamma-irradiated soils supplemented with nitrite under oxic and anoxic conditions in serum vials at 15°C. Anoxic conditions were secured by repeated evacuation and He-filling. Each vial, containing 2 g soil, was amended with nitrite by spreading 0.1 mL of 10 mM KNO_2 onto the soil surface by a syringe. For each of five soils (2 replicates of L and H, a single for M), we prepared six 120 mL vials (3 oxic, 3 anoxic) which were monitored for gas production (NO, N_2O and N_2), and 22 small replicate vials (11 oxic and 11 anoxic) which were sacrificed consecutively (every 5 h) to determine the concentration of nitrite. The nitrite addition to the 120 mL vials for determination of the gas kinetics was done <1 min before the first sampling of each vial: nitrite was added to one vial at a time as the robot took gas samples. The 22 small vials were 12 mL vials that were prepared and treated the same way as the larger vials. Nitrite was determined by rapid water extraction of all the soils within the vial (adding 5 mL distilled water), corrected for the partitioning due to ion exchange (F = 0.49, 0.57 and 0.64 for soil L, M and H, respectively).

2.6 Kinetics of denitrification in live soils

Prior to the determination of denitrification kinetics in unsterilised soils, they were revitalised from cold storage as described by Liu et al. (2014): soils were amended with 5 mg dried, powdered clover g^{-1} soil fw and incubated at 15 °C for 72 h. The soils were then transferred to 120 mL serum vials; the amount of soil adjusted to have 1.5 g soil organic C per vial (fw equivalent to 3.06, 3.33 and 3.75 g soil dw $vial^{-1}$ for L, M and H, respectively). After sealing the vials with butyl-rubber septa and aluminium crimps, nitrate solutions were added by syringe onto the soil surface. The vials were then gently agitated to assist in mixing the soil (so not all the nitrate would be on the surface). The volumes and nitrate concentrations were adjusted for each soil to achieve a final water content of 80 % of the WHC (i.e. 66, 63 and 54 % moisture (w/w), soil L, M and H respectively) and 5 mM nitrate in soil moisture. This planning was based on nitrate concentration measured prior to revitalisation, which turned out to be lower than that at the onset of incubation (determined by subsamples that were analysed at the onset of incubation). The reason is most probably nitrification during the revitalisation period. Thus, at the onset of the incubation, the nitrate concentrations in the soil moisture was 6.2, 7.7, and 7.1 mM in soil L, M, and H, respectively, and the total amount of nitrate per vial was 37, 40 and 26 μmol nitrate (L, M and H respectively).

The vials were made anoxic by 6 cycles of gas evacuation and helium filling (Liu et al., 2010), and incubated at 15 °C. Gases (CO_2, O_2, NO, N_2O and N_2) in the headspace were measured every three hours using an autosampler linked to a GC and NO analyser (Molstad et al., 2007). At each gas sampling time point, one replicate vial of each soil type was opened and soil nitrite was measured.

3 Results

3.1 Comparison of sterilisation methods

Autoclaving and gamma-irradiation effectively sterilised both soils (H, and L), as evidenced by the absence of colony-forming bacteria (plate counting, results not shown) and extremely low oxygen consumption rates which were not enhanced by adding glutamate; tested 2 months after sterilisation. In the gamma-irradiated soils L, M and H incubated without glutamate, the oxygen consumption rates (μmol g^{-1} dw h^{-1}) were 0.018 (0.003), 0.24 (0.016) and 0.35 (0.028), respectively (standard error in parenthesis), and very similar and stable rates were recorded when incubated with glutamate.

Chloroform fumigation effectively eliminated aerobic respiration in soil L for the entire incubation period (immediately after sterilisation), but in soil H the effect was transient: respiration was practically zero during the first 20 h, and then increased exponentially. The flooding with glutaraldehyde failed to eliminate respiration. Thus, autoclaving and gamma-irradiation were the only methods that permanently eliminated microbial activity in both soils, while chloroform fumigation had a transient effect: the metabolic activity was effectively close to zero only during the first 20 h.

To further evaluate the effect of the sterilisation methods, we incubated soil anaerobically with glutamate and nitrite. The NO production during anaerobic incubations of sterilised soils to which nitrite was injected are shown in Fig. 1. Soil L (pH 3.4) showed rapid accumulation of NO reaching 900-1000 nmol $vial^{-1}$ during the first 1-2 h of anaerobic incubation for both the gamma-irradiated and chloroform-fumigated soils. The gradual decline thereafter is due to autoxidation (Nadeem et al. 2013). In comparison, the NO production by the autoclaved soil L was only ≈15 % of that in the chloroform fumigated and gamma-irradiated soil L (Fig. 1). For soil H, practically no NO was produced in any of the sterilised samples, except for a sudden burst in NO from the chloroform fumigated soil after ≈35 h. The latter was ascribed to the escalating metabolism in the chloroform fumigated soil, starting around 20 h after incubation (in the aerobic incubation used to test sterility, see above).

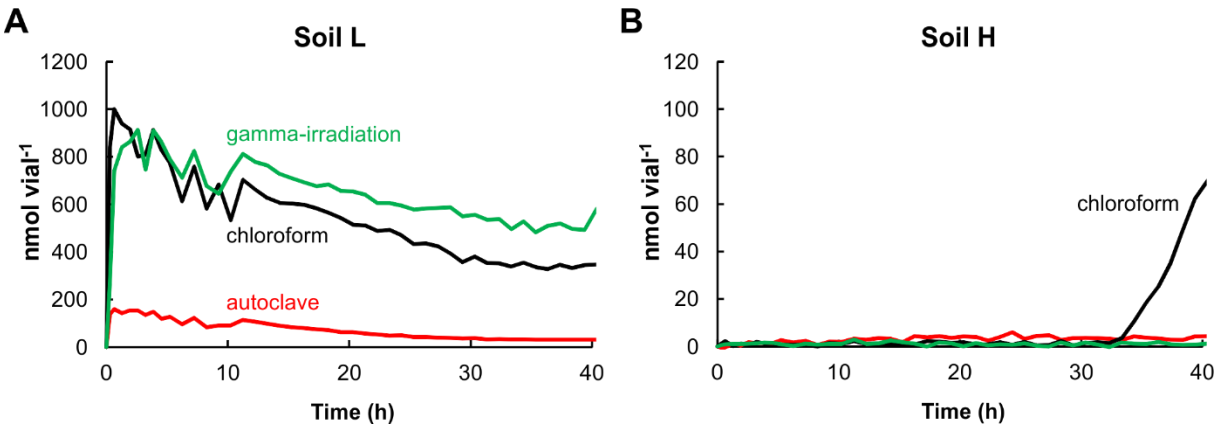


Fig. 1. Production of NO (nmol per vial) in autoclaved (red), chloroform-fumigated (black) and gamma-irradiated (green) soils incubated with glutamate and nitrite at 1 vol% O_2 in headspace. **A)** soil L (pH 3.4), **B)** soil H (pH 7.1).

Our purpose with soil sterilisation was to assess the kinetics of abiotic nitrite decomposition to NO (and possibly N_2O and N_2), and the results shown in Fig. 1 were taken to indicate that gamma irradiation was preferred over autoclaving, based on the following reasoning: None of the sterilisation techniques will leave the soil matrix unaffected (physically and chemically), thus there is a risk of biased assessment of the nitrite decay with any of the methods. However, chloroform fumigation had perceivably the least impact (compared to autoclaving and gamma sterilisation). The gamma-irradiated and chloroform fumigated soils showed practically identical NO kinetics in soil L, while autoclaved soil produced miniscule amounts of NO. We therefore assume that gamma irradiation had a less severe effect on relevant physical and chemical properties compared to autoclaving, which is known to induce quite profound changes both of structure and chemistry, as reviewed by Trevors (1996).

In summary, gamma-irradiation was the only of the four methods that was able to suppress microbial respiration in both soils L and H, and which had an apparent marginal interference with the abiotic nitrite decomposition. Additionally, soil pH was only marginally lowered by gamma-irradiation (3.44→3.40, 5.54→4.90, 7.24→7.06). Thus, gamma-irradiation was used to sterilise soils in all other experiments.

3.2 Nitrite decay and N gas kinetics in gamma-irradiated soils

The measured kinetics of nitrite anion exchange with the soils demonstrated that it took less than 10 min to reach equilibrium between free and adsorbed nitrite (Supplementary Fig. S1). In principle, the kinetic constants for ion exchange and nitrite decay could be determined by fitting a model that includes both phenomena, as demonstrated in Supplementary Fig. S3. This

exercise established, however, that the necessity of taking the kinetics of ion exchange into account is limited to the first 10 min after addition of nitrite. Hence, the measured nitrite >10 min after nitrite addition could be corrected for the soil specific partitioning at equilibrium. Table 1 summarises the partitioning and the estimated first order decay rates of nitrite in the gamma-irradiated soils (graphical presentation in Supplementary Fig. S4). The decay during oxic incubation appeared to be somewhat faster than for anoxic incubation (Fig. S5).

Plotting the first order decay rates against the fraction of un-dissociated HNO_2 (given $pK_a = 3.398$) revealed a linear relationship ($r^2 = 0.999$, Supplementary Fig. S6), suggesting that the decay of nitrite in all soils can be described by a first order decay of un-dissociated HNO_2 with the decay rate constant $k_{dHNO2}=1.43\ h^{-1}$. Thus the decay rate of total nitrite (TONI = NO_2^- + HNO_2) in a soil is given by

$$d(TONI)/dt = 1.43*[HNO_2]/([HNO_2]+[NO_2^-])$$

where $[HNO_2]$ and $[NO_2]$ is given by the total nitrite concentration and the soil pH (given that $[HNO_2]/([HNO_2]+[NO_2^-]=1/(1+10^{pH-pKa})$, where $pK_a = 3.398$).

Table 1. Decay rate of NO_2^- in gamma-irradiated soils. The table shows soil pH, the partitioning of nitrite ions during water extraction (R = estimated ratio between NO_2^- in the distilled water and NO_2^- adsorbed to soil particles after extraction with distilled water, WF = fraction of NO_2^- in the water ($=R/(R+1)$), and k_d = the estimated first order decay rate constant (h^{-1}) under anoxic conditions (standard error in parenthesis)

Lime treatment	pH	R	WF	k_d (h^{-1})
L	3.44	0.77	0.44	0.73 (0.065)
M	4.90	0.74	0.43	0.057 (0.007)
H	7.24	1.37	0.58	0.00055 (0.002)*

** the decay rate for soil H is not significantly different from zero.*

Gamma-irradiated samples of soil L, M and H, with and without nitrite, were incubated in a He (O_2-free) atmosphere and monitored for NO, N_2O and N_2 emissions by sampling every 5 h for 135 h. The N_2 production was essentially below detection limit for all soils: estimated cumulated N_2 production over the entire 135 h period ranged from -0.15 to +0.23 µmol N_2-N $vial^{-1}$ for the soils amended with nitrite (2.5 µmol NO_2^- $vial^{-1}$) and 0.17-0.31 µmol N_2-N $vial^{-1}$ for the soils without nitrite. Thus, there was a trend of soils with nitrite emitting less N_2 than those without amendment (Supplementary Table S2). In contrast, nitrite clearly enhanced the emission of NO and N_2O from the gamma-irradiated soil, as shown in Fig. 2, where cumulated production of the two gases are plotted against time, together with the cumulated nitrite

decomposition as predicted by the first order decay rates (Table 1). The nitrite-induced NO production clearly coincided with the decay of nitrite, while the nitrite-induced N_2O production continued beyond the depletion of nitrite (soil L and M). The fraction of nitrite decay recovered as NO was remarkably similar for all three soils (≈50 %), while the nitrite-induced N_2O production was clearly different: In soil H, the nitrite-induced N_2O production rate was similar to the nitrite decay rate; in the acidic soil L, nitrite-induced N_2O production was marginal; and the soil with the intermediate pH (soil M) stood out with a nitrite-induced N_2O production that was an order of magnitude higher than that of the two other soils.

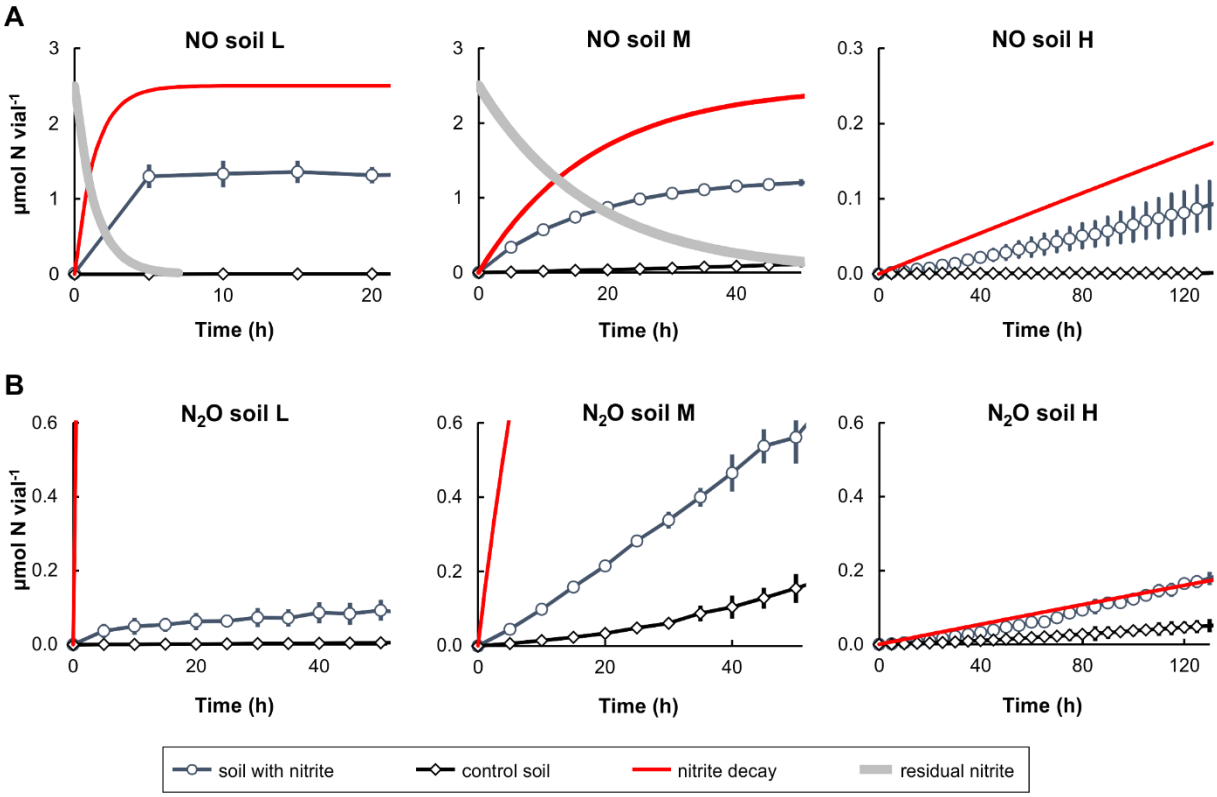


Fig. 2. Nitrite (NO_2^-) decay, NO and N_2O production in gamma-irradiated soil L (pH 3.4), M (pH 4.9) and H (pH 7.1). The panels show cumulated production of NO (**A**) and N_2O (**B**) in control soil (no nitrite added) and in nitrite amended soil (2.5 µmol NO_2 to 10 g soil fw in each vial). The residual nitrite, as predicted by the first order decay is shown as grey curves, and the red curves show the cumulated nitrite decay. Note that the scales are different and only the first part is reported for soil M and L to enhance visibility. Results for the entire incubation for all soils is found in Supplementary Fig. S7.

The fraction of nitrite decay recovered as NO during the first 10 h of incubation was 0.53, 0.52 and 0.20, for soil L, M and H, respectively. The fraction remained stable for soil L, declined slightly towards the end of the 135 h incubation for soil M (Supplementary Fig. S7), and for soil H there was an increasing trend. The fraction of nitrite decay recovered as N_2O

during the first 10 h was 0.02, 0.078 and 0.17 for soil L, M and H, respectively. This fraction increased gradually with time for all soils.

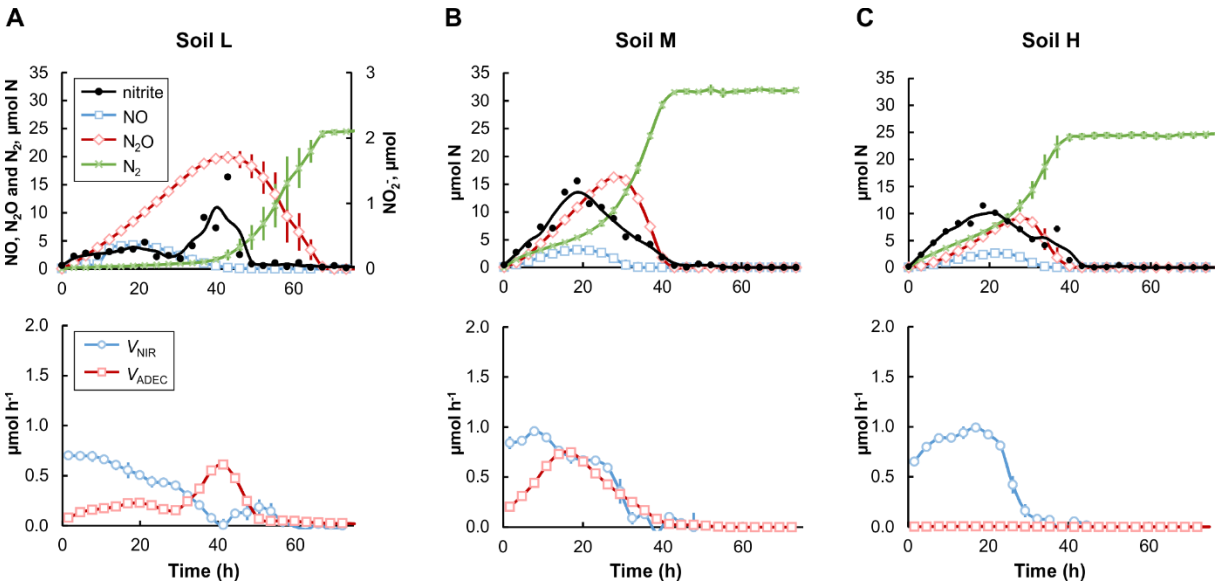
In order to use the abiotic nitrite decay kinetics (and the N gas production) when analysing the result of the nitrite kinetics in live soil (see below), we had to assume a constant product stoichiometry (NO and N_2O), and decided to use the fractions recovered as NO and N_2O at the time when nitrite decay exceeded 50 % for soil L and M, and after 10 h incubation for soil H.

3.3 Kinetics of denitrification in unsterilised soils, enzymatic reduction of nitrate versus abiotic decomposition.

Samples of unsterilised soil L, M and H were incubated under anoxic conditions with nitrate, and monitored for N-gas production. Parallel soil samples were treated identically in a series of vials which were analysed for nitrite (destructive sampling) at regular intervals.

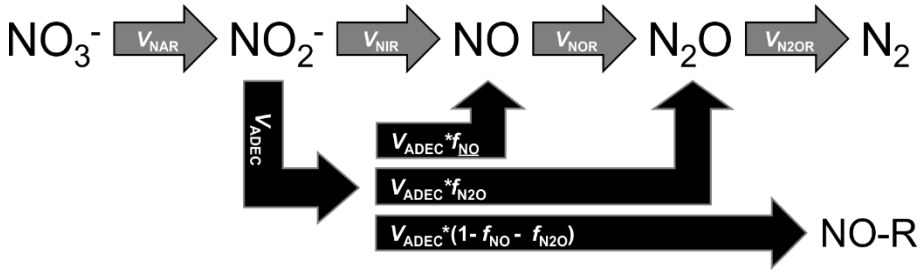
The kinetics of NO_2^-, NO, N_2O and N_2 for the three soils are shown in the top panels in Fig. 3. The cumulated N_2 reached plateaus at 24.5, 32 and 25 μmol N_2-N $vial^{-1}$ for soil L, M and H, respectively. In comparison, the initial amounts of nitrate was 37, 40 and 26 μmol $vial^{-1}$. Thus, for soil H, the cumulated N_2-N accounted for 96 % of the initial amount of nitrate N. The cumulated N_2-N as calculated is corrected for the N_2 lost by sampling, but not for the sampling loss of NO and N_2O. Taking these losses into account, which were 0.24 μmol NO and 0.81 μmol N_2O-N $vial^{-1}$, the recovery of nitrate-N as N-gases is 25+1=26.1 μmol N for soil H, which is 100 % of the initial nitrite-N.

For soils L and M, the recovery of nitrate-N as N_2 appears very much lower. In these soils; the sampling loss of NO and N_2O were much higher than for soil M, due to the high and long lasting peaks of NO and N_2O concentrations: The cumulated sampling loss of N_2O-N for L and M were 3.6 and 1.7 μmol N $vial^{-1}$ respectively, and the cumulated sampling loss of NO were 0.47 and 0.27 μmol N $vial^{-1}$. Thus, for soil L, the cumulated total recovery of N gas (N_2 + gas losses as N_2O and NO) was 24.5+3.6+0.47= 28.57 μmol N $vial^{-1}$, which is 77 % of the initial amounts of nitrite. The equivalent calculation for soil M gives 80 % recovery of nitrite N as N-gas production.



386

387 **Fig. 3.** Kinetics of denitrification and evaluation of abiotic NO_2^- decomposition versus enzymatic
388 reduction of NO_2^-. Top panels show the measured NO_2^- (single measurements and floating average as
389 black circles and lines, respectively), together with measured NO and N_2O and cumulated N_2 production
390 (i.e. corrected for dilution by sampling), and are averages of three replicate vials (standard deviation as
391 vertical lines). The lower panels show the estimated rates of enzymatic nitrite reduction (V_{NIR}) and the
392 rate of abiotic nitrite decomposition (V_{ADEC}); see text for explanation.



393

394 **Fig. 4.** Calculations of enzymatic and abiotic transformations. Enzymatic transformations are denoted
395 by grey arrows. Abiotic transformations (black arrows) were estimated based on measured
396 concentrations of nitrite, the first order decay, and partitioning, as observed in gamma-irradiated soils.
397 This allowed the estimation of enzymatic reduction rates based on the measured rates of change in NO_2^-,
398 NO, N_2O and N_2 (equations 1-6). V_{NAR}, V_{NIR}, V_{NOR}, and V_{N2OR} are the rates of enzyme-mediated reactions.
399 V_{ADEC} is the predicted rate of abiotic nitrite decomposition.

400 The measured rate of change in NO_2^-, NO, N_2O and N_2 were assumed to be the net result
401 of abiotic nitrite decomposition and enzymatic reductions, as illustrated in Fig. 4. We assumed
402 abiotic nitrite decomposition to follow the first order decay and its partitioning (to NO, N_2O
403 and NO-R) as in gamma-irradiated soil, which was thus predicted by the measured
404 concentration of nitrite and the decay rate constants (Table 1). Thus, the measured rates of
405 change for each N species (dNX/d*t*) and the concentration of nitrite could be used to estimate

the rates of enzymatic reductions (V_{NAR}, V_{NIR}, V_{NOR} and V_{N2OR}, denoting the rates of enzymatic reduction of NO_3^-, NO_2^-, NO, and N_2O, respectively) for each time increment. This was done consecutively through equations 1-4:

$$dN_2/dt = V_{N2OR} \quad (1)$$

$$dN_2O/dt = V_{NOR} + V_{AN2O} - V_{N2OR} \quad (2)$$

$$dNO/dt = V_{NIR} + V_{ANO} - V_{NOR} \quad (3)$$

$$dNO_2^-/dt = V_{NAR} - V_{NIR} - V_{ADEC} \quad (4)$$

where V_{NAR}, V_{NIR}, V_{NOR} and V_{N2OR} are the unknowns, dNX/dt is the measured rate of change of compound N_X, V_{ADEC} is the rate of abiotic nitrite decomposition as predicted by the measured nitrite concentrations, and the first order decay rates ($[NO_2^-]*k$, V_{ANO} and V_{AN2O} are the rates of NO and N_2O production by abiotic nitrite decomposition and the fractions emitted as NO (f_{NO}) and N_2O (f_{N2O}), equations 5-6:

$$V_{ANO} = V_{ADEC}*f_{NO} \quad (5)$$

$$V_{AN2O} = V_{ADEC}*f_{N2O} \quad (6)$$

where f_{NO} = 0.53, 0.52 and 0.2 for soil L, M and H, respectively, f_{N2O} = 0.02, 0.078 and 0.17 for soil L, M and H, respectively.

The resulting V_{ADEC} and V_{NIR} are shown in the lower panels of Fig. 3. For soil L, abiotic decomposition accounted for only 20-30 % of the total nitrite reduction during the first 30 h, but as V_{NIR} declined (coinciding with the onset of N_2O reduction), abiotic decomposition became the dominant sink for nitrite. In soil M, we see a similar pattern, but here the abiotic decomposition gained momentum earlier, and essentially equalled V_{NIR} until depletion of nitrite. In contrast to these two soils, abiotic decomposition of nitrite in soil H was insignificant throughout.

To inspect if abiotic nitrite decomposition in soil L and M could explain why much less than 100 % of the nitrate-N was recovered as N-gas in these soils (see above), we calculated the nitrate-N balance for each soil, including the abiotic formation of nitrosated/nitrosylated organic compounds, NO-R (Fig. 4, Table 2). The latter was estimated as the integral of V_{ADEC} multiplied by the fraction which was not recovered as N gas ($= \int V_{ADEC} dt*(1 - f_{NO} - f_{N2O})$; $\int V_{ADEC}$ = 14 and 17.1 μmol N, and $f_{NO} + f_{N2O}$ = 0.55 and 0.6 μmol N for soil L and M respectively). Based on our calculations, we were able to account for all added nitrate-N in soil M and H, and 94 % of added nitrate-N in soil L (Table 2).

Table 2. Nitrate N mass balance. The table shows the recovery of NO_3^--N as N gases (NO, N_2O and N_2) and as NO-R (abiotic reactions with soil organic matter, Fig. 4). The bottom row shows the total recovery (as % in parenthesis).

			Soil L			**Soil M**			**Soil H**
Initial NO_3			37			40.0			26.0
N-gas			28.6			34.0			26.1
NO-R	14*0.45	=	6.3	17*0.4	=	6.8	0.14*0.4	=	0.06
N accounted for			34.9			40.8			26.16
(%)			**(94 %)**			**(102 %)**			**(101 %)**

To inspect the kinetics of the various reductase reactions and the total electron flow, equations 1-4 were used to calculate the rates of the individual reductases and the total electron flow to denitrification throughout the entire incubation (Fig. 5). A conspicuous phenomenon revealed by these graphs is that in soil L and M, V_{NIR} declined substantially at the time when N_2O-reduction gained momentum. This decline in V_{NIR} was clearly not a result of nitrite depletion (see Fig. 3).

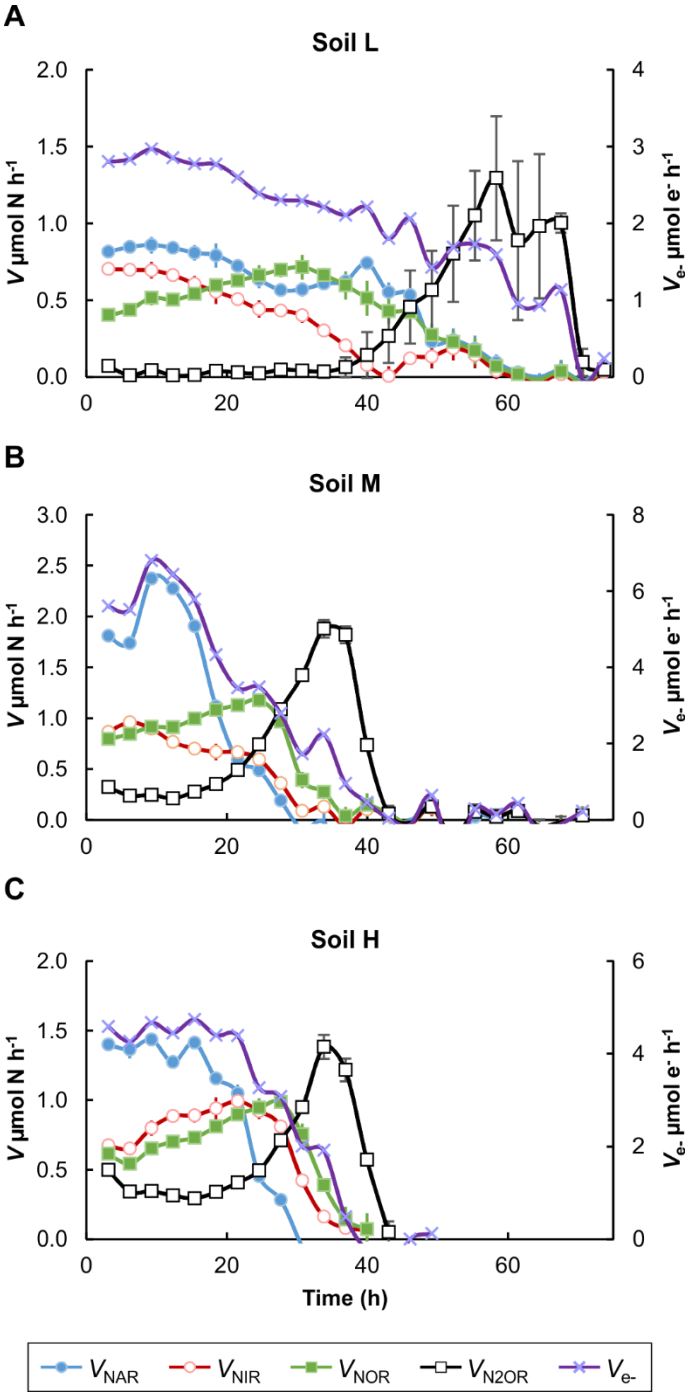


Fig. 5. Rates of individual reduction steps in denitrification. The panels show the rates of nitrate reduction (V_{NAR}), nitrite reduction (V_{NIR}), NO reduction (V_{NOR}) and N_2O reduction (V_{N2OR}), all as µmol N vial^{-1} h^{-1}. In addition, the total electron flow to denitrification is shown (V_{e-}, right axis), as µmol electrons vial^{-1} h^{-1}. The rates were based on measured gross transformations, solved for individual enzyme reaction through equations 1-4.

4 Discussion

Previous studies with soils of varying pH have often suggested that chemodenitrification plays a significant role in nitrite kinetics, with reference to "rapid" decomposition of nitrite under acidic conditions, but have not been able to precisely determine the magnitude of its effect and its dependency of soil pH (as reviewed by Spott et al., 2011 and Van Cleemput and Samater, 1996). In this paper, we have attempted to do so by meticulously determining the kinetics and product stoichiometry of abiotic nitrite decay and compared three soils of differing pH (ranging from highly acidic to near neutral) from the same field site. The kinetics of nitrite decomposition in these soils, as determined in gamma-irradiated soils, was convincingly first order, with decay rate constants that correlated strongly with the fraction of un-dissociated HNO_2, $F = [HNO_2]/([HNO_2]+[NO_2^-])$, as predicted by the soil pH. Thus, we have confirmed that soil pH is a good predictor of the abiotic nitrite decomposition rate in soil. The immediate gaseous products of HNO_2 was ≈50 % NO, a lower percentage of N_2O (that increased with soil pH), while N_2 production was marginal (not detectable). Thus, the formation of nitrosated soil organic N (R-ON) accounted for a significant fraction of the HNO_2 decay observed. The decay of R-ON could potentially account for the observed nitrite-induced N_2O emissions beyond the depletion of nitrite in soil L and M (Fig. 1 and Supplementary Fig. S7). This process has previously been defined as codenitrification, and since N_2O appeared to be the sole hybrid gaseous product (the other possible hybrid being N_2), this process is probably dominated by the nitrosation of amines, which are thought to decay to N_2O (Spott et al., 2011).

Using these abiotic nitrite decay rates, the biological enzymatic rates (V_{NIR}) of nitrite decomposition were determined for each soil (Fig. 3). These estimated rates of enzymatic versus abiotic nitrite decay demonstrated that abiotic nitrite decay could not account for the very low nitrite accumulation in the unsterilised acid soil L. In this soil, the microorganisms clearly kept nitrite concentrations low by high NIR activity compared to that of nitrate reductase (NAR), except for the brief period after 30 h. Interestingly, this coincided with the onset of N2OR activity, suggesting that N2OR was able to effectively compete with NIR for available electrons (since the total electron flow V_{e^-} remained essentially unchanged, Fig. 5). In soil M and H, NAR activity greatly exceeded that of NIR initially, resulting in the high transient nitrite accumulation observed (Fig. 3). As nitrite accumulated in soil M, the rates of abiotic nitrite decomposition increased to practically the same level as the enzymatic nitrite reduction. In soil H, however, the chemical decomposition of nitrite played no significant role and stayed at consistently negligible rates throughout.

Thus, in soil M and H, there was a preferential initial reduction of nitrate; either because nitrate-respiring organisms are more abundant than denitrifiers *sensu stricto*, or because the latter preferentially reduce nitrate to nitrite. This preference maybe either due to competition for electrons (NAR, stronger than NIR), or due to transcriptional regulation (*nar* gene expression preceding that of *nir*) as was previously observed in bacterial strains (Liu et al., 2013; Qu et al., 2016). The absence of such preferential reduction of nitrate to nitrite in the most acidic soil (be it caused by low numbers of nitrate-respiring organisms, or by the regulatory phenomena mentioned) probably reflects the high toxicity of nitrite at low pH due to un-dissociated HNO_2. These contrasting explanations for nitrite handling in soils M and L shed new light upon nitrite kinetics in acidic systems.

To date, it is not unusual in the literature to consider "acidic environments" (pH $\leq$5.5) as a whole, and there has been little indication of suspicion that abiotic nitrite transformations under such conditions may differ upon further increasing acidity (Spott et al., 2011; Van Cleemput and Samater, 1996). However, acid-tolerant specialist microorganisms (Parkin et al., 1985; Van Den Heuvel et al., 2010), diverse gene transcriptional regulation and phenotypes (Bergaust et al., 2011; Liu et al., 2013), and pH-dependent chemistry of soils and organic compounds (Stevenson et al., 1970; Thorn and Mikita, 2000), together have the potential to create complex and unpredictable whole-environment responses: The stronger biological control of nitrite observed in soil L could not have been predicted based on data derived from soil H and M, nor vice versa for soil M using data from soil H and L. Thus, one must take care not to ignore potentially dissimilar chemical-biological processes and interactions (even when dealing with soils from the same site), and that extrapolation of such processes may not always prove accurate.

Needless to say, the calculated nitrogen flows via denitrification and abiotic decomposition of nitrite is based on the assumption that the nitrite decomposition kinetics (and its product stoichiometry) observed in the gamma-irradiated soil is representative for the abiotic processes in the non-sterilised soil. We have no clear proof for this assumption, but find it rather plausible based on the nitrate N mass balance calculations: around 20 % of the nitrite N was not recovered as N-gas in soil L and M, but the inclusion of the estimated formation of nitrosated soil organic N could effectively account for this missing nitrate N. In soil H, the estimated nitrite decomposition was insignificant, and as expected, 100 % of the nitrite N was successfully recovered as N-gas. In theory, dissimilatory reduction of nitrite to ammonium (DNRA) could have accounted for some of the missing nitrate-N in the soil L and M. However, DNRA has

been found to be negligible in acidic soils compared to that in neutral and alkaline soils (Zhang et al., 2015). In our experiments, DNRA appears to be an insignificant sink, even in soil H (pH 7.24), considering the 100 % recovery of nitrate-N as N-gas. A reasonable conclusion is therefore that DNRA played a negligible role in our experiments.

5 Conclusions

Contrary to widespread assumption that chemical processes are likely the dominant source of nitrite scavenging under acidic conditions (Dail et al., 2001; McKenney et al., 1990; Nömmik and Thorin, 1972; Yamulki et al., 1997), we have provided strong evidence for biologically-driven control of nitrite levels in acidic environments during denitrification. However, abiotic nitrite decomposition did play a role, not only in keeping nitrite concentrations low, but also in having profound implications for the fate of nitrate-N: at low and intermediate pH, nitrite decomposition resulted in conversion of a significant fraction (10-20 %) of nitrate-N to nitroso-compounds. This underscores the need to take the abiotic nitrite kinetics into account in studies for nitrogen redox transformations in soils with pH $\leq$ 5.

Acknowledgements

The authors thank Bioforsk Vest Fureneset for providing the Fjaler soils, and Heidi Hillier (MSc student in KBM, NMBU) for sample collection and assisting in laboratory work.

References

Bancroft, K., Grant, I.F., Alexander, M., 1979. Toxicity of NO2: Effect of Nitrite on Microbial Activity in an Acid Soil. Appl. Envir. Microbiol. 38, 940–944.

Bergaust, L., Bakken, L.R., Frostegård, Å., 2011. Denitrification regulatory phenotype, a new term for the characterization of denitrifying bacteria. Biochemical Society Transactions 39, 207–212.

Braman, R.S., Hendrix, S.A., 1989. Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium(III) reduction with chemiluminescence detection. Analytical Chemistry 61, 2715–2718. doi:10.1021/ac00199a007

Cox, R.D., 1980. Determination of nitrate and nitrite at the parts per billion level by chemiluminescence. Analytical Chemistry 52, 332–335. doi:10.1021/ac50052a028

Dail, D.B., Davidson, E.A., Chorover, J., 2001. Rapid abiotic transformation of nitrate in an acid forest soil. Biogeochemistry 54, 131–146. doi:10.1023/A:1010627431722

Glass, C., Silverstein, J., 1998. Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. Water Research 32, 831–839. doi:10.1016/S0043-1354(97)00260-1

Homyak, P.M., Vasquez, K.T., Sickman, J.O., Parker, D.R., Schimel, J.P., 2015. Improving Nitrite Analysis in Soils: Drawbacks of the Conventional 2 M KCl Extraction. Soil Science Society of America Journal 79, 1237–1242. doi:10.2136/sssaj2015.02.0061n

Jacob, D.J., 2000. Heterogeneous chemistry and tropospheric ozone. Atmospheric Environment 34, 2131–2159. doi:10.1016/S1352-2310(99)00462-8

Kaiser, G., Heber, U., 1983. Photosynthesis of leaf cell protoplasts and permeability of the plasmalemma to some solutes. Planta 157, 462–470. doi:10.1007/BF00397204

Kulmala, M., Petäjä, T., 2011. Soil Nitrites Influence Atmospheric Chemistry. Science 333, 1586–1587. doi:10.1126/science.1211872

Labeda, D.P., Balkwill, D.L., Casida Jr., L.E., 1975. Soil sterilization effects on *in situ* indigenous microbial cells in soil. Canadian Journal of Microbiology 21, 263–269. doi:10.1139/m75-037

Liu, B., Frostegård, Å., Bakken, L.R., 2014. Impaired reduction of N2O to N2 in acid soils is due to a posttranscriptional interference with the expression of nosZ. mBio 5, e01383-14. doi:10.1128/mBio.01383-14

Liu, B., Mao, Y., Bergaust, L., Bakken, L.R., Frostegård, Å., 2013. Strains in the genus Thauera exhibit remarkably different denitrification regulatory phenotypes. Environmental Microbiology n/a-n/a. doi:10.1111/1462-2920.12142

Liu, B., Mørkved, P.T., Frostegård, Å., Bakken, L.R., 2010. Denitrification gene pools, transcription and kinetics of NO, N2O and N2 production as affected by soil pH. FEMS Microbiology Ecology 72, 407–417. doi:10.1111/j.1574-6941.2010.00856.x

Mania, D., Heylen, K., van Spanning, R.J.M., Frostegård, Å., 2014. The nitrate-ammonifying and nosZ -carrying bacterium Bacillus vireti is a potent source and sink for nitric and nitrous oxide under high nitrate conditions. Environmental Microbiology 16, 3196–3210.

578 doi:10.1111/1462-2920.12478

579 McKenney, D.J., Lazar, C., Findlay, W.J., 1990. Kinetics of the Nitrite to Nitric Oxide Reaction
580 in Peat. Soil Science Society of America Journal 54, 106.
581 doi:10.2136/sssaj1990.03615995005400010016x

582 Molstad, L., Dörsch, P., Bakken, L.R., 2007. Robotized incubation system for monitoring gases
583 (O2, NO, N2O N2) in denitrifying cultures. Journal of Microbiological Methods 71, 202–
584 211. doi:http://dx.doi.org/10.1016/j.mimet.2007.08.011

585 Nadeem, S., Dörsch, P., Bakken, L.R., 2013. Autoxidation and acetylene-accelerated oxidation of
586 NO in a 2-phase system: Implications for the expression of denitrification in ex situ
587 experiments. Soil Biology and Biochemistry 57, 606–614.
588 doi:http://dx.doi.org/10.1016/j.soilbio.2012.10.007

589 Nömmik, H., Thorin, J., 1972. Transformations of 15N-labelled nitrite and nitrate in forest raw
590 humus during anaerobic incubation, in: IAEA (Ed.), Isotopes and Radiation in Soil Plant
591 Relationships Including Forestry. IAEA-SM-151/58, Vienna, Austria, pp. 369–382.

592 Parkin, T.B., Sexstone, A.J., Tiedje, J.M., 1985. Adaptation of Denitrifying Populations to Low
593 Soil pH. Applied and Environmental Microbiology 49, 1053–1056.

594 Qu, Z., Bakken, L.R., Molstad, L., Frostegård, Å., Bergaust, L.L., 2016. Transcriptional and
595 metabolic regulation of denitrification in *Paracoccus denitrificans* allows low but significant
596 activity of nitrous oxide reductase under oxic conditions. Environmental Microbiology 18,
597 2951–2963. doi:10.1111/1462-2920.13128

598 Samouilov, A., Woldman, Y.Y., Zweier, J.L., Khramtsov, V.V., 2007. Magnetic resonance study
599 of the transmembrane nitrite diffusion. Nitric Oxide 16, 362–370.
600 doi:10.1016/j.niox.2006.12.006

601 Saunders, N.F.W., Houben, E.N.G., Koefoed, S., de Weert, S., Reijnders, W.N.M., Westerhoff, H.
602 V., De Boer, A.P.N., Van Spanning, R.J.M., 1999. Transcription regulation of the nir gene
603 cluster encoding nitrite reductase of Paracoccus denitrificans involves NNR and NirI, a
604 novel type of membrane protein. Molecular Microbiology 34, 24–36. doi:10.1046/j.1365-
605 2958.1999.01563.x

606 Shen, Q.., Ran, W., Cao, Z.., 2003. Mechanisms of nitrite accumulation occurring in soil
607 nitrification. Chemosphere 50, 747–753. doi:10.1016/S0045-6535(02)00215-1

608 Silva Aquino, K.A. da, 2012. Sterilization by Gamma Irradiation, in: Gamma Radiation. InTech.
609 doi:10.5772/34901

610 Sognnes, L.S., Fystro, G., Øpstad, S.L., Arstein, A., Børresen, T., 2006. Effects of adding moraine
611 soil or shell sand into peat soil on physical properties and grass yield in western Norway.
612 Acta Agriculturae Scandinavica, Section B - Soil & Plant Science 56, 161–170.
613 doi:10.1080/09064710500218845

614 Spott, O., Russow, R., Stange, C.F., 2011. Formation of hybrid N2O and hybrid N2 due to
615 codenitrification: First review of a barely considered process of microbially mediated N-
616 nitrosation. Soil Biology and Biochemistry 43, 1995–2011.
617 doi:10.1016/j.soilbio.2011.06.014

618

Stevens, R.J., Laughlin, R.J., Malone, J.P., 1998. Soil pH affects the processes reducing nitrate to nitrous oxide and di-nitrogen. Soil Biology and Biochemistry 30, 1119–1126. doi:10.1016/S0038-0717(97)00227-7

Stevenson, F.J., Harrison, R.M., Wetselaar, R., Leeper, R.A., 1970. Nitrosation of Soil Organic Matter: III. Nature of Gases Produced by Reaction of Nitrite with Lignins, Humic Substances, and Phenolic Constituents Under Neutral and Slightly Acidic Conditions. Soil Science Society of America Journal 34, 430–435. doi:10.2136/sssaj1970.03615995003400030024x

Su, H., Cheng, Y., Oswald, R., Behrendt, T., Trebs, I., Meixner, F.X., Andreae, M.O., Cheng, P., Zhang, Y., Pöschl, U., 2011. Soil nitrite as a source of atmospheric HONO and OH radicals. Science (New York, N.Y.) 333, 1616–8. doi:10.1126/science.1207687

Thorn, K.A., Mikita, M.A., 2000. Nitrite fixation by humic substances: Nitrogen-15 nuclear magnetic resonance evidence for potential intermediates in chemodenitrification. Soil Science Society of America Journal 64, 568–582.

Trevors, J.T., 1996. Sterilization and inhibition of microbial activity in soil. Journal of Microbiological Methods 26, 53–59. doi:10.1016/0167-7012(96)00843-3

Tuominen, L., Kairesalo, T., Hartikainen, H., 1994. Comparison of methods for inhibiting bacterial activity in sediment. Applied and Environmental Microbiology 60, 3454–7.

Van Cleemput, O., Samater, A.H., 1996. Nitrite in soils: accumulation and role in the formation of gaseous N compounds. Fertilizer Research 45, 81–89. doi:10.1007/BF00749884

Van Den Heuvel, R.N., Van Der Biezen, E., Jetten, M.S.M., Hefting, M.M., Kartal, B., 2010. Denitrification at pH 4 by a soil-derived Rhodanobacter-dominated community. Environmental Microbiology 12, 3264–3271. doi:10.1111/j.1462-2920.2010.02301.x

Yamulki, S., Harrison, R.M., Goulding, K.W.T., Webster, C.P., 1997. N2O, NO and NO2 fluxes from a grassland: Effect of soil pH. Soil Biology and Biochemistry 29, 1199–1208. doi:10.1016/S0038-0717(97)00032-1

Yoon, S., Cruz-García, C., Sanford, R., Ritalahti, K.M., Löffler, F.E., 2015. Denitrification versus respiratory ammonification: environmental controls of two competing dissimilatory NO3−/NO2− reduction pathways in Shewanella loihica strain PV-4. The ISME Journal 9, 1093–1104. doi:10.1038/ismej.2014.201

Zhang, J., Lan, T., Müller, C., Cai, Z., 2015. Dissimilatory nitrate reduction to ammonium (DNRA) plays an important role in soil nitrogen conservation in neutral and alkaline but not acidic rice soil. Journal of Soils and Sediments 15, 523–531. doi:10.1007/s11368-014-1037-7

Zhu-Barker, X., Cavazos, A.R., Ostrom, N.E., Horwath, W.R., Glass, J.B., 2015. The importance of abiotic reactions for nitrous oxide production. Biogeochemistry 126, 251–267. doi:10.1007/s10533-015-0166-4

Supplementary material

Soil pH dependent nitrite kinetics during anoxia; the role of abiotic reactions versus microbial reduction.

Natalie YN Lim [a], Åsa Frostegård [a] and Lars R Bakken [b]

[a] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, 1432 Ås, Norway

[b] Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, 1432 Ås, Norway

Corresponding author: Natalie YN Lim (Natalie.lim@nmbu.no)

Nitrite recovery by rapid extraction in water

The kinetics of anion exchange was investigated by rapid water extraction at time intervals during the first 10 min after addition of nitrite to soils (10 mL of 10 mM KNO_2, added to 0.2 g soil fresh weight). The result is shown in Fig. S1, together with modelled kinetics according to equation S1

$$\frac{dNO_2^-{}_w}{dt} = -k \cdot (NO_{2w} - NO_{2S} \cdot R) \qquad \text{(S1)}$$

where NO_{2w} is "free nitrite", NO_{2s} is adsorbed nitrite, k is the rate constant (min^{-1}) and R is the ratio NO_{2w}/ NO_{2s} at equilibrium.

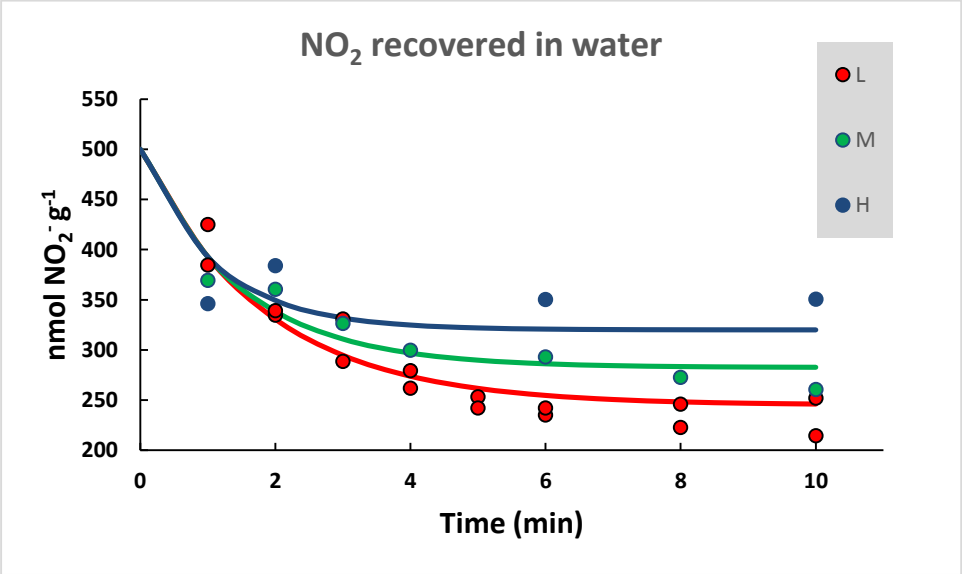


Fig. S1. Short term equilibration of nitrite by ion exchange with the soil matrix. The figure shows the measured nitrite (nmol g^{-1} soil fresh weight) in the supernatant after rapid extraction in microcentrifuge tubes (centrifuged immediately after vortexing for 10-15 sec), at time intervals after adding 500 nmol g^{-1} fresh weight (% dry weight was 25, 42 and 43 for soil L, M and H respectively) The curves show predicted values, assuming R = 0.96, 1.32 and 1.78 for the soils with pH 3.4, 4.9 and 7, respectively, and k = 0.21 min^{-1}. P is the fraction of adsorbed NO_2^- at equilibrium and k is the transfer coefficient; as defined by equation S1. The fraction of total nitrite at equilibrium is $R/(1+R)$.

To further elucidate the effect of ion exchange and to determine the exact partitioning at equilibrium, two types of experiments were conducted. First, nitrate was used as a surrogate for nitrite, and the efficiency of water extraction was evaluated by comparing with nitrate extracted by 2 M KCl. Table S1 summarises the recovery in water extracts compared to KCl. It shows a low recovery for the water extraction, confirming that anion exchange is significant.

Table S1. Nitrate extracted by the rapid water extraction procedure compared to extraction with 2 M KCl. Standard error is shown in parenthesis (n=4-6).

Soil	**NO_3^- in solution, µmol g^{-1}**	
	2 M KCl	**MilliQ water**
L	11.1 (0.6)	6.9 (0.3)
H	13.9 (0.2)	9.5 (0.6)

The fraction of nitrite extracted by water is theoretically affected by the total amount of nitrite present; it is expected to increase when nitrite concentrations approach the anion exchange capacity of the soil. To inspect this, we added a range of nitrite concentrations to two of the soils (gamma-irradiated soil L and H), and performed water extractions 10 min after addition. The measured nitrite in the water is shown in Fig. S2, plotted against the added amounts of nitrite.

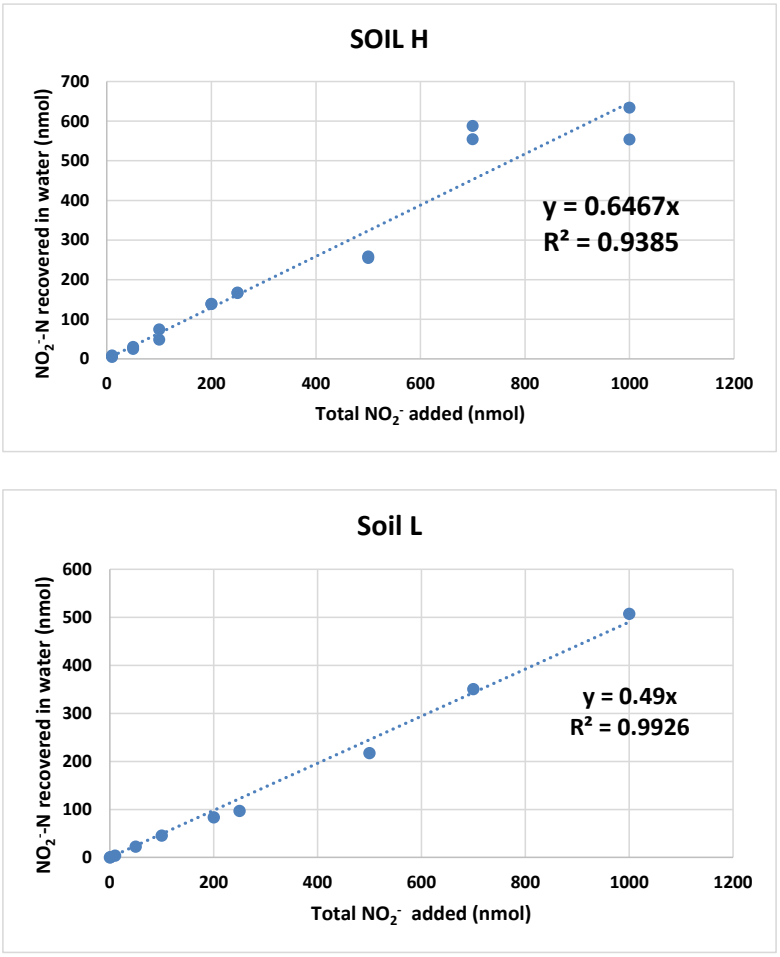


Fig. S2. Recovery of added NO_2^- by rapid water extraction, 10 min after addition. Experiment conducted in microcentrifuge tubes containing 0.2 g fresh weight soil [25% dry weight for soil L (pH 3.4) and 40 % dry weight for soil H (pH7.1)] to which 10 µL of KNO_2 (concentration range 1-100 mM) was added. Nitrite was extracted with 0.5 mL distilled water. Linear regression functions are shown; the regression coefficients estimating the fraction of total NO_2^- extracted, F = 0.49 for the soil L and 0.65 for soil L. An intermediate value of F = 0.57 was assumed for the soil with intermediate pH (soil M). These values were used for the simulation of the kinetics shown in Fig. S1 (R in equation 1 is equal to $F/(1-F)$).

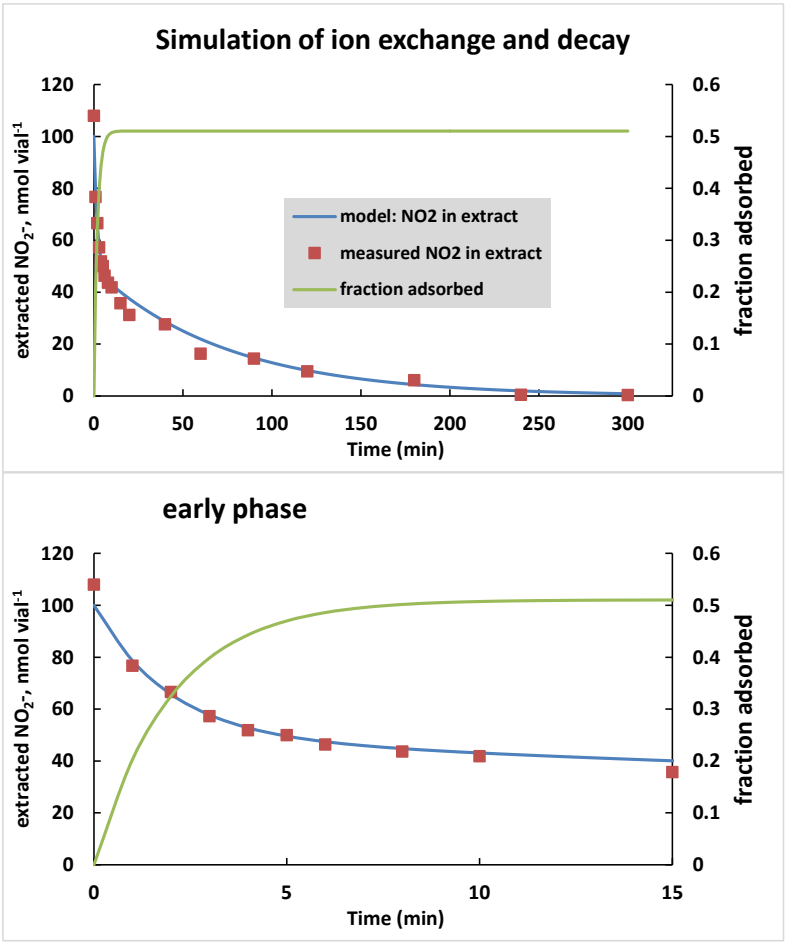


Fig. S3. Simulation of ion exchange and decay during the 0-5 h oxic experiment with soil L. The panel shows measured nitrite in water extract (nmol $vial^{-1}$), and the simulation of the kinetics of nitrite in water extracts based on the combined kinetics of ion exchange (Fig. S1) and first order nitrite decay. The ion exchange rate is given by equation S1. The decay rate is assumed to be first order with respect to total NO_2^-; $d(NO_{2w}+NO_{2s})/dt=-k(NO_{2w}+NO_{2s})$. The green line shows fraction of total NO_2^- adsorbed; i.e. $1/(1+R)$ (equation S1). The model was fitted to data, and the parameter values are t = 0.2 min^{-1} and k = 0.013 min^{-1}, equivalent to 0.78 h^{-1} , which is slightly higher than that determined for anoxic incubations of the same soil (0.73 h^{-1}; Fig. S5).

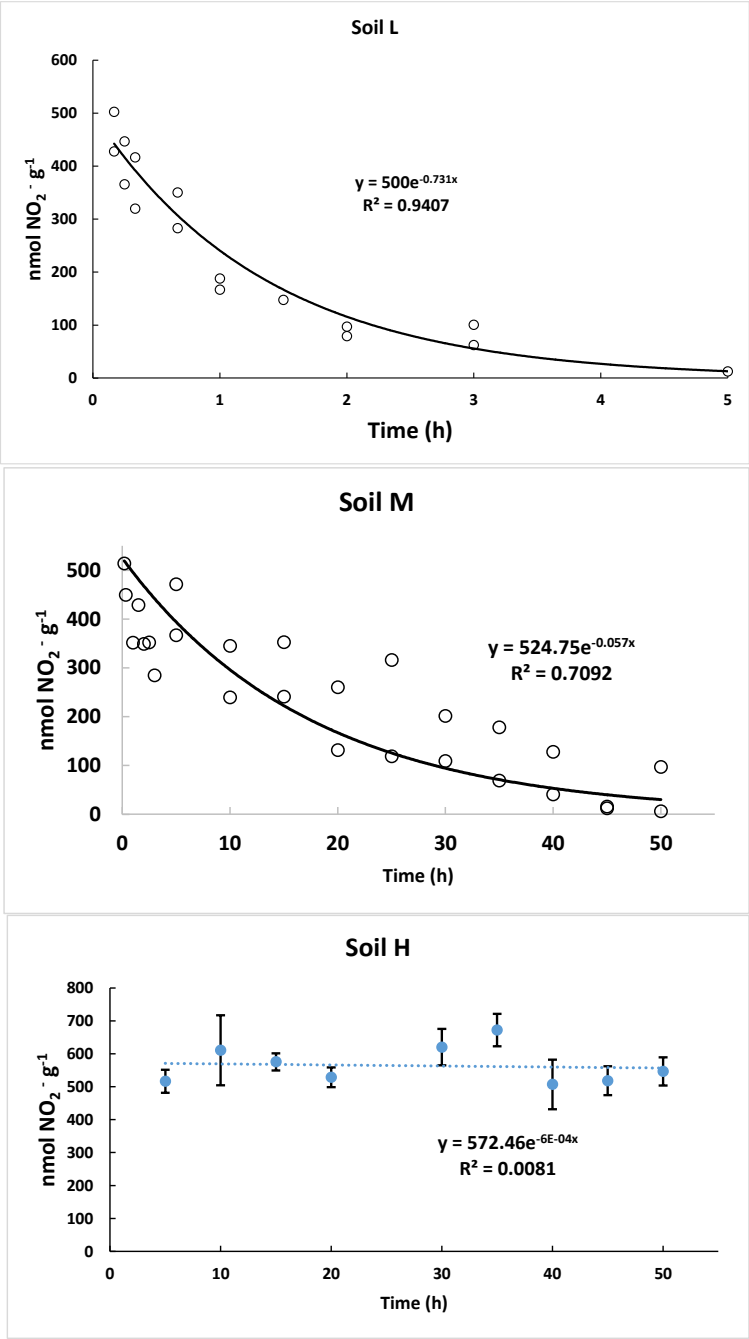


Fig. S4. Nitrite decay during anoxic incubations of gamma-irradiated soils. The panels show residual nitrite (nmol NO_2^- g^{-1} fresh weight soil against time. The top panel shows the result for the 0-5 h experiment with soil L, excluding the data for the first 10 min (due to lack of equilibration between adsorbed and extractable nitrite, see Fig. S1). The lower two panels show the results for soil M and H. Single measurements are shown for soil L and M, and average for 4 replicates are shown for soil H. First order decay functions fitted to data are shown for each soils. Residual nitrite is calculated from measured nitrite in water (fast extraction), corrected for the fraction of extractable nitrite for each soil (see Fig. S2). Estimated decay rates constants (h^{-1}) for each soil are:
Soil L: 0.73 h^{-1} (SE: 0.065)
Soil M: 0.057 h^{-1} (SE: 0.007)
Soil H: 0.00055 h^{-1} (SE: 0.002)

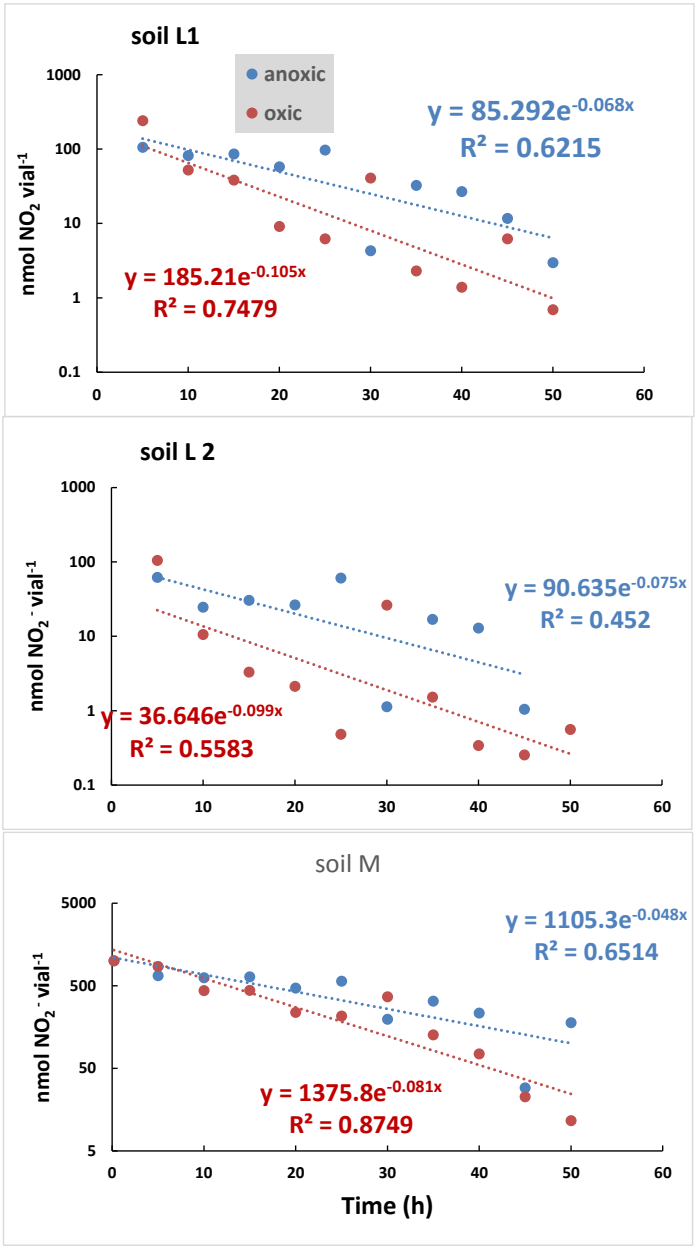


Fig. S5. Comparison of aerobic and anaerobic nitrite decay in gamma-irradiated soils. 2 g soil (fresh weight) was incubated in 12 mL vials crimp sealed with butyl rubber septa. One set was kept aerobic, the other was anaerobised (replaced atmosphere with He) prior to injection of nitrite (spreading 0.1 mL 10 mM KNO_2 onto the surface). At time intervals, vials were sacrificed to measure residual nitrate. The panels show the result for three soils (2 replicates of soil L and one for M), and the fitted exponential functions.

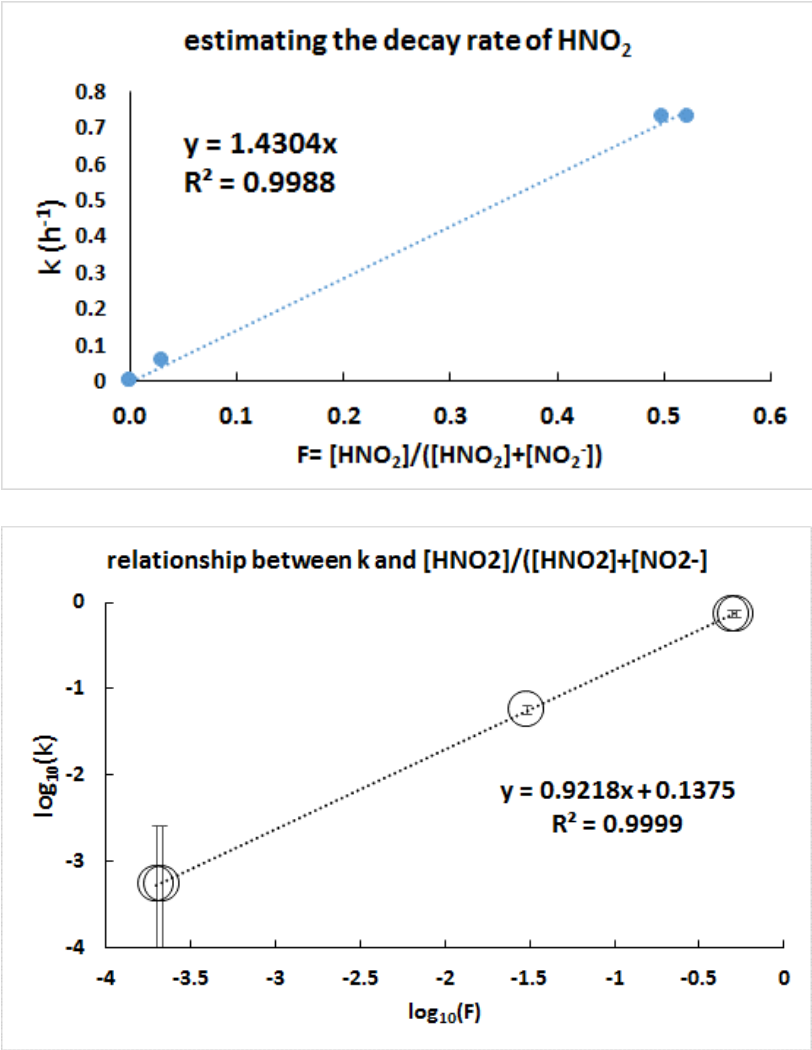


Fig. S6. Relationship between un-dissociated HNO_2 and observed decay rates of total nitrite (TONI=NO_2^-+HNO_2) in the three soils. The two panels show the estimated first order decay rates of nitrite (i.e. NO_2^-+HNO_2) plotted against the fraction of un-dissociated HNO_2. Top panel is a linear plot, the lower panels shows a log-log plot. The regression function in the top plot effectively estimates the first order decay rate of un-dissociated HNO_2 in the soils (k_{HNO2} = 0.143 h^{-1}, since we assume that d[TONI]/dt=[TONI]$*F*k_{HNO2}$). The regression function for the lower plot should in theory be y=$\log_{10}(F*k_{HNO2})$ =$\log_{10}(F)$+ $\log_{10}(k_{HNO2})$, thus the estimated k_{HNO2} is $10^{0.1375}$ = 1.37 h^{-1}.

Table S2. Measured N_2-N production (µmol $vial^{-1}$); cumulated production during the entire 135 hour anaerobic incubation of gamma-irradiated soils, with and without 2.5 µmol NO_2^- $vial^{-1}$ (=1 µmol g^{-1} soil fresh weight; soil moisture = 50% w/w). The average values for three replicate vials of each soil are shown, with standard deviation. The last column (Δ) shows the difference between vials with and without NO_2^-.

	with NO_2^-		**Control**		Δ
	avg	**stdev**	**avg**	**stdev**	
Low pH	0.04	0.12	0.20	0.13	-0.15
Mid pH	0.23	0.24	0.31	0.29	-0.08
High pH	-0.15	0.22	0.17	0.10	-0.31

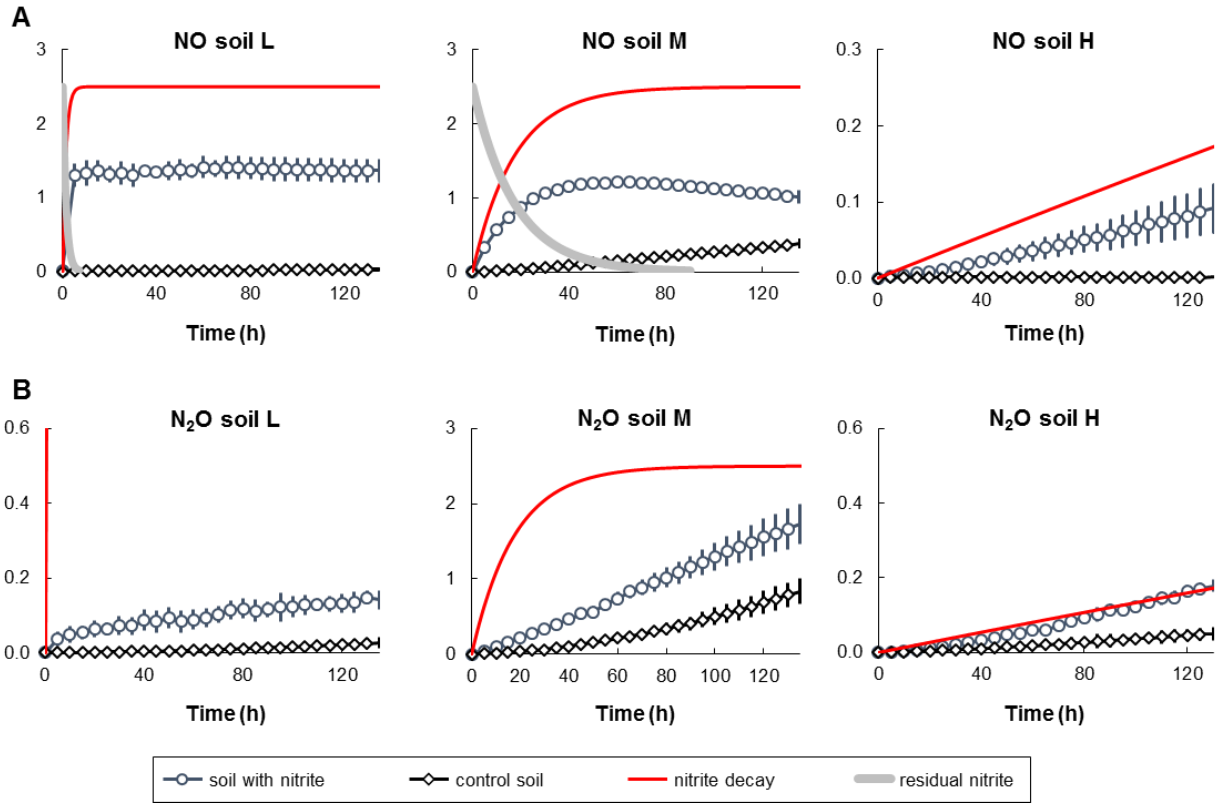


Fig. S7. NO production by NO_2^- decay in gamma-irradiated soil during anoxic incubation (2.5 µmol $NaNO_2$ was added to 5 g soil fresh weight). Entire incubation shown for all soils (equivalent to Fig. 2 in the main paper). The panels show cumulated production of NO (panel A) and N_2O (panel B) in control soil (no nitrite added) and in nitrite amended soil (2.5 µmol NO_2 to 10 g soil fresh weight in each vial). The residual nitrite, as predicted by the first order decay is shown as grey curves, and the red curves show the cumulated nitrite decay. The decline in NO in soil M after 50 h is due to neither sampling nor autoxidation.

Paper III

Linking meta-omics to the kinetics of denitrification intermediates reveals pH-dependent causes of N_2O emissions and nitrite accumulation in soil.

Natalie YN Lim, James P Shapleigh, Lars R Bakken, and Åsa Frostegård

Manuscript in preparation

This page intentionally left blank

Linking meta-omics to the kinetics of denitrification intermediates reveals pH-dependent causes of N_2O emissions and nitrite accumulation in soil

Natalie YN Lim[1]*, James P Shapleigh[2], Lars R Bakken[3] and Åsa Frostegård[1]

[1] *Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway*

[2] *Department of Microbiology, Cornell University, Ithaca, NY, USA*

[3] *Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, Ås, Norway*

* Corresponding author: Natalie YN Lim (natalie.lim@nmbu.no)

Abstract

Nitrite and nitric oxide are central molecules in multiple N-transformations. Soils emit substantial amounts of HONO (nitrous acid, derived from nitrite) and NO, both playing key roles in tropospheric chemistry. Under hypoxic conditions, their concentrations are controlled by denitrification reductases for NO_2^- (NirK and NirS), NO (cNor and qNor). The third denitrification intermediate is the greenhouse gas N_2O, regulated by NosZ. The regulatory network of denitrification is known for a few model bacteria, while transcription of denitrification genes in intact soil communities is less well documented, partly because PCR primers targeting denitrification genes only capture a fraction of the community. One major factor affecting the net production and accumulation of denitrification intermediates is soil pH. Here, two soils (pH 3.8 and 6.8) from the same experimental field-site were incubated under anoxia with NO_3^-, revealing transient accumulation of NO_2^- and NO. Complete denitrification to N_2 was only observed in soil 6.8, with soil 3.8 accumulating N_2O instead. The metagenome/-transcriptomes were sequenced, and denitrification-related genes were annotated. With the exception of high *qnor* in soil 3.8, the two soils had similar denitrification genetic potential. Contrary to qPCR results, metagenomics/-transcriptomics showed clear dominance of *nirK* and *qnor* over *nirS* and *cnor*. Transcription of *nar+nap* in soil 6.8 was higher than *nirK+nirS*, possibly explaining the transient accumulation of ≈50% of NO_3^--N as NO_2^-. Lack of NO_2^- accumulation in soil 3.8 could not be explained solely from transcriptomics, and was tentatively ascribed to a combination of metabolic control and chemodenitrification. This is the first metatranscriptomic study providing evidence that a wide range of denitrifiers transcribe *nosZ* genes in acid soil, but fail to produce functional N_2O reductase, indicating a species-independent overarching post-transcriptional phenomenon. The overall taxonomic profiles of genes and transcripts in both soils suggest a tendency towards modular denitrification by multiple organisms, instead of a singular process by complete denitrifiers.

1 Introduction

Starting with the soluble N-oxyanions nitrate (NO_3^-) and nitrite (NO_2^-), denitrification is the stepwise reduction through the intermediate nitric oxide (NO), ending with either nitrous oxide (N_2O) or dinitrogen gas (N_2). Nitrite and NO are central to several N-transforming processes and are biologically dangerous if not properly regulated. The former, which is reduced in denitrification by the nitrite reductases (NIR) NirK or NirS, is potentially toxic to microorganisms in acidic environments due to its propensity to form nitrous acid (HNO_2), which is able to pass through cell membranes (Bancroft et al., 1979; Kaiser and Heber, 1983). However, it is widely acknowledged that nitrite does not accumulate in highly acidic environments due to chemodenitrification, the abiotic degradation of nitrite under decreasing pH (Bancroft et al., 1979; Schreiber et al., 2012). Unfortunately, reports on nitrite levels in soils are scarce and the possible ecological consequences of nitrite accumulation in soils have not been thoroughly analysed. Such consequences may include transcriptional activation of denitrification genes in the presence of O_2 (Bergaust et al., 2011), which could hamper the activity of N_2O reductase (N2OR), leading to increased N_2O emissions. On the other hand, nitrite under anoxic conditions may also be used by microorganisms performing dissimilatory nitrite reduction to ammonia (DNRA), thus diverting the flow of nitrite-N from denitrification.

The other denitrification intermediate, NO, is an important signalling molecule with the potential to severely harm microorganisms, and is reduced by the NO reductases (NOR) cNor and qNor (Medinets et al., 2015). Involved in the regulation of various denitrification-related gene operons, NO activates regulators such as NsrR and the Crp-Fnr superfamily of transcriptional regulators (which includes DNR, NNR, and NnrR) to provide wide-ranging effects in the denitrification process (Bergaust et al., 2012; Körner et al., 2003; Torres et al., 2016; Vollack and Zumft, 2001).

Additionally, both nitrite and NO are important compounds in atmospheric chemistry as precursors to the reactive gaseous nitrous acid (HONO), which is believed to be responsible for much of the formation of highly oxidative OH in the troposphere and the subsequent production of harmful ozone (Kulmala and Petäjä, 2011; Li et al., 2012; Su et al., 2011). Soil-based nitrification and denitrification have been suggested to majorly contribute to HONO emissions, particularly from neutral and alkaline soils which potentially emit higher quantities of HONO than acid soils (Oswald et al., 2013; Spataro and Ianniello, 2014; Su et al., 2011).

A third intermediate of denitrification, N_2O, is a very potent greenhouse gas, and is estimated to have >300 times the global warming potential of the more widely-known CO_2 (Lashof and Ahuja, 1990). N_2O is also recognised as the main anthropogenic destructor of stratospheric ozone (Portmann et al., 2012; Ravishankara et al., 2009). Hence, it is important to determine the factors that contribute to the propensity of a soil to preferentially emit N_2O or inert N_2 as the principal product of denitrification.

One well-studied and key controlling variable of denitrification is pH. It has long been observed that acidic soils tend to emit mostly N_2O under denitrification-friendly conditions, whereas a lower N_2O:N_2 ratio is observed in near-neutral pH soils (Bakken et al., 2012; Nömmik and Thorin, 1972; Raut et al., 2012). Despite this, recent studies of pure cultures (*in vitro*) and extracted cells (*ex situ*) have shown that the transcription of the N2OR gene, *nosZ*, is detectable in the absence of a functional enzyme, suggesting post-transcriptional regulation (Bergaust et al., 2010; Liu et al., 2010, 2014). However, in the absence of *in situ* analyses of soil microbial communities, there is little evidence of similar regulation of *nosZ* in acidic environments. This is also further complicated by evidence of adaptive denitrifying communities and microorganisms that are able to complete denitrification to N_2 under acidic conditions (Lycus et al., 2017; Parkin et al., 1985; Šimek et al., 2002).

Moreover, denitrification intermediates are not only generated by classical denitrifiers such as the model organisms *Paracoccus denitrificans* and *Pseudomonas stutzeri*, but also by nitrifiers such as *Nitrosomonas europaea*, which produce NO and N_2O via a process termed "nitrifier denitrification" (Wrage et al., 2001) and DNRA organisms such as *Wolinella succinogenes* (Luckmann et al., 2014). Nitrifiers and DNRA organisms are capable of producing all three denitrification intermediates (nitrite, NO and N_2O), but only the latter has been confirmed to produce N_2 (Malm et al., 2009; Mania et al., 2014; Russow et al., 2009; Shen et al., 2003). However, while the NIR used in nitrifier denitrification is the same as that of classical denitrification, DNRA organisms use either NrfA or NirB for nitrite reduction (Cantera and Stein, 2007; Decleyre et al., 2015; Sun et al., 2016).

Given the complexity of these inter-linked biological and chemical processes that produce the same nitrogen compounds under similar conditions, many studies to date have only been able to demonstrate the results of coupled processes (Burns et al., 1996; Russow et al., 2009; Stevens et al., 1998). Experiments that are able to successfully separate these processes and focus on anaerobic nitrate-stimulated activities (denitrification and DNRA are difficult to uncouple) are far and few between, and often involve the use of extracted cells or soil slurries

and amplicon-based analyses (Brenzinger et al., 2015; Liu et al., 2014; Palmer and Horn, 2012). However, the control gained over gas exchange (agitated slurries) and gene specificity (targeted amplification) come at a price: Severely altering the natural physical attributes of the soil in the former, and overlooking bacteria without the same conserved genetic sequences for the latter.

Hence in this paper, we characterised the metagenome (MG), metatranscriptome (MT), and phenome of denitrification-mediated nitrite, NO and N_2O production/consumption in intact soils of contrasting pH (pH 3.8 and 6.8) from the same experimental field site. We employed the use of bioinformatics methods coupled with an automated sample incubation and gas measurement system (Molstad et al., 2007) to disentangle denitrification from related anaerobic nitrogen processes. The MG and MT were characterised using self-curated custom datasets, and were compared to the N-compound kinetics to detect links between biological potential and response with nitrite accumulation and N-gas (NO, N_2O, and N_2) emissions.

2 Materials and Methods

Soils. Two high organic content peat soils (40-45 % soil organic C, 2 % organic N) (Liu et al., 2010) with different pH, pH 3.80 (soil 3.8) and 6.80 (soil 6.8), were sampled from a long-term field experimental site in Fjaler in western Norway (61°17'42", 5°03'03"). Soil 3.8 is the original un-limed soil, and soil 6.8 was limed in 1978 with 800 m^3 of shell sand per hectare of soil (Sognnes et al., 2006). Both soils were immediately transported to the laboratory, sieved (4.5 mm) upon arrival, then stored in sealed plastic bags at 4 °C. All pH values were measured in 0.01 M $CaCl_2$ (1:5 ratio, soil to $CaCl_2$) immediately prior to using the soil.

Soil treatment. The soils were revitalised from cold storage by addition of 5 mg dried, powdered clover g^{-1} soil wet weight (ww) then incubated at 15 °C for 72 h. A small amount of a natural C source, in the form of clover, was added to standardise the conditions and to ensure that the organisms would have enough energy to induce transcription of the targeted denitrification genes (Liu et al., 2010). The soils were aliquoted (5-8 g of soil ww, corresponding to 1.5 g soil organic C) into air-tight glass vials and sealed with butyl-rubber septa and aluminum crimps, then nitrate was added to 80 % of the soil's water holding capacity (WHC) and 6.2-7.1 mM nitrate in soil moisture. The vials were immediately made anoxic by 6 cycles of gas evacuation and helium filling (Liu et al., 2010), and incubated anoxically at 15 °C to stimulate the production of denitrification gene transcripts. Gases (CO_2, O_2, NO, N_2O and N_2) produced in the headspace were measured every three hours using an autosampler linked to a GC and NO analyser (Molstad et al., 2007). At each gas sampling time point, one replicate vial of each soil type was opened and soil nitrite was measured. The nitrite was reduced to NO gas using 1 % w/v sodium iodide in acetic acid (Braman and Hendrix, 1989; Cox, 1980), then measured by chemiluminescence using a Sievers Nitric Oxide Analyzer NOA 280i. A portion of the same replicate vial was snap frozen in liquid nitrogen then stored at -80 °C until nucleic acid extraction.

Nucleic acid extraction. DNA and RNA were extracted from frozen samples using the method detailed in Lim et al. (2016). Briefly, 0.2 g of soil was lysed with glass beads, CTAB extraction buffer, and phenol-chloroform-isoamyl alcohol (25:24:1), using a FastPrep-24 instrument. The nucleic acids were washed with ethanol, precipitated and dried, then resuspended in 200 µL of DEPC-treated nuclease-free water. The nucleic acid extract was purified with the *OneStep* PCR Inhibitor Removal Kit (Zymo Research), then split into two fractions – one for DNA, and one for RNA. The DNA fraction was further purified using the Genomic DNA Clean & Concentrator kit (Zymo Research), then kept at -20 °C until use. The RNA fraction was digested

using TURBO DNA-*free* DNase kit (Ambion, Life Technologies) according to manufacturer's instructions, then purified using the RNA Clean & Concentrator-5 kit (Zymo Research). Quantitative PCR (qPCR) using primers targeting the 16S rRNA gene (described below) was used to assess the presence of residual genomic DNA (gDNA) in the purified RNA fractions (defined by signal detected in the qPCR at ≤ 35 cycles), and only RNA fractions free of gDNA was used for further analysis. The purified and DNA-free RNA fractions were reverse transcribed using the Maxima Reverse Transcriptase with random hexamer primers (Thermo Scientific), according to manufacturer's instructions. Primers targeting the 16S rRNA or *nosZ* genes (described below) were used in qPCR to assess the quality (defined by uninhibited amplifiability) of purified DNA and reverse-transcribed cDNA.

Quantitative amplification-based analysis. The genes encoding 16S rRNA and three denitrification enzymes (*nirK*, *nirS* and *nosZ*) were quantified using qPCR. The primers used were: 27F and 518R for the 16S rRNA gene (Muyzer et al., 1993; Weisburg et al., 1991), 517F and 1055R for the *nirK* gene (Chen et al., 2012), cd3aF and R3cd for the *nirS* gene (Hallin and Lindgren, 1999), and Z-F and 1622R for the *nosZ* gene (Kloos et al., 2001; Throbäck et al., 2004). DNA samples were diluted to 1-10 ng of DNA per reaction. All cDNA and RNA samples (DNase-digested) were used without dilution. Each 20 µL qPCR reaction contained SYBR *Premix Ex Taq* II (Tli RNaseH Plus) (Takara Bio) used according to manufacturer's instructions, and included 0.4 µM of each primer and 2 µL of template. The optimised qPCR cycling conditions for all primer sets were 95 °C for 5 min, 40 cycles of 95 °C for 30 s, *x* for 60 s, 72 °C for 30 s, 82 °C for 20 s, and a final melting curve analysis from 60 °C to 95 °C to determine the specificity of amplicons, where *x* = 54 °C (16S rRNA gene), or 60 °C (denitrification genes). To reduce background signals from primer dimers and unspecific PCR products, the fluorescence signal was measured during the final step of each cycle, at 82 °C. The detection limit of each qPCR run was 5 copies per microliter of reaction (Lim et al., 2016), which was approximately 4×10^5 copies g^{-1} soil (ww). Results of qPCR analyses can be found in Supplementary Fig. S1 and accompanying text.

Sequencing the metagenome, metatranscriptome, and 16S rRNA genes. Triplicate DNA and duplicate RNA samples were sent for metagenomic and metatranscriptomic sequencing at The Roy J. Carver Biotechnology Center (CBC) / W. M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-Champaign, using HiSeq 2500 technology. All nucleic acids were shipped in a liquid nitrogen vapour dry shipper (Cryoport), and arrived within 5 days (the Cryoport Express dewar is able maintain the temperature

at -150 °C during shipment for 10 days). The RNA integrity (including confirmation of the absence of gDNA) was also independently verified by the CBC prior to sequencing the samples.

DNA samples were sent for 16S rRNA community analysis using Illumina MiSeq technology (2 × 300 bp paired-end sequencing with V3 chemistry) at StarSEQ GmbH, Mainz (Germany). The primers used targeted the V4 region of the 16S, 515f and 806rB (Apprill et al., 2015; Parada et al., 2016), as detailed by the Earth Microbiome project (http://www.earthmicrobiome.org/emp-standard-protocols/16s/).

Annotation and taxonomic classification of genes. Custom datasets were used to identify reads assigned to the denitrification and DNRA genes (*nap*, *nar*, *nirK*, *nirS*, *cnor*, *qnor*, *nosZ*, *nirB*, and *nrf*). Datasets consisted of a manually curated set of full length protein sequences derived from sequenced genomes in the IMG database (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). These datasets were manually curated to contain diverse sequences while at the same time limiting multiple sequences from heavily sequenced species.

The sequenced Illumina HiSeq reads were quality controlled using BBDuk from the BBMap package version 35.66 (http://sourceforge.net/projects/bbmap/). For functional annotation, reads were aligned against using DIAMOND with an e-value cutoff of 1×10^{-3} (Buchfink et al., 2015). The DIAMOND output was converted to m8 blast format and analysed in R. Reads must have had a matching region of >30 amino acids and an identity of >60 % to be considered matching. Output of matching reads were normalised to reads per million of total reads, RPM (see below). Denitrification genes of interest were identified using DIAMOND using the reads as query and the custom denitrification library as the database. Taxonomic assignment was performed using Kraken with k=27 (Wood and Salzberg, 2014).

Contrary to common assumption, we observed that high-throughput sequencing did not necessarily yield reads that spanned the entire genome. Instead, a large majority of the reads were found to overlap in relatively conserved regions (data not shown). Thus, we decided that the current sequencing depth and read-lengths did not provide sufficient certainty to determine lower taxonomic hierarchies. Taxonomy was thus only reported to the level of order.

16S rRNA amplicon community sequencing and analysis. Processing of the sequenced 16S rRNA amplicons was performed by StarSEQ Gmbh, Mainz (Germany). Briefly, the sequences were demultiplexed and the adapters were trimmed locally on the MiSeq instrument with the Illumina Metagenomics 16S rRNA application, using default settings. The median library size was 400 bp prior to trimming, and 268 bp post-trim (the adapters were a total length

of 132 bp). Reads were annotated using the Greengenes 16S rRNA gene database as a reference (DeSantis et al., 2006). The processed and annotated sequences were manually checked for their accuracy and reliability.

Statistical and quantitative analysis of meta-omic data. All reads counts were normalised for sequencing depth, generating RPM values: (number of reads)/(total reads that passed quality control)$\times 10^6$. All statistical analyses and graphing were performed using in-house R scripts custom created for this purpose.

3 Results

Kinetics of gaseous denitrification intermediates depict a pH-dependent response to anoxia. In soil 6.8, there was an immediate net production of nitrite, NO, and N_2O, leading to their accumulation (Fig. 1). By 30 h, there was a net consumption of all three intermediates, and the only net production was N_2 gas. In contrast, soil 3.8 showed very low interim accumulation of nitrite (<0.5 μmol-N $vial^{-1}$, except 36-40 h that reached a maximum of 0.9 μmol-N $vial^{-1}$). The production rate of NO gas peaked at 6 h in soil 3.8 and at 15 h in soil 6.8, then decreased into a net consumption at ≈20 h in both soils.

Soil 6.8 completed denitrification of the provided nitrate to N_2 gas within 43 h, reducing N_2O to N_2 at a rate of 0.1-0.2 μmol-N h^{-1} (Fig. 1B). After 27 h, the N_2O reduction rate increased to a maximum of 0.8 μmol-N h^{-1} (40 h) until all of the added nitrate-N was recovered as N_2 gas, which is indicative of strong denitrification and minimal DNRA activity. Soil 3.8 accumulated principally N_2O with no reduction to N_2 until 37 h, after which a low rate of N_2O reduction was observed (<0.1 μmol-N h^{-1}).

Based on the total nitrite-N (TNN), we calculated the theoretical quantity of undissociated HNO_2 (aq) in the soil matrix (using the Henderson-Hasselbalch approximation, see Supplementary material), which potentially forms a chemical equilibrium with HONO (gas) in the atmosphere (thus predicting the potential emission of HONO to the atmosphere). Despite the high accumulation of TNN in soil 6.8 (up to 3.6 mM), the concentration of undissociated HNO_2 was ≤ 1.4 μM, which was almost two orders of magnitude lower than that in soil 3.8 (Fig. 1C).

Soil bacterial community differed strongly by pH but was stable over time. The microbial composition of the two soils was compared using 16S rRNA amplicon community analysis. Both soils had approximately the same proportions of classified and unclassified reads, and >93 % of all sequenced reads were annotated as bacterial (Supplementary Table S1-2). Archaeal representation was not high in either soil (≤0.6 %), but was relatively lower in soil 3.8 (≤0.3 %). The soils also had vastly different archaeal groups, with ammonia-oxidising archaea present only in soil 6.8 (Supplementary Fig. S2 and Table S1-2). The same major bacterial phyla/classes were present in both soils: Actinobacteria, Firmicutes, and Alphaproteobacteria each made up 10-14 % of the populations in both soils (Fig. 2, Supplementary Table S2). However, the community profiles based on phyla (with Proteobacteria split by class as shades of green) showed distinct pH-dependent microbial community fingerprints (Fig. 2).

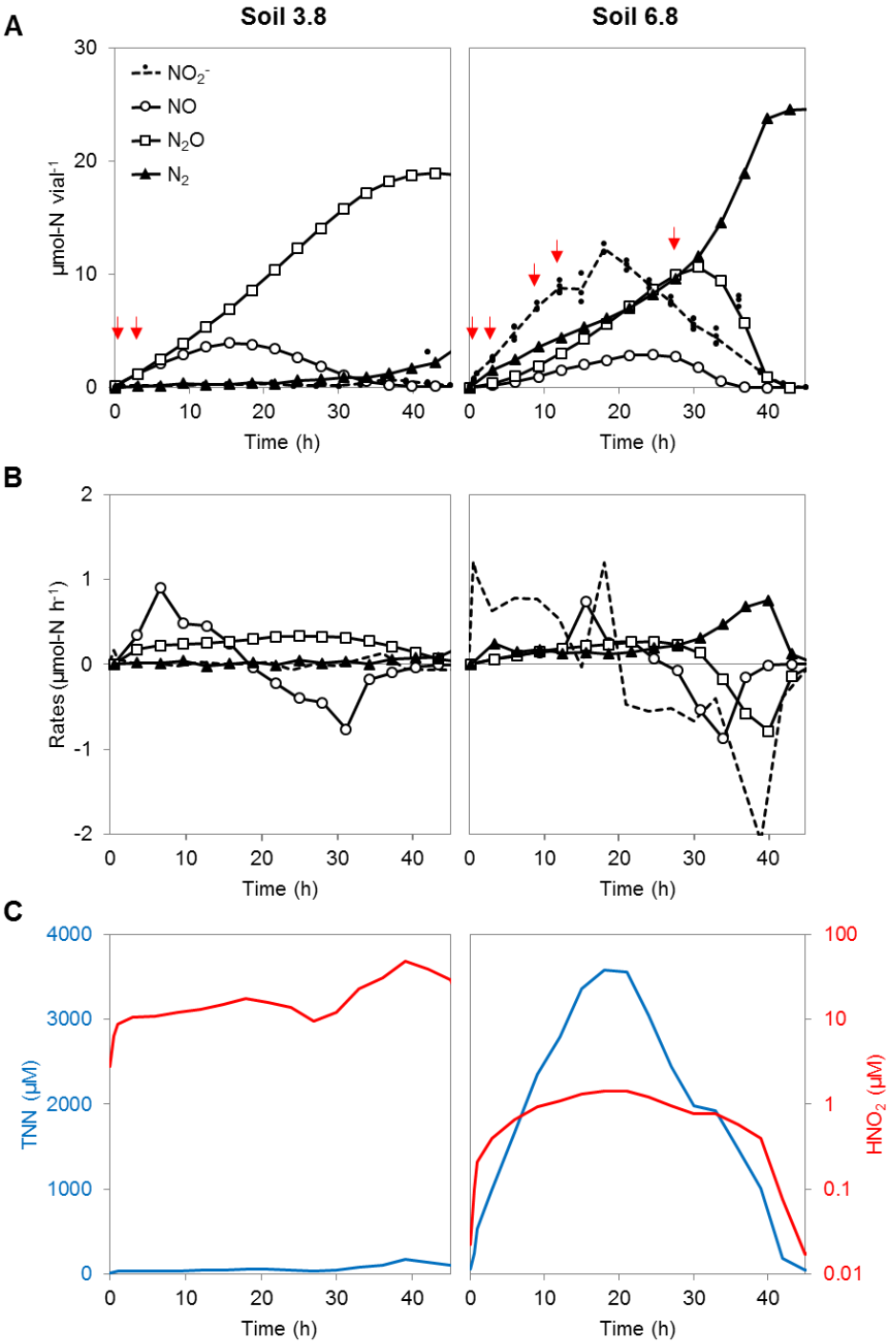


Figure 1 | Nitrite and gas kinetics of soil 3.8 and 6.8 during anoxic incubation in the presence of nitrate. Nitrate was added to 5-8 g soil ww (corresponding to 1.5 g soil organic C per vial) to a final concentration of 6.2-7.1 mM nitrate in soil moisture. **(A)** Kinetics of nitrite, NO, N_2O, and N_2 in soil 3.8 and 6.8 during a 45 h incubation under anoxic conditions. **(B)** Net production (positive values) and consumption (negative values) rates. Red arrows indicate samples taken for RNA analysis. **(C)** Total nitrite-N (TNN) in soil moisture (blue, left axis) vs. the amount of undissociated HNO_2 (red, right axis), calculated based on measured nitrite levels and soil pH: $[HNO_2]/([HNO_2]+[NO_2^-]=1/(1+10^{pH-pKa})$, where pK_a= 3.398.

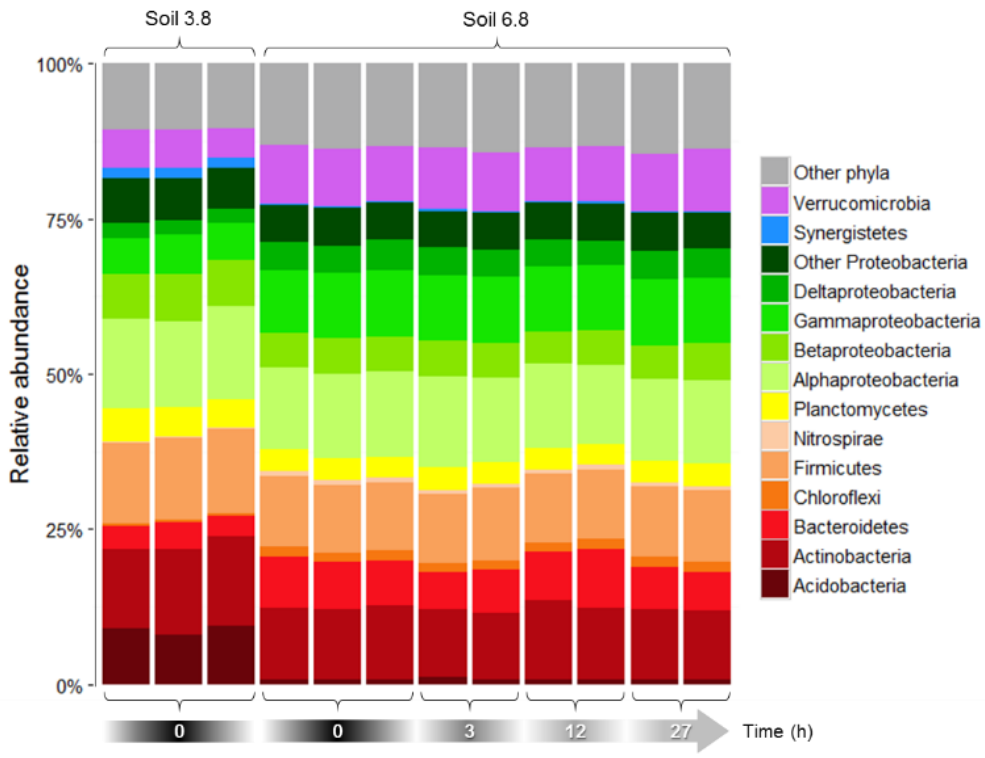


Figure 2 | Taxonomic distribution of bacteria in soil 3.8 and 6.8 based on 16S rRNA genes. Samples were sequenced using primers targeting the 16S rRNA gene (515f/806rB) and annotated using the GreenGenes database as reference. A detailed breakdown of bacterial phyla and archaeal groups is available in Supplementary Table S1-2 and Fig. S2.

Nevertheless, the microbial community profile of soil 6.8 was stable during the experiment. Thus, any differences observed in the metatranscriptome (MT) can be reasonably attributed to transcription regulation patterns, and not due to bacterial growth causing a resulting shift in the population.

Nitrogen cycling potential and transcription. DNA and RNA sequences were analysed with a custom annotation pipeline utilising a manually-curated dataset of nitrogen cycle-related genes (Materials and Methods). In the metagenome (MG) and metatranscriptome (MT) of soil 3.8 and 6.8, denitrification-related genes [encoding nitrate reductase (NAR), NIR, NOR and N2OR] made up 83 and 65 % of the assigned genes respectively, with the DNRA-related genes (*nirB* and *nrf*) together accounting for the remainder (Supplementary Table S3). In contrast, soil 6.8 transcribed both DNRA genes at levels comparable to the NIR and NOR genes, with strong transcription of *nirB* that was at times even higher than NIR. Yet, the complete recovery of all added nitrate-N as N_2 gas indicated minimal conversion of nitrite to NH_4^+ (and thus little effect of the DNRA-related genes *nirB* and *nrfA*). Thus, this paper shall henceforth focus on denitrification genes only.

Potential for denitrification. Of the denitrification genes, *nar* was the most abundant in the MG of both soils (Fig. 3A), and was more than double that of the next most abundant genes, *nap* (soil 6.8) and *qnor* (soil 3.8). The order of genes from most abundant were, for soil 3.8: *nar* > *qnor* > *nirK* > *nap* > *nosZ* > *cnor* > *nirS*; and for soil 6.8: *nar* > *nap* > *nirK* + *qnor* > *nosZ* > *cnor* > *nirS*. Levels of NIR (*nirK*+*nirS*) were comparable in the two soils with RPM values of 36.5±1.7 and 44.6±1.4 for soils 3.8 and 6.8, respectively (Table 1). *nirK* genes were 7-fold that of *nirS* in soil 6.8, and completely dominated over *nirS* in soil 3.8. The NOR genes (*cnor*+*qnor*) were more abundant in soil 3.8, where RPM values were 74.9±0.5 compared to 48.1±0.3 for soil 6.8 with *qnor* ≈11 and 4 fold higher than *cnor* in soil 3.8 and 6.8, respectively.

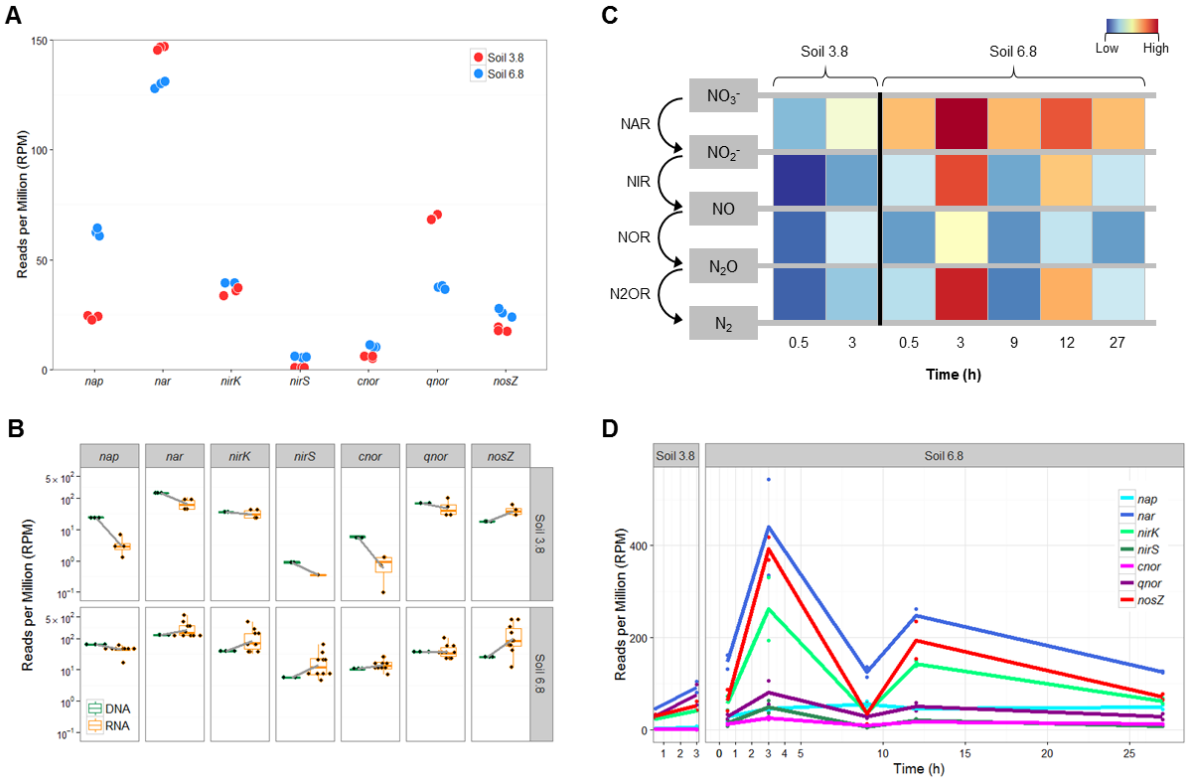


Figure 3 | Genetic potential and transcription of selected genes. Triplicate soil samples were taken at the start of the incubation for metagenomics analysis. Duplicate soil samples were taken at selected time intervals (Fig. 1) for metatranscriptomic analysis. **(A)** Comparison of the abundance of denitrification genes in soil 3.8 (red) and soil 6.8 (blue). **(B)** Abundance of genes (triplicates, green) vs. transcripts (all sampling times combined, orange) in soil 3.8 and soil 6.8. A similar comparison of only the 0.5 and 3 h transcripts from both soils is available as Supplementary Fig. S3. **(C)** Overview of denitrification gene transcription. Block colours correspond to transcription level (log_2 RPM), with red the highest expressed and blue the least expressed. NAR: nitrate reductase (*nap*+*nar*), NIR: nitrite reductase (*nirK*+*nirS*), NOR: nitric oxide reductase (*cnor*+*qnor*). N2OR: nitrous oxide reductase (*nosZ*). **(D)** Changes in the abundance of denitrification gene transcripts during incubation, presented on a linear time scale.

Table 1 | Occurrence of nitrite [*nirK* (K) and *nirS* (S)], nitric oxide [*cnor* (C) and *qnor* (Q)], and nitrous oxide reductases [*nosZ* (Z)] in the metagenome and metatranscriptome of soils 3.8 and 6.8 during anoxic incubation with nitrate (all values in RPM).

Soil pH	Time (h)	NIR			NOR			N2OR	NIR:NOR	NIR:N2OR	NOR:N2OR
		K	S	K:S	C	Q	Q:C	Z			
DNA											
3.8	-	35.58 (1.74)	0.88 (0.06)	40.82 (4.92)	5.70 (0.63)	69.16 (1.16)	12.27 (1.65)	18.18 (0.95)	0.49 (0.03)	2.01 (0.12)	4.13 (0.22)
6.8	-	38.92 (1.14)	5.69 (0.35)	6.85 (0.28)	10.69 (0.53)	37.41 (0.78)	3.51 (0.24)	25.79 (1.99)	0.93 (0.03)	1.74 (0.14)	1.87 (0.13)
RNA		ns	s	s	s	ns	ns	ns	s	ns	s
3.8	0.5	23.49	0.00	Inf	1.32	31.73	24.08	33.37	0.71	0.70	0.99
3.8	0.5	23.39	0.00	Inf	0.71	27.31	38.62	25.90	0.83	0.90	1.08
3.8	3	40.85	0.35	115.25	1.28	98.23	76.45	62.03	0.41	0.66	1.60
3.8	3	42.11	0.00	Inf	0.10	52.82	545.00	42.01	0.80	1.00	1.26
6.8	0.5	72.33	21.87	3.31	10.22	23.19	2.27	86.69	2.82	1.09	0.39
6.8	0.5	42.41	6.86	6.18	14.12	32.57	2.31	40.47	1.06	1.22	1.15
6.8	3	330.22	62.99	5.24	28.09	105.98	3.77	417.81	2.93	0.94	0.32
6.8	3	192.91	35.81	5.39	22.11	55.81	2.52	368.16	2.94	0.62	0.21
6.8	9	40.63	7.96	5.11	10.80	31.04	2.87	56.18	1.16	0.86	0.74
6.8	9	32.31	4.50	7.18	7.07	25.52	3.61	12.30	1.13	2.99	2.65
6.8	12	137.46	18.49	7.43	16.44	58.44	3.55	153.97	2.08	1.01	0.49
6.8	12	149.13	22.60	6.60	18.43	40.78	2.21	234.87	2.90	0.73	0.25
6.8	27	56.50	7.07	7.99	10.62	21.54	2.03	65.43	1.98	0.97	0.49
6.8	27	67.38	7.08	9.52	12.60	33.99	2.70	77.83	1.60	0.96	0.60

Averages of DNA triplicates are listed, standard deviation in parenthesis. RNA replicates were not averaged due to wide variations. Differences between the two soils (0.5+3 h) are indicated in red as s (significant) or ns (not significant). NIR = nitrite reductase; NOR = nitric oxide reductase; N2OR = nitrous oxide reductase; Inf = infinity (division by 0).

Denitrification response. As was observed previously (Bergaust et al., 2010; Henderson et al., 2010; Liu et al., 2010; Saleh-Lakha et al., 2009), denitrification genes are quickly transcribed upon onset of anoxia and/or addition of nitrate, regardless of environmental pH. Thus, RNA samples from both soils were taken 0.5 and 3 h after the start of the experiment to characterise the immediate community response (indicated by red arrows in Fig. 1). Additional RNA samples were taken from soil 6.8 prior to the peaks in nitrite, NO, and N_2O (Fig. 1) to determine if the switch from net production to consumption of each denitrification intermediate was due to secondary bursts of gene transcription, or if there was a shift in the actively transcribing denitrifying population within the microbial community.

Despite the relative metagenomic stability (seen in the consistent RPM values of the MG triplicates), there was a time-sensitive differential transcription of denitrification genes in both

soils (Fig. 3C-D). In particular, the first response of both soils (at 0.5 and 3 h) displayed the strongest increase in almost all transcripts (Fig. 3D). As may be predicted by the general high occurrence of non-denitrifying nitrate reducers in soil environments (Gamble et al., 1977; Lycus et al., 2017), NAR was the highest expressed in both soils and at all time points (Fig. 3C). Additionally, the order of denitrification reductase abundance did not change over time: In soil 6.8, it was NAR > N2OR > NIR > NOR; whereas soil 3.8 was NAR > NOR > NIR + N2OR.

Relative to their respective MG and MT, there was a proportionally higher but varying representation of denitrification transcripts in the soil 6.8 MT than in the MG that was most varied at 0.5-3 h (Fig. 3B, Supplementary Fig. S3). Nevertheless, the overall soil 6.8 trend in transcript abundance remained as *nar* > *nosZ* > *nirK* > *qnor* > *nap* > *nirS* > *cnor*, almost without exception throughout the incubation (Fig. 3D). This highlights the strong and immediate response of *nosZ* upon anaerobiosis in neutral pH soils. Similarly, the trend of soil 3.8 transcript abundance (*nar* > *qnor* > *nosZ* > *nirK* > *cnor* + *nirS*) stayed throughout, despite varying between higher and lower relative abundances than in the MG (Fig. 3B, D). Interestingly, although *nosZ* transcription was at comparable levels to the other denitrification genes (Fig. 3B), there was no corresponding N_2O reduction to N_2 (Fig. 1). The high NIR:NOR transcription ratio for soil 6.8 (≈2.4) compared to that in soil 3.8 (≈0.7) (Table 1) suggests less stringent NO control in the former. We also calculated the NAR:NIR transcript ratios for the two soils (based on Table S3), and found almost equally high ratios for the first 3 h (≈2.2 and 2.1 for soil 3.8 and 6.8, respectively).

The MT of soil 6.8 displayed two distinct spikes in denitrification gene transcription, which increased for all genes at 3 and 12 h (Fig. 3D and Table 1). Despite this, the transcript ratios for individual time points were not significantly different (rightmost columns in Table 1). Moreover, there was a strong positive correlation ($p < 0.01$) between all transcripts except *nap* (Supplementary Fig. S4 and Table S4). Soil 3.8 also showed a similar trend with a correlation ($p < 0.1$) between *nar*, *nirK*, *qnor* and *nosZ* genes (*nirS* and *cnor* were quantitatively irrelevant due to their low numbers).

Integration of taxonomic annotation with denitrification genes and transcripts. The sequenced reads were taxonomically annotated using our curated dataset as reference (Materials and Methods). Each gene revealed a different taxonomic profile that varied by soil pH (Fig. 4), i.e. the phyla present were not always shared by the soils. However, the soils shared the most abundant phyla in both MG and MT (Actinobacteria and Proteobacteria). Although the distribution among phyla for the different gene transcripts show some congruence between the

two soils, several phyla that were represented in soil 6.8 were not detected among the transcripts from soil 3.8, despite the presence of their genes. This could be due to late expression, thus missed because transcripts were only analysed during the first 3 h in soil 3.8. Such late transcription was indeed observed for some phyla in soil 6.8, most notably *nosZ* from Acidobacteria and Bacteriodetes, *qnor* from Proteobacteria and *nirK* from several phyla.

Proteobacteria was the only phylum consistently possessing all genes in both soils, and was the only active phylum with *nirS* and *cnor* in soil 3.8 (Fig. 4). It was also the most abundant phylum for all genes and transcripts excepting *nar*, where potential and transcription in both soils was matched or exceeded by Actinobacteria. However, *nosZ* transcription in soil 6.8 was not solely dominated by Proteobacteria, but was shared by a range of phyla, including Acidobacteria, Bacteroidetes, and Verrucomicrobia.

Proteobacterial denitrification. Because Proteobacteria was the only phylum present and active for all genes, the thus-assigned reads were further analysed at the levels of class and order. A deeper analysis of the only ubiquitous phylum revealed pH-dependent class representation in the two soils' MG and MT (Fig. 5). Both soils contained members of the Alpha- (α, shades of red), Beta- (β, shades of brown-yellow), Gamma- (γ, shades of green), and Deltaproteobacteria (δ, shades of blue) classes, but Epsilonproteobacteria (ε, shades of purple) were almost exclusively detected in soil 6.8, and possessed only *cnor*.

Overall, the most dominating orders came from all four of the main classes: the β-proteobacteria Burkholderiales (dark brown) was present in the MG and MT of both soils for most genes; the α-proteobacteria Rhizobiales (dark red) in both soils for all genes but *nirS* and *qnor*; the γ-proteobacteria Pseudomonadales and Xanthomonadales (green) together were present in both soils for all genes but had the strongest presence in NIR and NOR; and the δ-proteobacteria Myxococcales (dark blue) was most strongly associated with genes related to the final steps of denitrification (*qnor* and *nosZ*) in both soils. The profiles of NAR (*nap* and *nar*) in the MG and MT were largely similar and more stable over time, with the exception of a shift in transcription profile towards the start of the incubation in soil 6.8. NIR (*nirK* and *nirS*) profiles between the soils differed by the dominance of γ-proteobacteria in soil 3.8. The largest difference in transcription profile between the soils was for NOR (*cnor* and *qnor*), where soil 6.8 displayed a more stable MG and MT profile (excepting the presence of ε-proteobacteria Campylobacterales) whereas soil 3.8's MT profile showed a strong class-level community shift within the first 3 h. *nosZ* profiles of both soils also exhibited a similar class-level shift: soil 6.8's fluctuating *nosZ* MT profile was comprised of early β- transcription, later α- transcription and

inconsistent δ-proteobacteria transcription; whereas soil 3.8's MT profile was due to order-level replacement within the β-, a reduction in α-, and the start of δ-proteobacteria activity.

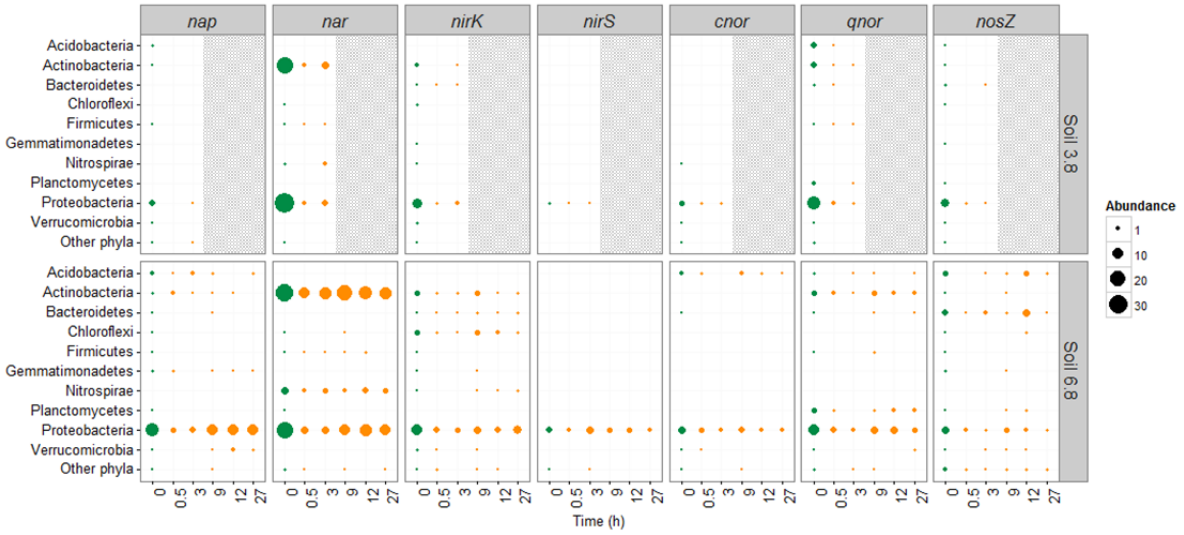


Figure 4 | Breakdown of genetic potential and expression of denitrification genes and transcripts by phyla. Annotated reads from the metagenome (green, 0 h) and metatranscriptome (orange, 0.5-27 h) were matched to our curated denitrification reference dataset for taxonomic annotation, and normalised for sequencing depth.

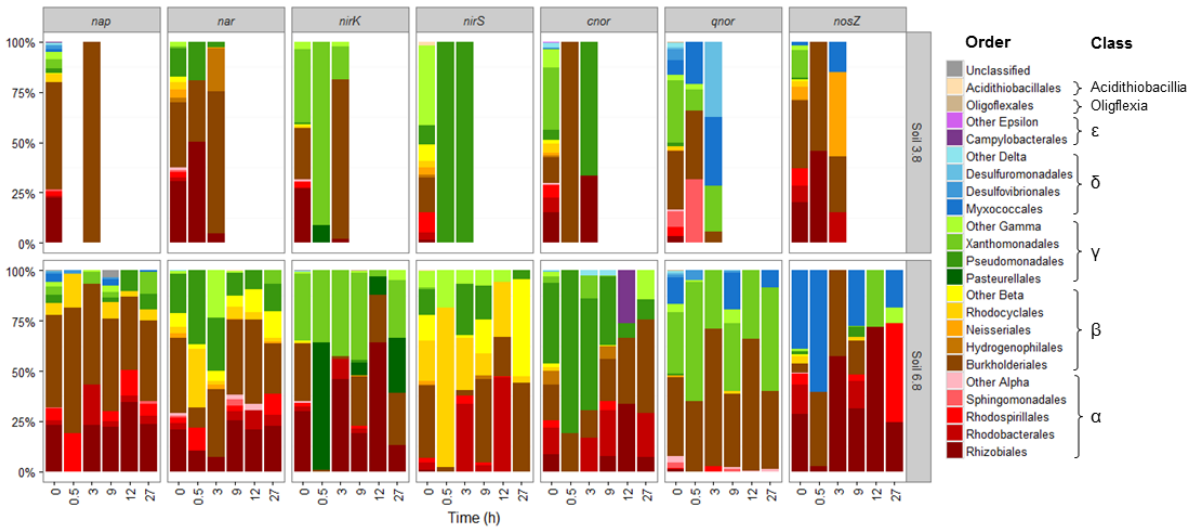


Figure 5 | Denitrification gene and transcript prevalence in the phylum Proteobacteria. Annotated genes (0 h) and transcripts (0.5-27 h) belonging to Proteobacteria were further analysed at the levels of class and order. Highly represented Proteobacterial orders were assigned individual colours, and lesser represented orders were grouped under class-specific "Other" categories. Note that the dominant "Other Beta" with *nirS* at 27 h in soil 6.8 is mainly unclassified Betaproteobacteria. Proteobacterial classes are indicated by their corresponding Greek letters.

4 Discussion

Strong capacities for denitrification. We investigated the immediate response to anoxia in two soils of differing pH from the same field site. Both soils 3.8 and 6.8 displayed a strong capacity for nitrate reduction to N_2O/N_2. Relatively large quantities of gaseous intermediates (NO and N_2O) accumulated (Fig. 1), indicating an unbalanced activity of denitrification enzymes (NIR>NOR>N2OR). The MG and MT largely reflected this strong denitrification potential and activity (Fig. 3). Interestingly, the taxonomic profile displayed a plausible trend of denitrification process modularity in both soils, where different phyla possessed different reductases and were active at different times (Fig. 4). In particular, none of the denitrification gene taxonomic profiles were identical, strongly suggesting that the full denitrification process was completed by multiple organisms throughout the incubation period, as proposed by Shapleigh (2013). However, it is important to note that the current sequencing depth is insufficient to verify or disprove the presence and/or activity of complete denitrifiers, and is only able to support the hypothesis of a modular denitrification process in complex communities without exempting complete denitrifiers.

Predicted HONO emissions decreased with increasing pH. Although it has previously been suggested that nitrite stemming from soil biological processes could make significant contributions to HONO emissions from neutral and alkaline soils (Oswald et al., 2013), we have not seen evidence of this in soil 6.8. Our calculations are based on the HNO_2 equilibrium concentration in the soil moisture using the Henderson-Hasselbalch approximation, and this suggested that the acid soil had a much higher potential HONO emission than the neutral soil, despite the high TNN content of the latter. Moreover, the proposed high biological HONO emissions from nitrite occurred under severe dry-out conditions of ≈0-30 % WHC (Oswald et al., 2013), whereas we had kept our soils at 80 % WHC, which is more relevant to the natural conditions at the field site. Thus, it seems improbable that denitrification in soil 6.8 could accumulate sufficient TNN to emit high levels of HONO at the field site in Fjaler, unless there exists an enzymatic reaction that is somehow able to bypass the pH-dependent chemical equilibrium between NO_2^- and HNO_2, which was the implicit assumption by the authors (Oswald et al., 2013). It is difficult to envisage such a microbial bypass mechanism in wet neutral pH soil, however, since microbes are well-embedded in the soil moisture. Interestingly however, our calculation of high concentrations of HNO_2 in the acid soil suggests that HONO emissions observed under acidic conditions may be attributed purely to abiotic processes such

as the natural achievement of chemical equilibrium of TNN with HNO_2 in soil, and aqueous HNO_2 with gaseous HONO.

Dominance of denitrification reductase functional homologues. Regardless of sample (MG or MT) or soil type, there was always one dominant reductase for NAR, NIR and NOR – *nar*, *nirK* and *qnor*, respectively (Fig. 3). Unlike *nap*, *nar* is synthesised under suboxic conditions and is rarely used for aerobic nitrate reduction (Moreno-Vivián et al., 1999; Zumft, 1997), which may explain the high *nar* transcription levels upon anoxia. Additionally, *nap* and *nar* are differentially expressed, where high concentrations of nitrate represses *nap* and induces *nar* expression (Wang et al., 1999). The lesser abundant NIR and NOR homologues (*nirS* and *cnor*) may have been negatively selected against by their need for accessory genes. Both reductases require cytochromes – functional NirS and cNor require genes for cytochrome synthesis in addition to the reductase genes themselves (Zumft, 1997). In contrast, *nirK* and *qnor* genes are sufficient for the synthesis of independently functional proteins. Coupled with the fact that denitrification genes may be acquired by horizontal gene transfer, the complexity hypothesis and gene deletion bias (e.g. selection-driven gene loss) argues for a higher probability of successful transfer and retention of *nirK* and *qnor* genes, since such newly acquired genes would be able to provide immediate function within the existing cell machinery and thus potentially increase overall cell fitness (Albalat and Cañestro, 2016; Koskiniemi et al., 2012; Kuo and Ochman, 2009; Mira et al., 2001).Interestingly, many primer-based studies have observed the opposite NIR trend, with a higher abundance of *nirS* than *nirK* genes and/or transcripts (Liu et al., 2010; Palmer and Horn, 2012; also Supplementary Fig. 1). However, *nirK* primers strongly favour α-proteobacteria (Coyotzi et al., 2017; Palmer and Horn, 2012; Penton et al., 2013), which may not always be a major population. In contrast, *nirS* primers are able to detect at least α-, β- and γ-proteobacteria (Palmer and Horn, 2012; Penton et al., 2013), leading to better detection of transcriptional patterns in soil 6.8 (Supplementary Fig. S1), but also the lack of detection in soil 3.8 since only γ-proteobacteria were present in the MT (Fig. 5).

Nitrite and nitric oxide control by NIR and NOR. Neither the genetic potential nor transcriptional activity of the two NIR genes (*nirK* and *nirS*) bore much similarity to each other, plausibly caused by their genomic mutually exclusivity (Graf et al., 2014). Additionally, Brenzinger et al. (2015) noted that *nirK* denitrifiers were more resilient to pH changes, unlike *nirS* denitrifiers that are suppressed by acidic conditions, likely explaining the pH-separated *nirS* taxonomic profiles of the soils, whereas *nirK* showed some similarity (Fig. 5). Regardless of specific NIR types, the overall levels of NIR (mainly *nirK*) present in the MG and MT of

both soil 3.8 and 6.8 were comparable (Fig. 3), indicating a similarly strong potential for a biological response to suppress potentially toxic nitrite. This suggests the existence of microbial control of nitrite levels in the acidic soil, and not sole reliance on chemical processes to keep levels of potentially toxic nitrite low. This is supported by other studies indicating that aerobic chemical degradation of nitrite accounts for ≤40 % of nitrite "loss" in soil 3.8 and none in soil 6.8 (Mørkved et al., 2007), and that the control of nitrite levels in soil 3.8 is biologically-driven during denitrification under anoxia (Lim et al., submitted for publication). Consequently, we postulate that most of the nitrite degradation in soil 3.8 is biotic, and that at least 60 % of gross NO produced is biological in origin.

The previously low *nirK* detection in both soil 3.8 and 6.8 (Liu et al., 2010) likely stemmed from the use of "universal" primers (Throbäck et al., 2004) that targeted relatively narrow groups of known NIR sequences (Coyotzi et al., 2017), as we observed first-hand in preliminary qPCR analyses (Supplementary Fig. S1 and accompanying text). Such primer limitations contribute to a consistent underestimation of less well-characterised environments that may harbour nitrite reducers with non-conserved priming regions. Nevertheless, nitrite degradation was still assisted by chemical processes at low pH, thus it is not surprising that *qnor* was higher upregulated in soil 3.8 than *nirK* (Fig. 3). This indicated a robust biological attempt at regulating nitrite-generated NO levels, which is in turn necessary because microorganisms risk inhibiting their own respiration under uncontrolled NO levels (Bergaust et al., 2008).

In contrast, since nitrite is increasingly stable close to pH 7 (Stevens et al., 1998), the high *nirK* transcription in soil 6.8 was not surprising, given the need to further reduce the accumulating nitrite (Fig. 3). Although it appears that microorganisms in soil 6.8 were apparently attempting to curb nitrite levels (albeit unsuccessfully as seen in Fig. 1), nitrite is known to accumulate at neutral pH without apparent toxicity to microorganisms (Glass and Silverstein, 1998). We hypothesise instead that the high *nirK* transcription may have been caused by NIR playing "catch-up" with the highly expressed and active NAR, which was generating very large quantities of nitrite (Fig. 1). Despite this high *nirK* transcription and the relatively low NOR (*cnor* and *qnor*) transcription (Table 1), NO levels were kept lowest of all the denitrification products during the entire incubation (Fig. 1). This inconsistency between transcription and activity may be attributed to different mRNA-protein translation ratios among the reductases. The relative NO suppression at both pH 3.8 and 6.8 sheds a different light on microbial control of potentially toxic denitrification intermediates, and may bear its roots in the complex regulatory relationship of NO-sensitive transcription regulators (Rodionov et al.,

2005; Torres et al., 2016). Nevertheless, it is worth noting that the amount of NO accumulated in both soils is very high when compared to the levels generally observed in pure cultures (Bakken et al., 2012; Liu et al., 2013), and the persistence of such high NO concentrations may result in denitrification inhibition (Bergaust et al., 2008).

Incomplete denitrification at acidic pH. As expected, larger quantities of N_2O were accumulated in soil 3.8 than 6.8, and the major end-products accumulated were N_2O and N_2 in soils 3.8 and 6.8, respectively. While it is known that acidic environments tend to accumulate N_2O instead of N_2 despite *nosZ* transcription (Brenzinger et al., 2015), previous (Liu et al., 2010) and preliminary studies (Supplementary Fig. S1) with soil 3.8 were unable to detect *nosZ* transcripts using primer-based techniques. Our current primer-free sequencing successfully revealed early *nosZ* transcription in soil 3.8 without corresponding N_2O reduction (Fig. 1, Table 1).

This disparity in function may have its roots in the dissimilar genetic potential and activity of the soils: Of all the denitrification genes (except for the low-occurring thus quantitatively irrelevant *nirS*), *nosZ* bore the most pH-differentiated MG profile. The MG of soil 3.8 was split between α-, β-, and γ-proteobacteria, unlike the α- and δ-proteobacteria dominated soil 6.8. Although similar orders were active, the active community was fluctuating and the proportions of transcripts from each order differed greatly between the soils. Since there was no observed N2OR activity in soil 3.8, one of two conclusions may be drawn: (i) all *nosZ*-possessing microorganisms had errors in their *nosZ* gene leading to non-function, or (ii) N2OR inactivity was due to a post-transcriptional failure, likely caused by the acidic pH. Since the former conclusion is unlikely to withstand the rigours of environmental selection [it is improbable that bacteria will maintain and actively transcribe non-functional genes over multiple generations (Albalat and Cañestro, 2016; Koskiniemi et al., 2012)], the latter conclusion is more probable. It has been suggested that post-transcriptional modifications were incomplete at low pH (Liu et al., 2014), which is supported by the recent isolation of NosZ apoenzymes lacking copper ions in its active site from *Paracoccus denitrificans* at pH <7 (Lycus et al., unpublished data). Alternatively, given the delayed and lower transcription of denitrification genes in soil 3.8, hindered transcription of regulators (e.g. *nos* operon element *nosR*, which directly affects NosZ synthesis and maintenance) would also result in similarly reduced N2OR activity (Wunsch et al., 2003; Wunsch and Zumft, 2005).

Because of the high gene sequence similarity and limited sequencing positions (see Materials and Methods), as well as relatively conserved *nosZ* active sites, we deemed the

separation of our reads to be insufficiently reliable to differentiate between the two types of *nosZ*: Z-type (zNos) or c-type (cNos), alternatively defined by taxonomic separation of *nosZ* clades I and II (Jones et al., 2012; Spiro, 2012). There is currently much interest in the often overlooked cNos-carrying microorganisms because they may be widely abundant in the environment (Jones et al., 2012; Orellana et al., 2014) and may also be correlated with the occurrence of other denitrification genes (Graf et al., 2014). However, very recent research has shown that systems with cNos-dominated DNA could still be dominated by active zNos organisms (Coyotzi et al., 2017). Since both zNos and cNos reduce N_2O to N_2, and the significance of the dominance of either type is still debatable, we felt no need to attempt further differentiation of the NosZ types in this study.

Trends in denitrification potential and response. Two of the strongest signalling molecules in denitrification regulation are nitrite and NO, via repressors and regulators such as NsrR and the aforementioned Crp-Fnr transcription regulator superfamily that activates denitrification-related gene clusters (Rodionov et al., 2005; Torres et al., 2016). Evidence of such global regulation was seen in the strong abundance correlation of all denitrification gene transcripts (except *nap*) in soil 6.8 in response to accumulated nitrite and NO (Fig. 1, Fig. S4). Similarly in soil 3.8, *nar*, *nirK*, *qnor* and *nosZ* transcript abundances were positively correlated (*nirS* and *cnor* abundances are uncertain due to their low numbers). In comparison, gene abundances of both soils revealed positive correlation only between NIR (*nirK*+*nirS*) and N2OR (*nosZ*) but a strong negative correlation with *qnor* (Fig. S4). Although NIR and N2OR abundances were positively correlated with *cnor*, this may be more indicative of the co-occurrence of *cnor* with NIR and N2OR within a single genome (Graf et al., 2014).

Abundances aside, MG and MT taxonomic profiles revealed trends within the microbial guilds. Both soils displayed similar *nirK* and *qnor*, but relatively distinct *nosZ* MG profiles (0 h bars in Fig. 5). Soil 6.8's *nirK* and *qnor* MT profile was also relative stable (compared to *nosZ*), supporting the hypothesis of a link between active *nirK* and *qnor* denitrifiers independent of *nosZ* organisms (Coyotzi et al., 2017). However, soil 3.8 bucks the trend with highly unstable and apparently unrelated NIR, NOR and N2OR transcriptional profiles, highlighting the influence of pH on the actively denitrifying population. Another previously hypothesised link described a higher correlation of *nirS* than *nirK* with NOR (*cnor*+*qnor*) and *nosZ* (Graf et al., 2014). Despite observing such an overall abundance correlation (Fig. S4), there was little evidence at the community level, with the presence of an unstable *nirS*- and *nosZ*-transcribing Burkholderiales population as the only indication in soil 6.8 (Fig. 5). Contrarily, Rhizobiales

was actively transcribing both *nirK* and *nosZ* in soil 6.8, and transcribed *nirK* at equal or higher levels than Burkholderiales *nirS* transcription. While the same Rhizobiales species may not have transcribed both *nirK* and *nosZ*, the MT profiles nevertheless fail to support greater co-occurrence of *nirS* than *nirK* with *nosZ*. Similarly in soil 3.8's MT, *nirS* was transcribed only by Pseudomonadales which did not transcribe *nosZ* at all. Importantly, the co-occurrence observations (Graf et al., 2014) stem from a DNA-based *in silico* study without associated transcriptional or phenotypical data, and as observed in this paper, genetic potential can strongly differ from activity. Therefore, this underscores the importance of phenome-linked meta-omic (MG+MT) studies. On the other hand, the present paper analyses a mixed soil community, where transcripts may be derived from different members of the same bacterial class, and may therefore not be easily comparable with organism-specific studies.

Disconnect between meta-omes and phenome. Despite largely similar levels in soil 6.8's MG, there was a persistent dominance of *nirK* and *nosZ* over *qnor* transcripts (Fig. 3 and Table 1). In theory, this should result in the accumulation of NO and N_2 gases with low quantities of intermediate nitrite and N_2O (owing to the low NOR transcription and strong simultaneous upregulation of all genes). However, we detected large quantities of interim nitrite and N_2O (Fig. 1). Similarly in soil 3.8, comparable levels of *nirK*, *qnor* and *nosZ* transcripts did not result in a "balanced" denitrification gas profile, but in the accumulation of large quantities of NO and N_2O that failed to yield N_2 (Fig. 1, 3). This complex relationship between genetic potential, transcript abundance, and denitrification phenotype is further highlighted by comparing the net production/consumption rates (Fig. 1B) to the transcription profile (Fig. 3D). Given that all genes were up- and downregulated simultaneously, if we assumed similar translation rates and enzyme activity, any change in enzymatic activity caused by bursts of transcription (indicated as net production/consumption) should be reflected as a significant increase in end-product accumulation (N_2O in soil 3.8 and N_2 in soil 6.8) since there was no loss of end-product. However, increases in gene transcription were not always accompanied by a corresponding change in net production/consumption. Such change was only seen post-0.5 h in soil 3.8 NO and soil 6.8 N_2 production (observed as spikes in production). The peak in net NO production in soil 6.8 at 15 h may also be attributed to the 12 h transcription burst (Fig. 3D). Even assuming that proteins may have been transcribed at different rates, the stability of all substrate production/consumption rates were largely unperturbed throughout the incubation (Fig. 1B). Contrarily, the only major change in end-product occurred around 30 h in soil 6.8, but is likely due to an exhaustion of nitrate instead. Thus, the MG, MT and phenome of these soils together

point towards post-transcriptional regulation or the regulation of associated essential genes (such as *nosR* of the *nosZ* operon) for most genes involved in denitrification.

Concluding remarks. In the last decade, falling costs associated with sequencing analyses and better bioinformatics tools have resulted in many MG and MT studies, but few are well-linked to phenotypic measurements. This paper details the effort of such close MG, MT and phenome associations, which are necessary together to fully comprehend the complexity of microbial community response to nitrate addition under anoxic conditions. Denitrification in these soils was clearly divided by environmental pH, most outstanding of all is the simultaneity of gene transcription (Fig. 3), the control of nitrite in soil 3.8 (Fig. 1), and the present and responding bacteria (Fig. 4-5). Jointly, these results clearly depict a process modularity expected in complex environments, as well as the fallibility of relying on only MG/MT for process predictions. In particular, this study conclusively shows that the effect of N2OR non-functionality at low pH ranges across a diverse microbial community, strongly arguing for a general post-transcriptional effect at low pH, rather than a pH-dependent composition of the community.

References

Albalat, R., and Cañestro, C. (2016). Evolution by gene loss. *Nat. Rev. Genet.* 17, 379–391. doi:10.1038/nrg.2016.39.

Apprill, A., McNally, S., Parsons, R., and Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. *Aquat. Microb. Ecol.* 75, 129–137. doi:10.3354/ame01753.

Bakken, L. R., Bergaust, L., Liu, B., and Frostegård, Å. (2012). Regulation of denitrification at the cellular level: a clue to the understanding of N2O emissions from soils. *Philos. Trans. R. Soc. London B Biol. Sci.* 367.

Bancroft, K., Grant, I. F., and Alexander, M. (1979). Toxicity of NO2: Effect of Nitrite on Microbial Activity in an Acid Soil. *Appl. Envir. Microbiol.* 38, 940–944. Available at: http://aem.asm.org/content/38/5/940.short [Accessed April 13, 2016].

Bergaust, L., Bakken, L. R., and Frostegård, Å. (2011). Denitrification regulatory phenotype, a new term for the characterization of denitrifying bacteria. *Biochem. Soc. Trans.* 39, 207–212. Available at: http://europepmc.org/abstract/MED/21265774.

Bergaust, L., Mao, Y., Bakken, L. R., and Frostegård, Å. (2010). Denitrification Response Patterns during the Transition to Anoxic Respiration and Posttranscriptional Effects of Suboptimal pH on Nitrogen Oxide Reductase in Paracoccus denitrificans. *Appl. Environ. Microbiol.* 76, 6387–6396. doi:10.1128/aem.00608-10.

Bergaust, L., Shapleigh, J., Frostegård, Å., and Bakken, L. R. (2008). Transcription and activities of NOx reductases in Agrobacterium tumefaciens: the influence of nitrate, nitrite and oxygen availability. *Environ. Microbiol.* 10, 3070–3081. doi:10.1111/j.1462-2920.2007.01557.x.

Bergaust, L., van Spanning, R. J. M., Frostegård, A., and Bakken, L. R. (2012). Expression of nitrous oxide reductase in Paracoccus denitrificans is regulated by oxygen and nitric oxide through FnrP and NNR. *Microbiology* 158, 826–34. doi:10.1099/mic.0.054148-0.

Braman, R. S., and Hendrix, S. A. (1989). Nanogram nitrite and nitrate determination in environmental and biological materials by vanadium(III) reduction with chemiluminescence detection. *Anal. Chem.* 61, 2715–2718. doi:10.1021/ac00199a007.

Brenzinger, K., Dörsch, P., and Braker, G. (2015). pH-driven shifts in overall and transcriptionally active denitrifiers control gaseous product stoichiometry in growth experiments with extracted bacteria from soil. *Front. Microbiol.* 6. doi:10.3389/fmicb.2015.00961.

Buchfink, B., Xie, C., and Huson, D. H. (2015). Fast and sensitive protein alignment using DIAMOND. *Nat. Methods* 12, 59–60. doi:10.1038/nmeth.3176.

Burns, L. C., Stevens, R. J., and Laughlin, R. J. (1996). Production of nitrite in soil by simultaneous nitrification and denitrification. *Soil Biol. Biochem.* 28, 609–616. doi:10.1016/0038-0717(95)00175-1.

Cantera, J. J. L., and Stein, L. Y. (2007). Molecular diversity of nitrite reductase genes (nirK) in nitrifying bacteria. *Environ. Microbiol.* 9, 765–776. doi:10.1111/j.1462-2920.2006.01198.x.

Chen, Z., Liu, J., Wu, M., Xie, X., Wu, J., and Wei, W. (2012). Differentiated response of denitrifying communities to fertilization regime in paddy soil. *Microb. Ecol.* 63, 446–59.

650 doi:10.1007/s00248-011-9909-5.

651 Cox, R. D. (1980). Determination of nitrate and nitrite at the parts per billion level by
652 chemiluminescence. *Anal. Chem.* 52, 332–335. doi:10.1021/ac50052a028.

653 Coyotzi, S., Doxey, A. C., Clark, I. D., Lapen, D. R., Van Cappellen, P., and Neufeld, J. D. (2017).
654 Agricultural soil denitrifiers possess extensive nitrite reductase gene diversity. *Environ.*
655 *Microbiol.* doi:10.1111/1462-2920.13643.

656 Decleyre, H., Heylen, K., Van Colen, C., and Willems, A. (2015). Dissimilatory nitrogen reduction
657 in intertidal sediments of a temperate estuary: small scale heterogeneity and novel nitrate-to-
658 ammonium reducers. *Front. Microbiol.* 6, 1124. doi:10.3389/fmicb.2015.01124.

659 DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., et al. (2006).
660 Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with
661 ARB. *Appl. Environ. Microbiol.* 72, 5069–72. doi:10.1128/AEM.03006-05.

662 Gamble, T. N., Betlach, M. R., and Tiedje, J. M. (1977). Numerically Dominant Denitrifying
663 Bacteria from World Soils. *Appl. Environ. Microbiol.* 33, 926–939. Available at:
664 http://aem.asm.org/content/33/4/926.abstract.

665 Glass, C., and Silverstein, J. (1998). Denitrification kinetics of high nitrate concentration water: pH
666 effect on inhibition and nitrite accumulation. *Water Res.* 32, 831–839. doi:10.1016/S0043-
667 1354(97)00260-1.

668 Graf, D. R. H., Jones, C. M., and Hallin, S. (2014). Intergenomic Comparisons Highlight
669 Modularity of the Denitrification Pathway and Underpin the Importance of Community
670 Structure for N2O Emissions. *PLoS One* 9, e114118. doi:10.1371/journal.pone.0114118.

671 Hallin, S., and Lindgren, P.-E. (1999). PCR Detection of Genes Encoding Nitrite Reductase in
672 Denitrifying Bacteria. *Appl. Environ. Microbiol.* 65, 1652–1657. Available at:
673 http://aem.asm.org/content/65/4/1652.abstract.

674 Henderson, S. L., Dandie, C. E., Patten, C. L., Zebarth, B. J., Burton, D. L., Trevors, J. T., et al.
675 (2010). Changes in Denitrifier Abundance, Denitrification Gene mRNA Levels, Nitrous Oxide
676 Emissions, and Denitrification in Anoxic Soil Microcosms Amended with Glucose and Plant
677 Residues. *Appl. Environ. Microbiol.* 76, 2155–2164. doi:10.1128/aem.02993-09.

678 Jones, C. M., Graf, D. R. H., Bru, D., Philippot, L., and Hallin, S. (2012). The unaccounted yet
679 abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. *ISME J*
680 7, 417–426. doi:http://www.nature.com/ismej/journal/v7/n2/suppinfo/ismej2012125s1.html.

681 Kaiser, G., and Heber, U. (1983). Photosynthesis of leaf cell protoplasts and permeability of the
682 plasmalemma to some solutes. *Planta* 157, 462–470. doi:10.1007/BF00397204.

683 Kloos, K., Mergel, A., Rösch, C., and Bothe, H. (2001). Denitrification within the genus
684 Azospirillum and other associative bacteria. *Funct. Plant Biol.* 28, 991–998.
685 doi:http://dx.doi.org/10.1071/PP01071.

686 Körner, H., Sofia, H. J., and Zumft, W. G. (2003). Phylogeny of the bacterial superfamily of Crp-
687 Fnr transcription regulators: exploiting the metabolic spectrum by controlling alternative gene
688 programs. *FEMS Microbiol. Rev.* 27, 559–592. doi:10.1016/S0168-6445(03)00066-4.

689 Koskiniemi, S., Sun, S., Berg, O. G., Andersson, D. I., and Boxer, D. (2012). Selection-Driven

690 Gene Loss in Bacteria. *PLoS Genet.* 8, e1002787. doi:10.1371/journal.pgen.1002787.

691 Kulmala, M., and Petäjä, T. (2011). Soil Nitrites Influence Atmospheric Chemistry. *Science (80-.
692).* 333, 1586–1587. doi:10.1126/science.1211872.

693 Kuo, C.-H., and Ochman, H. (2009). The fate of new bacterial genes. *FEMS Microbiol. Rev.* 33,
694 38–43. doi:10.1111/j.1574-6976.2008.00140.x.

695 Lashof, D. A., and Ahuja, D. R. (1990). Relative contributions of greenhouse gas emissions to
696 global warming. *Nature* 344, 529–531. doi:10.1038/344529a0.

697 Li, X., Brauers, T., Häseler, R., Bohn, B., Fuchs, H., Hofzumahaus, A., et al. (2012). Exploring the
698 atmospheric chemistry of nitrous acid (HONO) at a rural site in Southern China. *Atmos. Chem.
699 Phys.* 12, 1497–1513. doi:10.5194/acp-12-1497-2012.

700 Lim, N. Y. N., Frostegård, Å., and Bakken, L. R. (2017). Soil pH dependent nitrite kinetics during
701 anoxia; the role of abiotic reactions versus microbial reduction. S*ubmitted for publication*.

702 Lim, N. Y. N., Roco, C. A., and Frostegard, A. (2016). Transparent DNA/RNA co-extraction
703 workflow protocol suitable for inhibitor-rich environmental samples that focuses on complete
704 DNA removal for transcriptomic analyses. *Front. Microbiol.* 7, 1588.
705 doi:10.3389/FMICB.2016.01588.

706 Liu, B., Frostegård, Å., and Bakken, L. R. (2014). Impaired reduction of N2O to N2 in acid soils is
707 due to a posttranscriptional interference with the expression of nosZ. *MBio* 5, e01383-14.
708 doi:10.1128/mBio.01383-14.

709 Liu, B., Mao, Y., Bergaust, L., Bakken, L. R., and Frostegård, Å. (2013). Strains in the genus
710 Thauera exhibit remarkably different denitrification regulatory phenotypes. *Environ.
711 Microbiol.*, n/a-n/a. doi:10.1111/1462-2920.12142.

712 Liu, B., Mørkved, P. T., Frostegård, Å., and Bakken, L. R. (2010). Denitrification gene pools,
713 transcription and kinetics of NO, N2O and N2 production as affected by soil pH. *FEMS
714 Microbiol. Ecol.* 72, 407–417. doi:10.1111/j.1574-6941.2010.00856.x.

715 Luckmann, M., Mania, D., Kern, M., Bakken, L. R., Frostegard, A., and Simon, J. (2014).
716 Production and consumption of nitrous oxide in nitrate-ammonifying Wolinella succinogenes
717 cells. *Microbiology* 160, 1749–1759. doi:10.1099/mic.0.079293-0.

718 Lycus, P., Kjendseth, Å. R., Bergaust, L., Bakken, L. R., Frostegård, Å. (2017). [Impaired N2OR
719 assembly at low pH causes N2O emissions]. *Unpublished data*.

720 Lycus, P., Bøthun, K., Bergaust, L., Shapleigh, J., Bakken, L., and Frostegård, Å. (2017).
721 Phenotypic and genotypic richness of denitrifiers revealed by a novel isolation strategy. *ISME
722 J.* In Press.

723 Malm, S., Tiffert, Y., Micklinghoff, J., Schultze, S., Joost, I., Weber, I., et al. (2009). The roles of
724 the nitrate reductase NarGHJI, the nitrite reductase NirBD and the response regulator GlnR in
725 nitrate assimilation of Mycobacterium tuberculosis. *Microbiology* 155, 1332–1339.
726 doi:10.1099/mic.0.023275-0.

727 Mania, D., Heylen, K., van Spanning, R. J. M., and Frostegård, Å. (2014). The nitrate-ammonifying
728 and nosZ -carrying bacterium Bacillus vireti is a potent source and sink for nitric and nitrous
729 oxide under high nitrate conditions. *Environ. Microbiol.* 16, 3196–3210. doi:10.1111/1462-

730 2920.12478.

731 Medinets, S., Skiba, U., Rennenberg, H., and Butterbach-Bahl, K. (2015). A review of soil NO
732 transformation: Associated processes and possible physiological significance on organisms.
733 *Soil Biol. Biochem.* 80, 92–117. doi:10.1016/j.soilbio.2014.09.025.

734 Mira, A., Ochman, H., and Moran, N. A. (2001). Deletional bias and the evolution of bacterial
735 genomes. *Trends Genet.* 17, 589–596. doi:10.1016/S0168-9525(01)02447-7.

736 Molstad, L., Dörsch, P., and Bakken, L. R. (2007). Robotized incubation system for monitoring
737 gases (O2, NO, N2O N2) in denitrifying cultures. *J. Microbiol. Methods* 71, 202–211.
738 doi:http://dx.doi.org/10.1016/j.mimet.2007.08.011.

739 Moreno-Vivián, C., Cabello, P., Martínez-Luque, M., Blasco, R., and Castillo, F. (1999).
740 Prokaryotic nitrate reduction: molecular properties and functional distinction among bacterial
741 nitrate reductases. *J. Bacteriol.* 181, 6573–84. Available at:
742 http://www.ncbi.nlm.nih.gov/pubmed/10542156 [Accessed February 10, 2017].

743 Mørkved, P. T., Dörsch, P., and Bakken, L. R. (2007). The N2O product ratio of nitrification and
744 its dependence on long-term changes in soil pH. *Soil Biol. Biochem.* 39, 2048–2057.
745 doi:10.1016/j.soilbio.2007.03.006.

746 Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. (1993). Profiling of complex microbial
747 populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-
748 amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695–700. Available at:
749 http://aem.asm.org/content/59/3/695.abstract.

750 Nömmik, H., and Thorin, J. (1972). "Transformations of 15N-labelled nitrite and nitrate in forest
751 raw humus during anaerobic incubation," in *Isotopes and Radiation in Soil Plant Relationships*
752 *Including Forestry*, ed. IAEA (Vienna, Austria: IAEA-SM-151/58), 369–382.

753 Orellana, L. H., Rodriguez-R, L. M., Higgins, S., Chee-Sanford, J. C., Sanford, R. A., Ritalahti, K.
754 M., et al. (2014). Detecting nitrous oxide reductase (NosZ) genes in soil metagenomes: method
755 development and implications for the nitrogen cycle. *MBio* 5, e01193-14.
756 doi:10.1128/mBio.01193-14.

757 Oswald, R., Behrendt, T., Ermel, M., Wu, D., Su, H., Cheng, Y., et al. (2013). HONO Emissions
758 from Soil Bacteria as a Major Source of Atmospheric Reactive Nitrogen. *Science (80-.).* 341,
759 1233–1235. doi:10.1126/science.1242266.

760 Palmer, K., and Horn, M. A. (2012). Actinobacterial nitrate reducers and proteobacterial denitrifiers
761 are abundant in N2O-metabolizing palsa peat. *Appl. Environ. Microbiol.* 78, 5584–96.
762 doi:10.1128/AEM.00810-12.

763 Parada, A. E., Needham, D. M., and Fuhrman, J. A. (2016). Every base matters: assessing small
764 subunit rRNA primers for marine microbiomes with mock communities, time series and global
765 field samples. *Environ. Microbiol.* 18, 1403–1414. doi:10.1111/1462-2920.13023.

766 Parkin, T. B., Sexstone, A. J., and Tiedje, J. M. (1985). Adaptation of Denitrifying Populations to
767 Low Soil pH. *Appl. Environ. Microbiol.* 49, 1053–1056.

768 Penton, C. R., Johnson, T. A., Quensen, J. F., Iwai, S., Cole, J. R., and Tiedje, J. M. (2013).
769 Functional genes to assess nitrogen cycling and aromatic hydrocarbon degradation: primers
770 and processing matter. *Front. Microbiol.* 4. doi:10.3389/fmicb.2013.00279.

771 Portmann, R. W., Daniel, J. S., and Ravishankara, A. R. (2012). Stratospheric ozone depletion due
772 to nitrous oxide: influences of other gases. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 367, 1256–
773 64. doi:10.1098/rstb.2011.0377.

774 Raut, N., Dörsch, P., Sitaula, B. K., and Bakken, L. R. (2012). Soil acidification by intensified crop
775 production in South Asia results in higher N2O/(N2 + N2O) product ratios of denitrification.
776 *Soil Biol. Biochem.* 55, 104–112. doi:http://dx.doi.org/10.1016/j.soilbio.2012.06.011.

777 Ravishankara, A. R., Daniel, J. S., and Portmann, R. W. (2009). Nitrous Oxide (N2O): The
778 Dominant Ozone-Depleting Substance Emitted in the 21st Century. *Science (80-.).* 326.
779 Available at: http://science.sciencemag.org/content/326/5949/123.full [Accessed April 4,
780 2017].

781 Rodionov, D. A., Dubchak, I. L., Arkin, A. P., Alm, E. J., and Gelfand, M. S. (2005). Dissimilatory
782 Metabolism of Nitrogen Oxides in Bacteria: Comparative Reconstruction of Transcriptional
783 Networks. *PLoS Comput. Biol.* 1, 0415–0431. doi:10.1371/journal.pcbi.0010055.

784 Russow, R., Stange, C. F., and Neue, H.-U. (2009). Role of nitrite and nitric oxide in the processes
785 of nitrification and denitrification in soil: Results from 15N tracer experiments. *Soil Biol.*
786 *Biochem.* 41, 785–795. doi:10.1016/j.soilbio.2009.01.017.

787 Saleh-Lakha, S., Shannon, K. E., Henderson, S. L., Goyer, C., Trevors, J. T., Zebarth, B. J., et al.
788 (2009). Effect of pH and Temperature on Denitrification Gene Expression and Activity in
789 Pseudomonas mandelii. *Appl. Environ. Microbiol.* 75, 3903–3911. doi:10.1128/aem.00080-
790 09.

791 Schreiber, F., Wunderlin, P., Udert, K. M., and Wells, G. F. (2012). Nitric oxide and nitrous oxide
792 turnover in natural and engineered microbial communities: biological pathways, chemical
793 reactions, and novel technologies. *Front. Microbiol.* 3, 372. doi:10.3389/fmicb.2012.00372.

794 Shapleigh, J. P. (2013). "Denitrifying Prokaryotes," in *The Prokaryotes* (Berlin, Heidelberg:
795 Springer Berlin Heidelberg), 405–425. doi:10.1007/978-3-642-30141-4_71.

796 Shen, Q. ., Ran, W., and Cao, Z. . (2003). Mechanisms of nitrite accumulation occurring in soil
797 nitrification. *Chemosphere* 50, 747–753. doi:10.1016/S0045-6535(02)00215-1.

798 Šimek, M., Jíšová, L., and Hopkins, D. W. (2002). What is the so-called optimum pH for
799 denitrification in soil? *Soil Biol. Biochem.* 34, 1227–1234. doi:10.1016/S0038-
800 0717(02)00059-7.

801 Sognnes, L. S., Fystro, G., Øpstad, S. L., Arstein, A., and Børresen, T. (2006). Effects of adding
802 moraine soil or shell sand into peat soil on physical properties and grass yield in western
803 Norway. *Acta Agric. Scand. Sect. B - Soil Plant Sci.* 56, 161–170.
804 doi:10.1080/09064710500218845.

805 Spataro, F., and Ianniello, A. (2014). Sources of atmospheric nitrous acid: State of the science,
806 current research needs, and future prospects. *J. Air Waste Manage. Assoc.* 64, 1232–1250.
807 doi:10.1080/10962247.2014.952846.

808 Spiro, S. (2012). Nitrous oxide production and consumption: regulation of gene expression by gas-
809 sensitive transcription factors. *Philos. Trans. R. Soc. B Biol. Sci.* 367, 1213–1225.
810 doi:10.1098/rstb.2011.0309.

811 Stevens, R. J., Laughlin, R. J., and Malone, J. P. (1998). Soil pH affects the processes reducing

nitrate to nitrous oxide and di-nitrogen. *Soil Biol. Biochem.* 30, 1119–1126. doi:10.1016/S0038-0717(97)00227-7.

Su, H., Cheng, Y., Oswald, R., Behrendt, T., Trebs, I., Meixner, F. X., et al. (2011). Soil nitrite as a source of atmospheric HONO and OH radicals. *Science* 333, 1616–8. doi:10.1126/science.1207687.

Sun, Y., De Vos, P., and Heylen, K. (2016). Nitrous oxide emission by the non-denitrifying, nitrate ammonifier Bacillus licheniformis. *BMC Genomics* 17, 68. doi:10.1186/s12864-016-2382-2.

Throbäck, I. N., Enwall, K., Jarvis, Å., and Hallin, S. (2004). Reassessing PCR primers targeting nirS, nirK and nosZ genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol. Ecol.* 49, 401–417. doi:10.1016/j.femsec.2004.04.011.

Torres, M. J., Simon, J., Rowley, G., Bedmar, E. J., Richardson, D. J., Gates, A. J., et al. (2016). "Chapter Seven – Nitrous Oxide Metabolism in Nitrate-Reducing Bacteria: Physiology and Regulatory Mechanisms," in *Advances in Microbial Physiology*, 353–432. doi:10.1016/bs.ampbs.2016.02.007.

Vollack, K. U., and Zumft, W. G. (2001). Nitric oxide signaling and transcriptional control of denitrification genes in Pseudomonas stutzeri. *J. Bacteriol.* 183, 2516–26. doi:10.1128/JB.183.8.2516-2526.2001.

Wang, H., Tseng, C. P., and Gunsalus, R. P. (1999). The napF and narG nitrate reductase operons in Escherichia coli are differentially expressed in response to submicromolar concentrations of nitrate but not nitrite. *J. Bacteriol.* 181, 5303–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10464201 [Accessed March 15, 2017].

Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J. Bacteriol.* 173, 697–703. Available at: http://jb.asm.org/content/173/2/697.abstract.

Wood, D. E., and Salzberg, S. L. (2014). Kraken: ultrafast metagenomic sequence classification using exact alignments. *Genome Biol.* 15, R46. doi:10.1186/gb-2014-15-3-r46.

Wrage, N., Velthof, G. ., van Beusichem, M. ., and Oenema, O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biol. Biochem.* 33, 1723–1732. doi:10.1016/S0038-0717(01)00096-7.

Wunsch, P., Herb, M., Wieland, H., Schiek, U. M., and Zumft, W. G. (2003). Requirements for Cu(A) and Cu-S center assembly of nitrous oxide reductase deduced from complete periplasmic enzyme maturation in the nondenitrifier Pseudomonas putida. *J. Bacteriol.* 185, 887–96. doi:10.1128/JB.185.3.887-896.2003.

Wunsch, P., and Zumft, W. G. (2005). Functional domains of NosR, a novel transmembrane iron-sulfur flavoprotein necessary for nitrous oxide respiration. *J. Bacteriol.* 187, 1992–2001. doi:10.1128/JB.187.6.1992-2001.2005.

Zumft, W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* 61, 533–616. Available at: http://mmbr.asm.org/content/61/4/533.abstract.

Supplementary material

Linking meta-omics to the kinetics of denitrification intermediates reveals pH-dependent causes of N_2O emissions and nitrite accumulation in soil

Natalie YN Lim[1], James P Shapleigh[2], Lars R Bakken[3] and Åsa Frostegård[1]

[1] *Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway*

[2] *Department of Microbiology, Cornell University, Ithaca, NY, USA*

[3] *Faculty of Environmental Sciences and Natural Resource Management, Norwegian University of Life Sciences, Ås, Norway*

Quantification of transcripts using quantitative real-time PCR (qPCR).

Amplicon-based quantification of denitrification using primers targeting 16S rRNA (27F/518R), *nirK* (517F/1055R), *nirS* (cd3aF/R3cd) and *nosZ* (nosZF/1622R) genes were performed on both DNA and RNA samples from soil 3.8 and 6.8. There was consistently lower quantities of all tested genes in soil 3.8 than 6.8, but all were above the detection limit (4×10^5 copies g^{-1} soil, ww). Reverse transcription qPCR of the three denitrification genes revealed two peaks in transcription in soil 6.8 at 3 and 9-12 h, pointing towards two distinct bursts in transcription. The two bursts of transcription were confirmed with sequencing-based analyses (see main paper), and was not an artefact of extraction efficiency bias. In comparison, soil 3.8 signals were indistinguishable from the qPCR signal baseline. This either indicated a constitutive low expression of these genes, or the unsuitability of existing denitrification gene primers targeting the microflora in soil 3.8. Given the high expression detected using sequencing technologies and the dominance of *nirK* over *nirS* in soil 3.8 (Fig. 3, main text), the latter is the most likely reason.

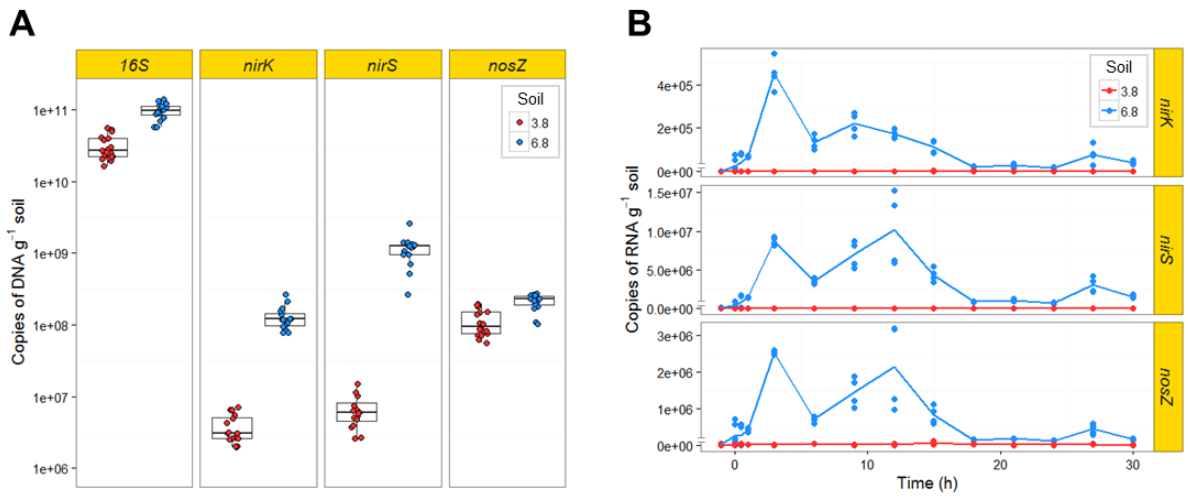


Figure S1 | Degenerate primers targeting *nirK, nirS* and *nosZ* were unsuitable for soil 3.8 and failed to detect gene transcripts in amplification-based analyses. *(A)* Primers targeting the 16S rRNA (27F/518R), *nirK* (517F/1055R), *nirS* (cd3aF/R3cd) and *nosZ* (nosZF/1622R) genes were able to detect gene copies in both soils 3.8 and 6.8. *(B)* mRNA transcripts of *nirK*, *nirS*, and *nosZ* were only detected in soil 6.8 (blue), revealing a "dual expression peak" pattern for all three genes. The qPCR fluorescence signal from soil 3.8 (red) samples could not be differentiated from baseline noise.

Calculating the concentration of HNO_2 as a function of pH and total nitrite concentration.

The Henderson-Hasselbalch equation (1) can be used to calculate the relative amount of undissociated nitrite as a function of pH (which is controlled by the buffer system of the soil):

$$K_a = [H^+]*[A^-]/[HA] \quad (1)$$

Where K_a is the base dissociation constant

Taking the $\log_{10}$ of both sides

$$\log_{10}(K_a) = \log_{10}[H^+] + \log_{10}([A^-]/[HA]) \quad (2)$$

defining $pX = -\log_{10}[X]$, (2) gives:

$$-pK_a = -pH + \log_{10}([A^-]/[HA]) \quad (3)$$

Replacing $[A^-]$ with $[NO_2^-]$ and $[HA]$ with $[HNO_2]$,

and solving equation (3) for $[NO_2^-]/[HNO_2]$:

$$[NO_2^-]/[HNO_2] = 10^{(pH-pKa)} \quad (4)$$

Equation (4) can be solved for $[HNO_2]/([HNO_2]+[NO_2^-])$, which is the molar fraction of total nitrite (as measured) that is un-dissociated:

$$[HNO_2]/([HNO_2]+[NO_2^-]) = 1/(1+[NO_2^-]) = 1/(1+10^{(pH-pKa)}) \quad (5)$$

Hence, we can calculate the concentration of un-dissociated HNO_2 in the soil

$$[HNO_2] = TNN/(1+10^{(pH-pKa)}) \quad (6)$$

where TNN is the measured concentration of total nitrite N ($[HNO_2]+[NO_2^-]$, pH is the measured soil pH and K_a is the dissociation constant for nitrous acid, which is 4E-4 (hence pK_a=3.3398).

Needless to say, soil pH is the most problematic parameter, since the pH may vary throughout the soil matrix, and the bulk pH as measured depends on the cation concentration in the soil slurry. Our pH measurements were done in 0.01 M $CaCl_2$, which is thought to give pH values close to the average of the intact soil. Higher salt concentrations will give lower pH values and vice versa.

Table S1 | Kingdom-based relative abundance of taxonomies based on 16S rRNA analysis.

Soil pH	Time (h)	Relative abundance (%)		
		Archaea	Bacteria	Unclassified
3.8	0	0.3	94.2	5.5
3.8	0	0.3	94.2	5.5
3.8	0	0.2	94.5	5.3
6.8	0	0.6	93.9	5.5
6.8	0	0.5	94.1	5.4
6.8	0	0.4	94.2	5.4
6.8	3	0.5	94.1	5.4
6.8	3	0.2	94.5	5.4
6.8	12	0.6	93.8	5.5
6.8	12	0.6	94.0	5.5
6.8	27	0.4	94.2	5.4
6.8	27	0.3	94.3	5.4

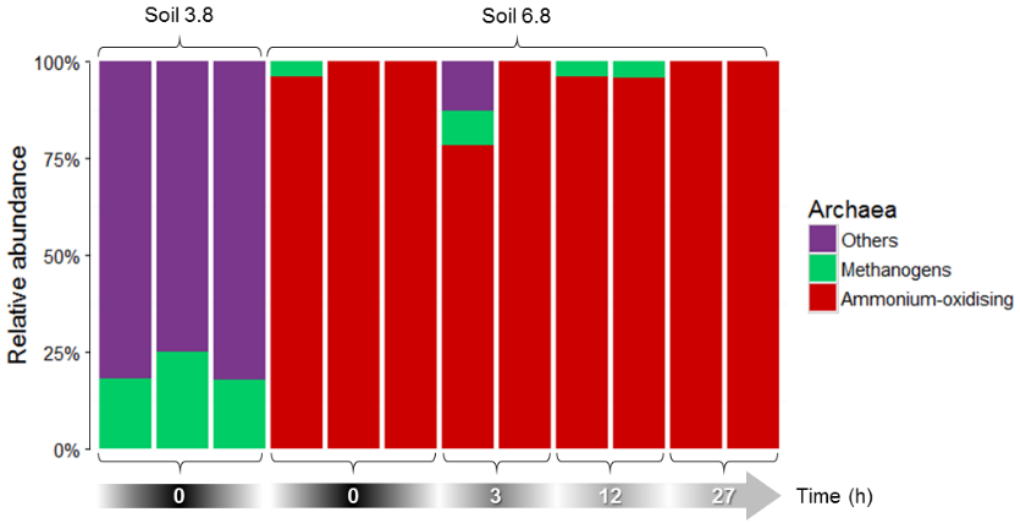


Figure S2 | The taxonomic distribution of archaea in soil 3.8 and 6.8 based on 16S rRNA sequencing revealed contrasting communities. Samples were sequenced using primers targeting the 16S rRNA gene (515f/806rB) and annotated using the GreenGenes database as reference. Values are relative to total archaeal 16S rRNA. Detailed breakdown found below (Supplementary Table S1).

Table S2 | Taxonomic distribution of 16S rRNA genes present in soil 3.8 and 6.8 during anoxic incubation.

Kingdom	Phylum	Soil 3.8			Soil 6.8								
		0 h	0 h	0 h	0 h	0 h	0 h	3 h	3 h	12 h	12 h	27 h	27 h
Archaea	Ammonia-oxidising (Crenarchaeota: Thaumarchaeota)	0.00	0.00	0.00	0.57	0.48	0.42	0.40	0.15	0.59	0.53	0.40	0.35
Archaea	Methanogens (Euryarchaeota: Methanomicrobia)	0.06	0.08	0.04	0.02	0.00	0.00	0.04	0.00	0.03	0.02	0.00	0.00
Archaea	Other Archaea (Crenarchaeota: Thermoprotei)	0.07	0.06	0.04	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.00	0.00
Archaea	Unclassified Archaea	0.19	0.18	0.14	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteria	Acidobacteria	8.45	7.57	8.85	0.74	0.77	0.73	1.05	0.67	0.71	0.71	0.74	0.73
Bacteria	Actinobacteria	12.07	12.85	13.62	10.70	10.54	11.14	10.21	10.21	11.92	10.78	10.71	10.49
Bacteria	Armatimonadetes	0.12	0.11	0.10	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.00
Bacteria	Bacteroidetes	3.49	4.07	3.21	7.79	7.19	6.94	5.72	6.53	7.33	8.99	6.33	5.77
Bacteria	Caldithrix	0.00	0.00	0.00	0.08	0.10	0.08	0.08	0.11	0.07	0.08	0.09	0.08
Bacteria	Chlamydiae	0.00	0.07	0.00	0.05	0.03	0.03	0.02	0.03	0.05	0.05	0.05	0.04
Bacteria	Chlorobi	0.06	0.06	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bacteria	Chloroflexi	0.32	0.42	0.37	1.57	1.36	1.47	1.42	1.44	1.51	1.44	1.58	1.49
Bacteria	Cyanobacteria	0.13	0.13	0.20	0.05	0.08	0.06	0.09	0.04	0.06	0.05	0.10	0.07
Bacteria	Deferribacteres	0.09	0.07	0.08	0.06	0.07	0.06	0.07	0.07	0.06	0.07	0.06	0.06
Bacteria	Firmicutes	12.25	12.43	12.88	10.73	10.40	10.37	10.45	10.96	10.39	10.60	10.56	10.94
Bacteria	Gemmatimonadetes	0.13	0.11	0.12	0.04	0.04	0.05	0.11	0.05	0.05	0.05	0.05	0.06
Bacteria	Nitrospirae	0.21	0.15	0.15	0.68	0.70	0.65	0.61	0.60	0.63	0.62	0.71	0.65
Bacteria	Planctomycetes	4.97	4.55	4.21	3.29	3.36	3.24	3.33	3.33	3.19	3.14	3.28	3.42
Bacteria	Proteobacteria (Alphaproteobacteria)	13.72	13.08	14.28	12.32	12.79	12.89	13.81	12.81	12.76	12.05	12.34	12.72
Bacteria	Proteobacteria (Betaproteobacteria)	6.71	7.08	6.95	5.23	5.35	5.38	5.44	5.45	4.90	5.15	5.15	5.59
Bacteria	Proteobacteria (Deltaproteobacteria)	2.46	2.24	2.16	4.27	4.15	4.64	4.23	4.18	3.94	3.74	4.25	4.32
Bacteria	Proteobacteria (Epsilonproteobacteria)	0.00	0.00	0.00	0.07	0.06	0.06	0.06	0.03	0.06	0.06	0.05	0.03
Bacteria	Proteobacteria (Gammaproteobacteria)	5.42	5.99	5.68	9.53	9.93	9.96	9.92	9.96	9.83	9.92	10.12	9.98
Bacteria	Proteobacteria (Unclassified)	6.73	6.31	6.16	5.49	5.62	5.57	5.42	5.58	5.61	5.57	5.74	5.44
Bacteria	Spirochaetes	0.05	0.06	0.05	0.15	0.15	0.15	0.15	0.14	0.12	0.14	0.14	0.14
Bacteria	Synergistetes	1.47	1.52	1.63	0.29	0.27	0.26	0.28	0.28	0.26	0.27	0.27	0.28
Bacteria	Tenericutes	0.00	0.00	0.00	0.03	0.00	0.08	0.00	0.00	0.02	0.06	0.05	0.00
Bacteria	Thermotogae	0.22	0.20	0.19	0.16	0.16	0.16	0.16	0.23	0.17	0.15	0.18	0.15
Bacteria	Unclassified Bacteria	9.28	9.29	9.16	11.76	12.28	11.84	12.03	12.95	12.04	11.87	13.07	12.45
Bacteria	Verrucomicrobia	5.86	5.80	4.39	8.83	8.76	8.43	9.37	8.86	8.17	8.43	8.62	9.40
Unclassified	Unclassified	5.49	5.52	5.32	5.51	5.36	5.36	5.44	5.36	5.55	5.52	5.39	5.37

All values are listed as relative abundance (% of total sequences)

Table S3 | Occurrence of assigned denitrification and DNRA genes and transcripts in soil 3.8 and 6.8 (all values in RPM).

Soil pH	Time (h)	*nap*	*nar*	*nirK*	*nirS*	*cnor*	*qnor*	*nosZ*	*nirB*	*nrf*	Total	Denitrification	DNRA
DNA													
3.8	-	24.6	147.0	33.7	0.9	5.0	70.5	17.5	56.7	5.6	*361.5*	82.8%	17.2%
3.8	-	24.1	146.8	35.9	0.9	6.0	68.7	19.3	59.5	5.2	*366.5*	82.3%	17.7%
3.8	-	22.5	145.3	37.1	0.8	6.1	68.3	17.8	54.8	5.5	*358.2*	83.2%	16.8%
6.8	-	62.5	128.1	37.6	5.4	10.5	37.5	25.8	129.1	37.2	*473.5*	64.9%	35.1%
6.8	-	61.0	130.3	39.6	6.1	10.3	38.2	27.8	132.4	38.8	*484.4*	64.7%	35.3%
6.8	-	64.5	131.3	39.6	5.6	11.3	36.6	23.8	133.8	40.6	*487.0*	64.2%	35.8%
Total		259.2	828.8	223.5	19.7	49.2	319.7	131.9	566.3	132.7	*2531.0*	72.4%	27.6%
RNA													
3.8	0.5	3.0	46.0	23.5	0.0	1.3	31.7	33.4	8.8	0.0	*147.7*	94.1%	5.9%
3.8	0.5	1.3	45.1	23.4	0.0	0.7	27.3	25.9	3.0	0.0	*126.7*	97.6%	2.4%
3.8	3	2.8	104.7	40.9	0.4	1.3	98.2	62.0	2.7	0.2	*313.2*	99.1%	0.9%
3.8	3	6.6	79.9	42.1	0.0	0.1	52.8	42.0	6.3	1.5	*231.4*	96.6%	3.4%
6.8	0.5	16.3	162.2	72.3	21.9	10.2	23.2	86.7	29.6	9.1	*431.5*	91.0%	9.0%
6.8	0.5	41.1	131.9	42.4	6.9	14.1	32.6	40.5	63.0	20.3	*392.7*	78.8%	21.2%
6.8	3	50.0	544.6	330.2	63.0	28.1	106.0	417.8	17.8	11.7	*1569.2*	98.1%	1.9%
6.8	3	41.6	336.1	192.9	35.8	22.1	55.8	368.2	52.0	8.5	*1112.9*	94.6%	5.4%
6.8	9	49.9	114.5	40.6	8.0	10.8	31.0	56.2	102.1	21.8	*434.8*	71.5%	28.5%
6.8	9	60.8	136.5	32.3	4.5	7.1	25.5	12.3	125.8	27.2	*432.1*	64.6%	35.4%
6.8	12	48.0	261.9	137.5	18.5	16.4	58.4	154.0	67.0	27.5	*789.3*	88.0%	12.0%
6.8	12	44.7	235.1	149.1	22.6	18.4	40.8	234.9	68.1	32.9	*846.6*	88.1%	11.9%
6.8	27	43.8	127.6	56.5	7.1	10.6	21.5	65.4	87.1	16.6	*436.2*	76.2%	23.8%
6.8	27	53.1	124.2	67.4	7.1	12.6	34.0	77.8	92.5	19.3	*487.9*	77.1%	22.9%
Total		*462.8*	*2450.3*	*1251.1*	*195.6*	*153.9*	*639.0*	*1677.0*	*725.8*	*196.7*	*7752.1*	88.1%	11.9%

Denitrification = denitrification-related genes (nap+nar+nirK+nirS+cnor+qnor+nosZ); DNRA = DNRA-related nitrite reductases (nirB+nrf)

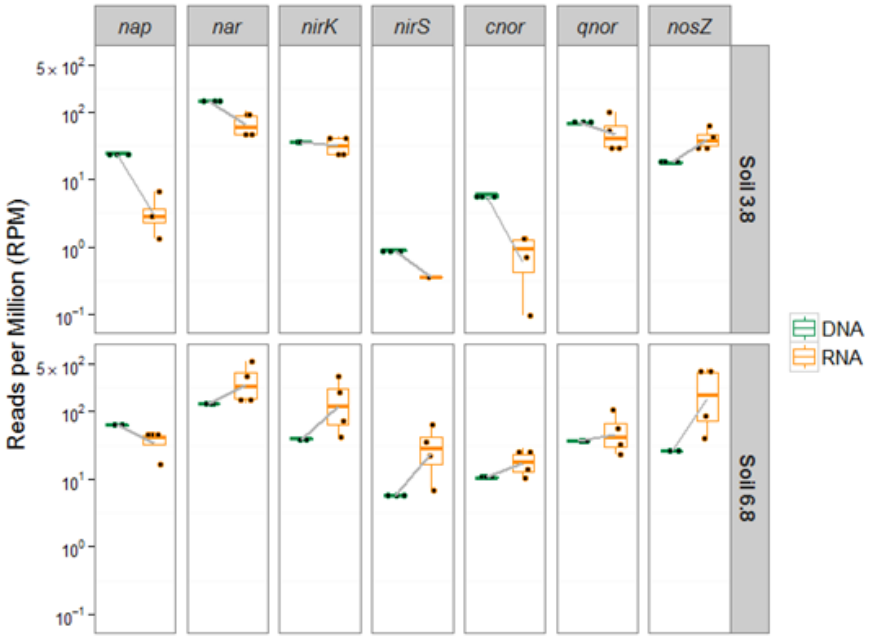


Figure S3 | Genetic potential vs. transcription at 0.5 and 3 h in soils 3.8 and 6.8. Gene abundance (triplicates, green) vs. transcript abundances (combined 0.5 and 3h, orange) in soil 3.8 (upper panel) and soil 6.8 (lower panel).

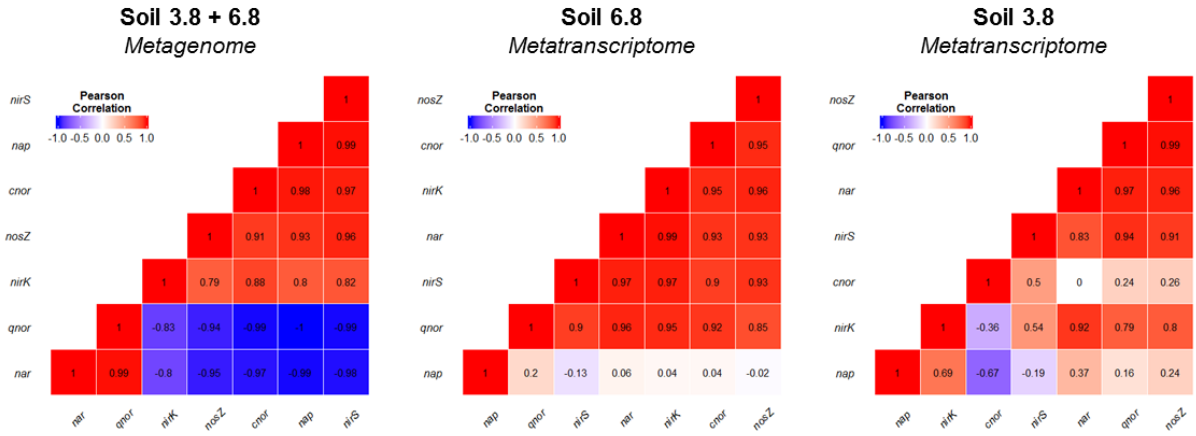


Figure S4 | Correlation of denitrification gene and transcript abundances from both soils depict a strong pH-dependent effect. The metagenomes of both soils 3.8 and 6.8 were analysed together to create a general denitrification gene abundance correlation pattern for the entire experimental field site. Metatranscriptomes were analysed separately to determine pH-influenced transcriptional abundance differences. Correlation matrices were sorted to group stronger correlations together. Correlation and probability values are reported in Supplementary Table S4.

Table S4 | Raw values for Pearson correlation of denitrification genes and transcripts (Fig. S4).

Correlation

DNA (Soil 6.8 + 3.8)

	nap	*nar*	*nirK*	*nirS*	*cnor*	*qnor*	*nosZ*
nap	1.00						
nar	-0.99	1.00					
nirK	0.80	-0.80	1.00				
nirS	0.99	-0.98	0.82	1.00			
cnor	0.98	-0.97	0.88	0.97	1.00		
qnor	-1.00	0.99	-0.83	-0.99	-0.99	1.00	
nosZ	0.93	-0.95	0.79	0.96	0.91	-0.94	1.00

RNA (Soil 6.8)

	nap	*nar*	*nirK*	*nirS*	*cnor*	*qnor*	*nosZ*
nap	1.00						
nar	0.06	1.00					
nirK	0.04	0.99	1.00				
nirS	-0.13	0.97	0.97	1.00			
cnor	0.04	0.93	0.95	0.90	1.00		
qnor	0.20	0.96	0.95	0.90	0.92	1.00	
nosZ	-0.02	0.93	0.96	0.93	0.95	0.85	1.00

RNA (Soil 3.8)

	nap	*nar*	*nirK*	*nirS*	*cnor*	*qnor*	*nosZ*
nap	1.00						
nar	0.37	1.00					
nirK	0.69	0.92	1.00				
nirS	-0.19	0.83	0.54	1.00			
cnor	-0.67	0.00	-0.36	0.50	1.00		
qnor	0.16	0.97	0.79	0.94	0.24	1.00	
nosZ	0.24	0.96	0.80	0.91	0.26	0.99	1.00

Probability

DNA (Soil 6.8 + 3.8)

	nap	*nar*	*nirK*	*nirS*	*cnor*	*qnor*	*nosZ*
nap	0.00						
nar	0.00	0.00					
nirK	0.06	0.06	0.00				
nirS	0.00	0.00	0.04	0.00			
cnor	0.00	0.00	0.02	0.00	0.00		
qnor	0.00	0.00	0.04	0.00	0.00	0.00	
nosZ	0.01	0.00	0.06	0.00	0.01	0.00	0.00

RNA (Soil 6.8)

	nap	*nar*	*nirK*	*nirS*	*cnor*	*qnor*	*nosZ*
nap	0.00						
nar	0.87	0.00					
nirK	0.91	0.00	0.00				
nirS	0.73	0.00	0.00	0.00			
cnor	0.92	0.00	0.00	0.00	0.00		
qnor	0.57	0.00	0.00	0.00	0.00	0.00	
nosZ	0.96	0.00	0.00	0.00	0.00	0.00	0.00

RNA (Soil 3.8)

	nap	*nar*	*nirK*	*nirS*	*cnor*	*qnor*	*nosZ*
nap	0.00						
nar	0.63	0.00					
nirK	0.31	0.08	0.00				
nirS	0.81	0.17	0.46	0.00			
cnor	0.33	1.00	0.64	0.50	0.00		
qnor	0.84	0.03	0.21	0.06	0.76	0.00	
nosZ	0.76	0.04	0.20	0.09	0.74	0.01	0.00

This page intentionally left blank