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Short-Term Emission of Nitrous Oxide from Oxic Denitrification in Soil

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Short-Term Emission of Nitrous Oxide from Oxic Denitrification in Soil

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Abstract

The reduction of nitrate (NO_3^-) to gaseous products in aerated environments (oxic denitrification) has been reported sporadically in the past, mostly in pure culture studies, but the extent to which it occurs in soil is not known. We investigated the emission of nitrous oxide (N_2O) due to oxic denitrification in bulk soil under continuous aeration in a soil slurry experiment. Out of 19 soils assayed, a subset of five, representing both cultivated and natural or unmanaged ecosystems, showed emissions of N_2O-N derived from NO_3^- ranging from 0.02 to 1.14 ng per gram of soil per hour. This emission occurred immediately after rewetting and continued during the 8 hour observation period. Although much less attention has been given to oxic denitrification compared to its anaerobic counterpart, our study indicates that the phenomenon, specifically the emission of N_2O, is active in a variety of soils. The periods during which denitrification in soils can occur may be far more extensive if both aerobic and anaerobic activities are considered, especially in soils that remain moist (but not necessarily anoxic) for long periods.

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Introduction

While "how to feed a growing population" is still a pertinent issue, innovations in plant science, agronomy, engineering, and soil science remind us that a holistic approach is required beyond increasing crop productivity. Simply feeding the population is not a solution to all the problems associated with food insecurity and should not be done without consideration of the social and environmental externalities. Agriculture is more than a tool to feed people. It should be viewed as an ecosystem that functions within itself and in the context of the landscapes that contain it. Agriculture involves cultivating a relationship with soils, crops, livestock, water resources, air quality, climate variability, wildlife, and biodiversity. Human beings cultivate relationships with soil and land through agriculture, in which soil nitrogen plays a key role—in biological N fixation, decomposition of soil organic matter, fertilization, and other processes. Over the past two decades, the scientific body representing multiple disciplines has provided mountainous evidence to warn about the escalating consequences of climate change, as well as provided information about how agricultural activities and associated changes in land-use patterns contribute to these problems. Gaining an understanding of greenhouse gas (GHG) emissions due to agriculture will close the nitrogen budget, allowing achievement of economic benefits, as well as design of proper management practices to mitigate the impact of climate change.

In this project, the production of nitrous oxide (N_2O), a potent greenhouse gas, through denitrification, the "last mile" of the terrestrial nitrogen cycle, is studied with particular consideration of its occurrence under aerobic conditions. In this Chapter, I summarize known

biological and abiotic N_2O production pathways to give context to the role of denitrification under aerobic conditions and background for the incubation study presented in Chapter 2. This study examined the occurrence and extent of aerobic denitrification in soil under natural conditions to demonstrate other potential loss pathways formally not considered in our attempts to understand N losses in soil.

Chapter 1

Literature review on denitrification and N_2O production pathways in the soil nitrogen cycle

1.1. Biotic and abiotic sources of N_2O

N_2O is a potent GHG with a global warming potential 298 times higher than that of CO_2 for a 100-year timescale. Apart from contributing to global warming, N_2O is also one of the important ozone-depleting agents in the stratosphere. Soil emits 6.6 Tg nitrogen annually as N_2O, accounting for about 60% of natural N_2O emission and about 37% of total N_2O emission[1]. N_2O is produced by a wide range of microbiological and abiotic processes (Figure 1.1.), with autotrophic nitrification and heterotrophic denitrification being the main pathways in soils[2]. Other microbial processes include nitrifier denitrification[3–5], co-denitrification, dissimilatory nitrate reduction to ammonia (DNRA), and heterotrophic nitrification. Firestone and Davidson[6] proposed the classic hole-in-the-pipe conceptual model to represent nitrogen trace gas production (NO, N_2O, and N_2). Since then, N_2O production pathways have been reviewed extensively, first by Bremner (1997) and Wrage (2001), then by Baggs (2011), Braker and Conrad (2011), and Butterbach-Bahl (2013), and more recently by Groenigen (2015) and Wrage (2018)[3,7–12]. However, source partitioning the various N_2O production pathways across different soil nitrogen cycling processes remains challenging. Source partitioning of N_2O production is further complicated by abiotic reactions such as N_2O production from hydroxylamine (NH_2OH), a nitrification intermediate[13]. The complex interactions that occur between microorganisms and

biotic and abiotic factors may suggest a biotic–abiotic reaction sequence[14] may be important to consider in studies of N_2O production and consumption.

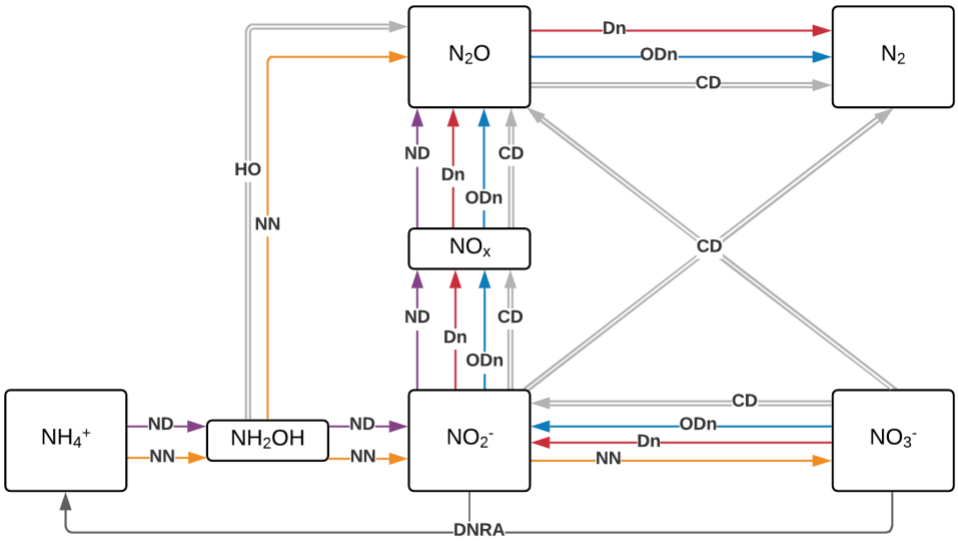


Figure 1.1. Major N_2O production pathways in soil. Nitrification (NN), nitrifier denitrification (ND), denitrification (Dn), aerobic/oxic denitrification (ODn), chemodenitrification (CD), nitrate reduction to ammonium (DNRA), hydroxylamine oxidation (HO).

1.2. Denitrification

1.2.1. Anaerobic denitrification

Heterotrophic denitrification is the reduction of nitrate (NO_3^-) to gaseous products, primarily N_2O and dinitrogen (N_2), but also including nitric oxide (NO) (equations [1]–[4]). The losses of the gaseous denitrification products are highly depended on soil moisture and their solubility. Denitrification represents a loss of soil fertility for plant growth; a means of reducing NO_3^- contamination of ground and surface waters; and a source of gaseous pollutants that contribute to the destruction of atmospheric ozone. Thus, it contributes to greenhouse gases.

Factors such as aerobicity, pH, and carbon or nitrogen availability control the instantaneous denitrification rate and the relative proportions of NO, N_2O, and N_2 production. These drivers act through the microbial community. The denitrifier community composition depends on the interactions between soil properties, soil microorganisms, climate variabilities, and management practices.

$$NO_3^- + 2e^- + 2H^+ \rightarrow NO_2^- + H_2O \quad (1)$$

$$NO_2^- + 2e^- + 4H^+ \rightarrow NO + H_2O + 2OH^- \quad (2)$$

$$2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O \quad (3)$$

$$N_2O + 2e^- + 2H^+ \rightarrow N_2 + H_2O \quad (4)$$

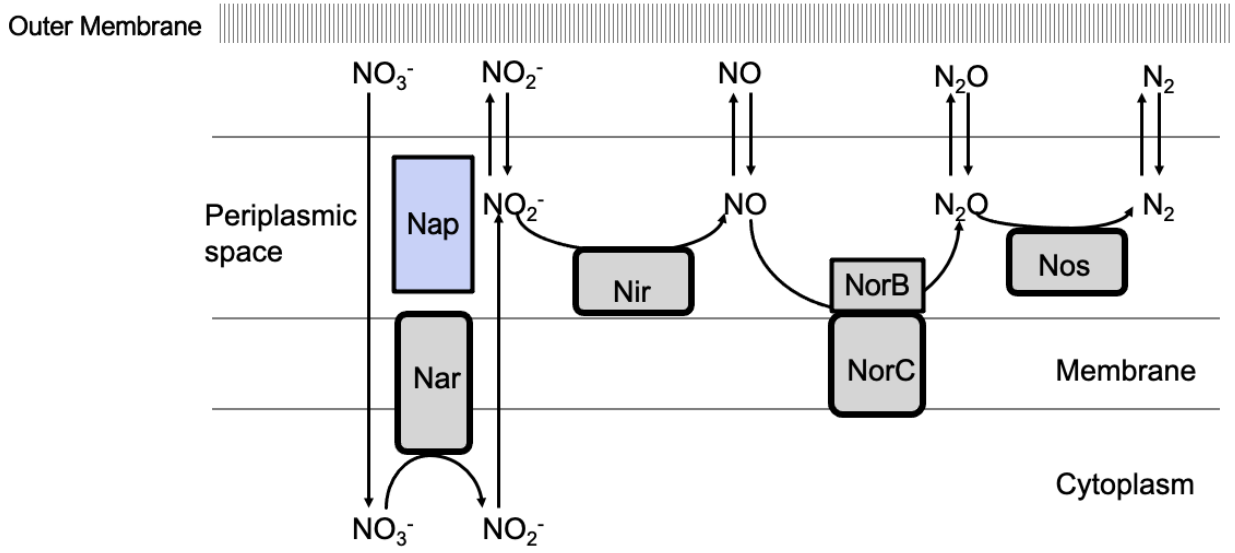
Denitrification is known as a facultative anaerobic respiration process where bacteria and other microorganisms substitute NO_3^- for oxygen as a terminal electron acceptor. It is commonly assumed that when oxygen is present as the superior terminal electron acceptor, NO_3^- respiration does not occur, due to the notion that oxygen represses or inhibits the synthesis of denitrifying enzymes and their activities[15,16,17]. Denitrifiers are ubiquitous in soil[18,19] but account for only 0.5–5% of the total bacterial community[19]. However, denitrifying activity has been shown to be prevalent in soils.[20] The process is not limited to bacteria; known denitrifiers include archaea, fungi, and protozoans from more than 60 genera[21] as well. In general, denitrifiers utilize nitrogen oxides as electron acceptors in the generation of energy. Chemolithotrophic denitrifying bacteria

and archaea are also known to be capable of reducing nitrogen oxides when oxidizing inorganic compounds[22,23]. In addition, denitrifiers, both bacteria[24,25] and fungi[26], are found to lack one or more enzymes to catalyze the entire reduction processes (equations [1]–[4]). In other words, denitrifiers are variable in their ability to completely denitrify NO_3^- to N_2. N_2O net production can be of particular concern in systems dominated by fungi and bacteria lacking N_2O reductase (Nos)[24,27]. A detailed review of fungal denitrification is beyond the scope of this work and has been considered recently elsewhere[28].

1.2.2. Enzymes involved in anaerobic denitrification

Denitrification is carried out by a suite of enzymes including NO_3^- reductase (Nar), nitrite (NO_2^-) reductase (Nir), NO reductase (Nor), and N_2O reductase (Nos) (Figure 1.2.), encoded by the genes narGH or napA, nirK or nirS, norB, and nosZ Clade I, respectively. Recently, Nos Clade II was identified among predominantly non-denitrifying N_2O reducers, but its role in regulating N_2O emissions has been overlooked[29]. The tight coupling of denitrification enzymes is not necessarily universal in soils[30], and the expression of each enzyme may have a different sensitivity to oxygen, for example[31]. Bonin (1991)[32] reported that the Nir and Nos of *Pseudomonas nautica* were more oxygen-sensitive than the Nar (with sensitivities of 0.25%, 0.15%, and 0.02%, respectively). Nos is highly sensitive to oxygen[8] and high NO_3^- concentration[33,34]. Evidence shows that denitrification enzymes can be induced rapidly in response to changes in aerobicity and substrate availability. Holtan-Hartwig (2000)[35] reported that Nar is activated within 2–3 h, Nir between 2 h and 12 h, and Nor between 24 h and 48 h.

Figure 1.2. Bacterial denitrification pathways with location of enzymes relative to the cytoplasmic membrane and periplasmic space. Nap: periplasmic nitrate reductase, Nar: nitrate reductase; Nir, nitrite reductase; Nor: nitric oxide reductase; Nos, nitrous oxide reductase. Adapted from Ji et al. (2015) and Wallenstein (2006).



1.2.3. Factors controlling anaerobic denitrification

Previous studies on environmental controls of denitrification often categorize factors as proximal and distal regulators[20,36]. Proximal regulators of denitrification refer to the factors that immediately affect denitrification rates and relative proportions of NO, N_2O, and N_2 production, such as moisture or oxygen (aerobicity) and carbon or nitrogen availability. Distal regulators refer to factors that influence the composition and diversity of denitrifying communities over spatial and temporal scales beyond those of proximal regulators. Common regulators include plant–soil–water interaction, management practices (e.g., tillage, compost, fertilization, and animal grazing), soil texture, disturbances (wetting or drying, freeze or thaw), and predation (soil or sediment fauna and viruses)[20,36–38]. Temperature and pH can affect the instantaneous denitrification rates, as well as denitrifier communication composition. Next, we discuss

proximal regulators of denitrification in soil and relative N_2O production that led into the incubation experiment presented in the following chapter of this thesis.

$$5C_6H_{12}O_6 + 24NO_3^- \rightarrow 12N_2 + 30CO_2 + 18H_2O + 24OH^- \quad (5)$$

1.2.3.1. Carbon availability

As a heterotrophic process (equation [5]), denitrification depends on the availability of labile carbon substrates. Water-soluble organic carbon is shown to be a good proxy of the denitrification rate of soil[39]. Carbon availability also controls oxygen consumption rates in the soil matrix, by either directly fueling aerobic heterotrophic respiration or indirectly supporting the anaerobic production of reductants such as Fe(II), which can react with oxygen subsequently. Actual carbon regulation on denitrification has not yet been fully understood[8]. The availability of carbon for microorganisms is an important factor in controlling denitrification, especially under field conditions. High potential for denitrification is reported for permanent pasture soils with high concentrations of organic material[40]. The amendment of soil with slurries and organic manures provides for a readily available energy source for denitrifiers under field conditions[39,41,42].

1.2.3.2. Nitrate availability

Availability of NO_3^- is known to be a strong factor controlling the rate and net N_2O production via denitrification. Furthermore, an increase in NO_3^- concentration is reported to inhibit N_2O consumption[43,44] and lead to higher net N_2O production[45–48]. At a larger scale, little

correlation has been found between NO_3^- concentrations and denitrifier abundance[49]. In other words, NO_3^- supply might not be a primarily control of denitrifier community composition in soil systems. This perhaps is because the dynamics of the NO_3^- pool are affected by other factors such as carbon availability, moisture, and pH. Fertilization is shown to promote N_2O emission, in a nonlinear pattern rather than a linear one[50]. Organic fertilizer application might promote N_2O reduction and thus contribute to lower N_2O emissions[51]. In addition, studies support an increase in the effect of NO_3^- on denitrification in unmanaged systems where net mineralization and nitrification are small. Conditions such as wet sites, clay-rich soils, and high precipitation often favor denitrification.

1.2.3.3. Soil pH

Soil pH is known to influence denitrification rates, as well as alter the microbial community over time. It may therefore indirectly regulate the predominate gaseous nitrogen product pathways. Overall, the denitrification rate decreases and the ratio of N_2O to N_2 increases in acidic soils compared with neutral or slightly alkaline soils[52,53], due to the high sensitivity of Nos to pH. In addition, soil pH indirectly affects organic carbon and mineral nitrogen availability to the denitrifying population. At low soil pH (i.e., pH < 6) a decrease of denitrification enzyme activity (DEA) is found[54,55]. Simek (2002)[56] debunked the idea of an optimum pH, finding that the denitrifier community exhibits the ability to adapt to acidic conditions.

1.2.3.4. Soil temperature

The occurrence of denitrification has been reported over a wide range of soil temperatures, ranging from 0°C to 75°C, with DEA especially sensitive to changes in temperature[46]. Q_{10} rates of 1.6–2.8 have been reported[57,58]. The Q_{10} of denitrification indicates the stimulation of denitrification rate following an increase in temperature by 10°C, and is higher than that of soil CO_2 emissions[59–61]. N_2O emitted from soil might have greater GHG impact than CO_2 in terms of global warming. However, substrate and moisture limitation of microbial nitrogen cycling processes under climate variability might offset such an effect. Low temperatures have been found to affect N_2O reduction[62] and consequently lead to higher N_2O:N_2 ratios. During a three-week soil slurry incubation at different temperatures (4°C, 15°C, 20°C, 25°C, and 37°C), Braker (2010)[63] observed not only short-term effects, such as increasing denitrification rates, but also shifts in denitrifier community composition over the long term. This finding contributes to the notion that temperature serves as both a proximal and distal control on denitrification.

1.2.3.5. Water content

Denitrification is generally believed to be an anaerobic process. In soil, its rate is often positively related to water content.[64,65] The relative contribution of N gas production (i.e., NO, N_2O, and N_2) varies with soil water content[66]. Water slows oxygen diffusion by a factor of 10^4 compared to air. It can reduce oxygen supply by blocking a fraction of the pores, thus increasing the effective distance an oxygen molecule has to travel to get to a given microsite[67]. Denitrification is speculated to explain N_2O pulse after rewetting[68]. In another study, the DEA in northern hardwood forests was found to vary with annual precipitation[69]. The highest NO fluxes are expected at 30–60% water-filled pore space (WFPS), when nitrification is most active. The

highest N_2O fluxes occur at 50–80% WFPS, when nitrification and denitrification occur simultaneously[70]. At higher WPFS ratios, denitrification continues to dominate while N_2O consumption increases. As a result, N_2 is expected to be the main gaseous product in saturated soils. Although this model is supported by observations from many studies[71], WFPS should not be used as a sole proxy to distinguish N_2O from nitrification and denitrification. WFPS controls not only oxygen diffusion but also substrate availability. Li (2019) found denitrification to be the main source of N_2O at 40–80% WFPS in arable soils with crop residue addition[72]. The net N_2O production from denitrification remains unclear, due to the enormous difficulties in the quantification of N_2 production[73,74].

Denitrification under presumed aerobic conditions has been reported from pure culture, coastal sediment, and soil studies, although the relative proportion of aerobic process in soils remains unclear (discussed in detail in later sections). Simultaneous oxygen consumption and denitrification form anaerobic sites in soil aggregates[75], referred to as "hot spots" of denitrification. In a soil core segmentation experiment, Parkin (1987)[76] found that 1% of the soil mass derived from plant residue contributed 85% of total denitrification activity of the intact soil core.. A high spatial variability in N_2O production is common in upland soil[76] and estuarine sediments[77]. Conrad (1996)[78] estimated that microsites in upland soil contribute 70% of global N_2O emissions. Despite the importance of soil denitrification and an abundance of knowledge on the topic, denitrification remains the most poorly understood process in the terrestrial nitrogen cycle[79]. Moreover, measurement of N_2 against high background atmospheric concentrations is challenging[80]. Furthermore, spatial and temporal variability in point measurements of denitrification lead to high uncertainty when scaling up the measurements in space and time[81].

1.3. Aerobic denitrification

1.3.1. Evidence in the literature

Denitrification is generally considered as an anaerobic process, despite the fact that aerobic or oxic denitrification has been reported since 1984 [8283]. Existing evidence of aerobic denitrification across disciplines and environmental conditions is summarized here to provide context for the incubation study presented in the following chapter.

Pure culture experiments have revealed the occurrence of aerobic denitrification involving the production of N_2O and N_2[83,84,85]. Lloyd (1987)[84] suggests that "aerobic bacterial denitrification…may, contrary to the beliefs of many, be as widespread and ecologically important as its anaerobic counterpart." Carter et al. (1995)[86] reported bacteria capable of aerobic NO_3^- respiration in greater abundance than those in its anaerobic counterpart. Patureau et al. (2000)[87] isolated 28 bacterial strains, among which 10 exhibited aerobic denitrifying activity in response to alternating aeration conditions. Indeed, aerobic denitrification as a novel biological nitrogen removal technology has motivated researchers to screen and isolate new aerobic denitrifiers and to understand the expression characteristics of key functional genes. This advancement has been reviewed by Ji et al. (2015)[31] and, more recently, with a focus on intracellular electron transfer, by Yang (2020)[88].

Through a combination of advancements in stable isotope techniques and enhanced temporal resolution, Gao et al. (2010)[89] challenged the role of oxygen as a primary control of N_2 production from denitrification in natural systems. This position was supported by Marchant (2017)[90], who reported simultaneous aerobic and anaerobic respiration from denitrifier communities as an important sink for anthropogenic nitrogen inputs. Co-respiration of NO_3^- and oxygen (discussed in more detail in the following paragraphs) is speculated to explain the rapid nitrogen loss, although the mechanisms of aerobic denitrification and whether N_2O is emitted remain unclear.

In soil studies, N_2O and N_2 from aerobic denitrification have been reported from laboratory studies using different methods. Using gas-flow soil core and acetylene (C_2H_2) inhibition, Parkin and Tiedje (1984)[91] reported 0.4–10.29 ng N_2O-N/g/h from a sandy loam. Wang[92] found 1 μg N/h/kg N_2 production when soil cores were under aerobic conditions for 40 h; NO and N_2O production were found to be one magnitude lower. Using the ^{13}N technique, Speir et al. (1995)[93] found 0.1–183 ng/g/h N_2O production from soil cores. Using the ^{15}N labeling technique, Batemen and Baggs (2005)[94] reported that 50% of N_2O was derived from denitrification under 20% WFPS (0.2 ng N_2O-N/g soil/h) in a silt loam soil. Zhu et al. (2013)[5] showed that 45% of N_2O emitted from a clay loam soil was derived from heterotrophic denitrification in an atmosphere of 20% oxygen (0.3 ng N_2O-N/g soil/h). In another study, denitrification under 21% oxygen concentration accounted for 10–40% from Spanish forest soils[95]. Similarly, low N_2 formation has been observed under aerobic conditions[96]. Finally, denitrification dominated N_2O production (0.015 and 0.02 mg/kg/d) in the first two days of a 14-day incubation under 0.35% and 20% oxygen concentrations[97].

1.3.2. Relationship with other denitrification pathways

The mechanism(s) of aerobic denitrification remains unclear. Co-respiration of nitrate and oxygen has been proposed to explain denitrification under aerobic conditions. The periplasmic nitrate reductase (Nap) enzyme (Figure 1.3) isolated from soil and sediment is involved in the first reduction step during aerobic denitrification. It is not inhibited by oxygen, unlike the membrane-bound nitrate reductase (Nar) of anaerobic denitrification[8698]. Roco (2016)[99] found a large diversity of bacteria capable of reducing nitrate to nitrite under oxic conditions in forest, agricultural, and wetland soils. Co-respiration provides a theoretical explanation to alleviate the oxygen bottleneck and facilitate denitrification, particularly in diffusion limited networks of soil and sediment micropores.

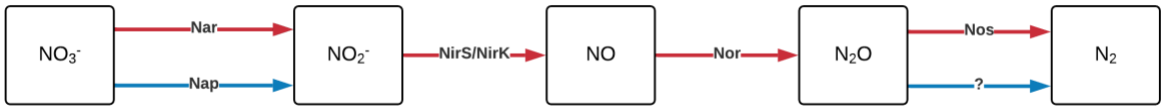


Figure 1.3. Enzymes involved in microbial denitrification. Red arrows indicate anaerobic processes; blue arrows indicate aerobic processes.

Nitrifier denitrification is technically denitrification, and it can occur under aerobic conditions. Unlike denitrification, however, nitrifier denitrification begins with ammonia (NH_3) oxidation followed by denitrification within the same organism. Nitrifier denitrification is dependent on initial oxidation of NH_3, unless an extra source of NO_3^- is produced or introduced. Similar to nitrifier denitrification, coupled nitrification–denitrification involves two steps: NO_3^- production and denitrification. Since these processes include the reduction of NO_3^- or NO_2^- under oxic conditions, both nitrifier denitrification and coupled nitrification–denitrification may be classified as oxic or aerobic denitrification. However, in both processes, denitrification

constitutes only half of the process. The two pathways are more accurately described as "ammonia oxidation–denitrification" processes, since "denitrification" in these cases depends on prior NH_3 oxidation. Unlike these two pathways, aerobic denitrification is strictly "denitrification" independent of ammonium (NH_4^+) as a precursor.

Clarification regarding these terms helps not only better design methods to quantify N_2O production from aerobic denitrification but also fills the gaps in N_2O production pathway terminology. On the one hand, N_2O production processes and relevant microbial contributors under sub-oxic conditions warrant further studies; nitrifier denitrification is considered the main N_2O production pathway in sub-oxic conditions[5], but aerobic denitrification challenges the notion that NH_4^+ oxidization is the only biological pathway for NO_2^- reduction to N_2O under sub-oxic conditions. Conversely, the net production of N_2O from (aerobic) denitrification is determined by both N_2O production and consumption. At present, N_2O consumption or uptake and N_2 production under aerobic conditions remain unclear. Nos is thought to be the most oxygen-sensitive step during denitrification. However, aerobic N_2 production has been reported. Wu (2013)[100] observed N_2O consumption in oxic, dry field soil using static flux chambers; this observation was supported by lab incubation showing significant N_2O consumption at 2% moisture. Qin (2017)[101] showed using soil extracts that aerobic pathways accounted for 29–51% of N_2 produced relative to the anaerobic denitrification. The extent of aerobic denitrification and whether this process functions as a sink or source of N_2O emission largely depends on the final N_2O:N_2 ratio.

1.3.3. Factors that potentially explain and/or control aerobic denitrification

1.3.3.1. Oxygen availability

Anaerobic microsites formed in aerated soil under low WFPS and or artificial oxygen headspaces, or due to a high respiration rate under high carbon availability, are often suggested to explain the seemingly "aerobic" denitrification. Microsites within soil aggregates are widely observed; however, the existence of anaerobic niches does not necessarily rule out the possibility of aerobic denitrification. Co-respiration of oxygen and NO_3^- might offer a physiological advantage in environments subjected to fluctuating oxygen availability[102]. NO_3^- might temporally function as an auxiliary oxidant to dispose of the excess reducing equivalents or achieve redox balance for cell growth when oxygen is temporarily limiting. In other words, transition between oxic and anoxic conditions might be viewed as the best selective criterion in environments where fluctuating oxygen concentrations, such as under conditions of high soil or plant root respiration and/or reduced carbon are available, such as wastewater treatment plants. Few speculations have been made on the sequential reduction steps. One explanation for this might be that the production of NO_2^- from aerobic NO_3^- reduction can induce denitrification under oxic conditions. In this regard, expression of Nir might have the advantage of protecting against toxic spikes of NO_2^-[103]. A similar detoxification hypothesis has been proposed to explain the nitrifier denitrification process[4]. In soil systems, the accumulation of NO_2^- depends on the interaction of several environmental factors, as discussed below.

1.3.3.2. Carbon availability

Most denitrifiers are chemo-organotrophic; thus, their activities are largely controlled by the reducing power of organic carbon compounds[46]. A correlation between carbon availability

and denitrification rate is often found. However, this relationship is confounding, since carbon utilization affects oxygen supply; this relationship can be further complicated in the presence of aerobic denitrification. The consequences of dissolved organic matter (DOM) for denitrification depend on the character of the DOM (i.e., quality), which is strongly influenced by the carbon source[104]. DOM as a carbon source can result in increased N_2O emissions—up to soil depths of 1.2 m[105]. Melero (2011)[106] found that soils with high DOM favor some Nir enzymes, while Barrett[107] reported that DOM favors Nos more than Nir. The effect of carbon sources on denitrifying microbial abundance and activities remains unclear, and even less is known about the extent of denitrification under aerobic conditions. Aerobic denitrification is reported in the presence of carbon sources with a carbon-to-nitrogen ratio of 5-10. Extremely low or high carbon concentration has been found to negatively affect bacteria growth rates, leading to lower denitrification activity[31]. Obligate heterotrophs constitute the bulk of the microbial biomass and compete for carbon in the presence of oxygen, but not in the absence of oxygen. This finding indicates the importance of studying the relationship between carbon and aerobic denitrification.

1.3.3.3. pH

Above, pH as both a proximal and distal control of denitrification has been summarized. Here, the potential control of pH on aerobic denitrification is discussed. The adaptation of microorganisms to environmental change might be viewed as evidence that aerobic denitrification can be common to various ecosystems. Nevertheless, there is scarce evidence regarding the effect of pH on aerobic denitrification[31], especially in soil systems[10]. We hypothesize that the general "rules" under anaerobic conditions will apply to aerobic denitrification. Despite the lack of evidence, the effect of pH on periplasmic nitrate reductase

(Nap) is of particular interest, as reduction of NO_3^- to NO_2^- is the first step in biological denitrification (aerobic and anaerobic); NO_2^- is therefore the "focal point" of most studies of N_2O production in soil.

1.3.3.4. Nitrate availability

The addition of NO_3^- either initiates or increases production of Nar. Roco (2016)[99] reported aerobic NO_3^- reduction in soils provided with both NO_3^- and a carbon source, with the highest rate in wetland sites. The reduction of NO_3^- was carried out mainly by gram-negative proteobacteria in this study, as in Ji et al. (2015)[31]. It was observed at 45–86% atmospheric oxygen concentration. The paucity of knowledge about bacteria capable of performing anaerobic NO_3^- reduction hinders the process of aerobic denitrification as well. The activity of NO_3^- reduction under oxic conditions, which may be related to its abundance, influences NO_2^- production, and therefore likely has an important role in N_2O production.

1.4. Ammonium oxidation and nitrifier denitrification

NH_4^+ oxidation and NO_3^- reduction processes complete the production of N_2O under aerobic and anaerobic conditions. It has been suggested that nitrification of NH_4^+ and denitrification of NO_3^- account for about 70% of the annual N_2O budget globally[108]. Autotrophic and heterotrophic nitrifiers oxidize reduced nitrogen forms (NH_3 or organic nitrogen) to NO_2^- under aerobic conditions (i.e., obligatorily requiring molecular oxygen).

1.4.1. Ammonia oxidation (nitrification)

In the first step of nitrification, NH_3 is oxidized to NH_2OH by ammonia monooxygenase[3,109]. N_2O is formed during the NH_3 oxidation process via chemical decomposition of NH_2OH, with NO as a precursor[110,111] ($NH_3^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow N_2O$, N_2O as byproduct). This process is often viewed as a special form of chemodenitrification[112] (discussed separately below). Nitrifying organisms are often slow in growth and require high amounts of NH_3[3]. For a long time, the contribution of NH_2OH to N_2O production was neglected, compared with the N_2O production from "classic" denitrification and nitrifier denitrification.

Through an improved method of measuring NH_2OH[113], Heli[13] showed that some soils have the potential to oxidize NH_2OH to N_2O in a purely abiotic reaction. The process was found to be dependent on soil properties (i.e., soil pH, carbon-to-nitrogen ratio and manganese content). Based on Heli's study, even a small fraction of NH_2OH produced in soil during nitrification could be transformed into N_2O via fast chemical oxidation. Thus, in conditions such as fertilized agricultural systems where a high nitrification rate is common, environmental consequences can be substantial. A recent study highlighted the greater role of NH_2OH in N_2O formation than in NO_2^- formation (i.e., nitrifier denitrification). Under conditions with low carbon and high manganese content, NH_2OH contributes more to N_2O formation than to NO_2^- production via biotic processes[114].

Although "classic" nitrification terminology includes both autotrophic and heterotrophic nitrifiers, some authors[115] consider heterotrophic nitrification as a separate N_2O production pathway. In acidic soils, heterotrophic nitrifiers have been shown to contribute substantially to

NO_3^- production[116–118], where the activity of autotrophic nitrifiers may be inhibited by the low pH. Heterotrophic nitrifiers are found to utilize both inorganic and organic substrates[119], as well as utilizing unidentified organic nitrogen compounds, not NH_4^+, as substrates[120–122]. Between 0% and 25% of N_2O production has been reported to arise from heterotrophic nitrification[95,123] in forest, pasture, and arable systems.

1.4.2. Nitrite oxidation (nitrifier denitrification)

In the second step of nitrification, NH_2OH is oxidized to NO_2^-. The reaction is catalyzed by the enzyme hydroxylamine oxidoreductase (HAO). NO_2^- is further oxidized by NO_2^--oxidizing bacteria to NO_3^-. During nitrification, NH_3 and NO_2^- serve as energy sources to fix CO_2. Most nitrifying bacteria are chemolitho-autotrophs and taxonomically belong to *Nitrobacteraceae*[124]. While N_2O is formed during NH_3 oxidation as a byproduct of a chemical process, NO_2^- is stepwise reduced to NO and N_2O within the same NH_3-oxidizing bacteria. Organisms processing enzymes responsible for this process (i.e., Nir and Nos) are not phylogenetically different from those that carry out denitrification, possibly because of lateral gene transfer[11,125,126]. Despite the similarity, denitrifiers are heterotrophic, while NH_3-oxidizing bacteria are chemoautotrophic.

The lack of a proper method to quantify N_2O emitted through nitrifier denitrification hinders evaluating the proportion of N_2O production via nitrifier denitrification in soils. The ^{15}N tracer method cannot distinguish N_2O from nitrification and nitrifier denitrification from the same NH_4^+ pool[70]. Inhibition methods were attempted in certain steps of (de)nitrification in one study but were found to be unreliable due to effusiveness and selectivity[11]. In another study, the

intramolecular distribution of ^{15}N within the N_2O molecule (site preference [SP]) was considered[127,128]. This endeavor showed that SP can potentially serve as a tool to distinguish nitrifier denitrification and denitrification from classical nitrification and fungal denitrification. Decock and Six[129] concluded that analysis of SP will likely remain only a qualitative tool and should be coupled with other approaches (e.g., tracer and/or molecular tools). Although this observation remains relevant, improvements in modeling and analytical capability have been shaping the natural-abundance approach into a promising tool[130,131]. Nevertheless, SP was not sufficient to unequivocally distinguish between the three pathways—nitrifier denitrification, denitrification, and nitrification—in the early 2010s. Kool (2007)[132] took a different approach and quantified oxygen exchange between H_2O and intermediates of the (de)nitrification pathways. On the basis of this breakthrough, a dual method using ^{18}O and ^{15}N was developed, which revealed that nitrifier denitrification exceeded nitrification as a dominant source of NH_4^+-derived N_2O emission in soils with low and intermediate moisture content[4,132]. Other studies coupled this novel labeling method with an artificial oxygen gradient in the incubation headspace and confirmed that nitrifier denitrification contributes significantly in agriculture soils with different types of NH_4^+ fertilizers under 3% and 0.15% oxygen concentrations[5]. Although this dual-isotope method includes a relatively large number of assumptions[11], it remains the only method to partition N_2O via nitrifier denitrification.

Using the methods described above, studies found that nitrifier denitrification and denitrification together accounted for 61–92% of N_2O production[133] and 50–100%[134] from agriculture systems. These results are consistent with laboratory incubations that found 10–40% of N_2O to be derived from nitrifier denitrification and/or coupled nitrification–denitrification[135],

with 54–100% from denitrification and nitrifier denitrification[136]. Variations in the relative contributions of nitrification and (nitrifier) denitrification to N_2O emission were found using an Andisol fertilized with NH_4^+ and poultry manure[137].

Nitrifier denitrification contributes considerably to N_2O emission from soils under conditions of low oxygen, low carbon, and low pH[138–142]. The ability of aerobicity to directly control N_2O production through nitrifier denitrification is under debate. Wrage (2018)[12] has suggested that changes in aerobicity are directly influenced not by oxygen but rather by accumulation of NO_2^- under suboxic conditions. Nitrifier denitrification is favored under high NO_2^- concentrations, while N_2O production via NH_4^+ oxidation (NH_2OH) might be triggered by high NH_3 and low NO_2^- in combination with high nitrogen oxidation rates[143]. It is not clear why NH_3-oxidizing bacteria perform nitrifier denitrification. Speculations around this phenomenon include the following: (a) the nitrifier denitrification by the bacteria could be a response to the toxicity caused by the accumulated NO_2^- under marginally aerobic conditions[144]; and (b) the oxidation of NH_4^+ might provide the electron source for NO_2^- reduction[4]. It remains unclear whether N_2 can be formed by nitrifier-denitrification[145].

1.5. Coupled nitrification–denitrification and fertilized denitrification

The term "coupled nitrification–denitrification" is used to describe the combination of two distinct processes, nitrification and denitrification, in a concerted action. The NO_2^- or NO_3^- produced by nitrifiers in aerobic microhabitats is rapidly reduced by nearby denitrifiers. The N_2O formation is carried out by "classical" nitrification and denitrification as defined earlier. In

environments where anaerobic and aerobic conditions coexist in microhabitats, coupled nitrification–denitrification is of high relevance (e.g., in hydromorphic soils[3]).

The term fertilizer denitrification was introduced to describe the denitrification occurring when NO_3^- was applied to soil or leaked into hydrological pathways—in other words, when NO_3^- was not of immediate microbial origin[142]. Fertilizer denitrification may surpass nitrifier denitrification as the predominate N_2O production pathway when soil moisture is high. Although these two N_2O production pathways are fundamentally a special form and/or combination of nitrification and denitrification, distinguishing these terminologies has applications to aid method choice to investigate denitrification. For example, the ^{15}N gas flux method is used to determine denitrification gas products. The ^{15}N gas flux method traces ^{15}N-enriched NO_3^- to determine denitrification gas products is usually based on the application and tracing of ^{15}N-enriched NO_3^-, and thus is an approach to directly measure fertilizer denitrification only. In contrast, the direct measurement of N_2 and N_2O in an (almost) N_2-free helium/oxygen environment provides data on N gas formation from all contributing processes.

1.6. Co-denitrification

Co-denitrification is performed by fungi and bacteria and involves the formation of N_2 or N_2O through an N-nitrosation reaction by one nitrogen atom of NO originating from denitrification and another nitrogen atom from a co-substrate, for example, ammonia hydroxylamine or a range of monomeric organic nitrogen compounds[146,147]. The process is termed as co-denitrification because it is simultaneous with denitrification and is at least partially

conducted by similar or the same microorganisms. During this reaction, co-denitrification was found to be a significant source of N_2 in a grassland study[27]. Microbial formation of N_2O was reported with NH_2OH, N_3^-, NH_4^+, hydrazine ($H_2N\text{-}NH_2$), and salicylhydroxamic acid ($C_7H_7NO_3$) as precursors (Spott et al.,[148] and the reference therein). Since co-denitrification is dependent on the microbial reduction of nitrogen oxides in denitrification, it might be relatively safe to assume that the process is restricted to anaerobic conditions. The actual relevance of co-denitrification in terrestrial environments as a source of N_2O remains unclear[147]. Co-denitrification might be an important process in environments where a closed nitrogen cycle is established by fungi and bacteria communities, such as permanent grasslands and undisturbed forest soils. Recently, Spott (2011)[148] proposed the term "BioNitrosation" to represent a wide range of possible biotic nitrosation reactions that can lead to either nitrogen gas release or nitrogen immobilization, independently from the main driving processes (e.g., nitrification or denitrification) and the current environmental conditions (e.g., aerobic or anaerobic). In terms of soils, it is assumed that N_2O formation via BioNitrosation is not significant compared with N_2 formation. However, the amount of research that has been conducted on this intriguing microbial process is not sufficient.

1.7. Dissimilatory nitrate reduction to ammonia, and anammox

DNRA is the dissimilatory reduction of NO_3^- to NO_2^- and NH_4^+. Similar to microbial immobilization and remineralization, this pathway can lead to nitrogen retention and nutrient conservation. DNRA is strictly an anaerobic process carried out by facultative and obligate anaerobic microorganisms[8]. Recent studies suggest that it can be a significant pathway in upland soils and is not being limited to wetland ecosystems[118,149]. Although DNRA is generally viewed

as a process that conserves nitrogen in the ecosystem, N_2O production in the process has been reported[149,150]. The use of $^{13}NO_3^-$ labelling proved the simultaneous production of NH_4^+ and N_2O via DNRA, whereby NH_4^+ accounted for 90% of the total production[151]. This finding was supported by soil studies confirming that N_2O accounted for 5–10% of the added NO_3^-[152]. Nonetheless, the N_2O production rate is lower, usually in the range of 1% of the NO_2^- or NO_3^- reduction[150]. It is worth noting that as both DNRA and denitrification use the same substrates (NO_3^- and NO_2^-), the contribution of these two processes to total N_2O production cannot be investigated based on $^{15}NO_3^-$ labelling alone. The accumulation of NO_2^- might explain the N_2O production by DNRA, especially under high pH[153,154]. The quantity of N_2O produced during DNRA varies with the carbon-to-nitrogen ratio and is not correlated with the production of NH_4^+[155].

Anammox refers to the microbial processes of anaerobic NH_3 oxidation, that is, the conversion of NO_2^- and NH_4^+ directly into dinitrogen gas. Anammox is estimated to account for 50% of the nitrogen removed from the global oceans[156]. Anammox bacteria have been reported from a wide range of terrestrial ecosystems, including temperate forest soil, peat soil, upland arable soils, paddy soil, and permafrost soils (Zhao (2021) [157] and the reference therein). Environmental conditions that favor anammox include low dissolved oxygen, low NO_2^-, moderate nitrogen loading rate (in the form of NH_4^+), and neutral to slightly alkaline pH[158]. Wang (2012)[159] reported riparian zones as hot spots for anammox abundance and biodiversity. A relationship between depth and anammox has been observed, with higher rates in deeper layers[156,160]. It is interesting that the highest rate of anammox occurs at oxic–anoxic interfaces in aquatic systems[161], despite the fact that anammox is considered a strictly anaerobic process.

Although N_2O formation has not been observed in these processes[162], anammox's role as a microbial nitrogen removal pathway in soil should not be overlooked. For example, in ecosystems where environmental conditions favor both anammox and denitrification, the extent of anammox may be expected to go hand in hand with the determination of N_2O production and consumption.

1.8. Chemodenitrification and abiotic decomposition of ammonium nitrate

$$4Fe^{2+} + 2NO_2^- + 5H_2O \rightarrow 4FeOOH + N_2O + 6H^+ \quad (5)$$

Chemodenitrification is viewed as the sum of nonenzymatic (i.e., abiotic) pathways involving the formation of NO, N_2O, and N_2 in soil. The process includes the chemical decomposition of NH_2OH and NO_2^- during nitrification. Chemodenitrification typically occurs in acidic soils with high NO_2^- concentrations. This is because nitrous acid (HNO_2), the protonated form of NO_2^-, has a pKa of 3.3 at 25°C. Additionally, HNO_2 can react with amino compounds and NH_4^+ and soil organic matter (SOM). Under anoxic conditions, humic acids and fulvic acids, lignins, and phenols were found to reduce NO and HNO_2 to N_2 and N_2O[163,164]. N_2O formation via NO_2^- reduction by Fe (II) (equation [5]) is stimulated by decreasing oxygen concentration[164] and increasing Fe(II)[165]. N_2O formation from chemodenitrification is most likely to occur in anoxic habitats with a high organic carbon supply, such as wetland and waterlogged soils. Because high NO_2^- concentration and low pH are not common in natural environments, chemodenitrification and N_2O production via this pathway were not considered important at a global scale in early studies[145]. However, increasing evidence has demonstrated that N_2O

emission from chemodenitrification can be substantial in various ecosystems, including extremely arid areas and cold lakes, ponds, and soils[166–168], temperate forests and arable lands[169,170], and sediments[171]. Wang (2019)[172] found that chemodenitrification accounted for 6.8–67.6% of the total N_2O emissions in rice paddy soils. Wei (2017)[170] reported 43.2–483.8 μmol N_2O/mol NO_2^-/kg soil/d from soils with lignin additions. Otte (2019)[173] reported 15–25% of total N_2O formation in coastal marine sediments to be via chemodenitrification. This new evidence indicates that the contribution of chemodenitrification to global N_2O emissions could have been substantially underestimated.

Other abiotic N_2O formation pathways include (1) the decomposition of soil nitrite through interaction with organic materials in the soil with NO as the main product[174] and (2) the decomposition of ammonium nitrate in the presence of light[175]. These reactions could easily be overlooked because of the simultaneous activity of biological and abiotic N_2O source processes in close proximity in the matrix of soil. Up to now, N_2O production is generally accepted to be a microbial process. Including biotic-abiotic reaction sequences will improve our understanding of N_2O production and development of better predictions[176].

Chapter 2

Short-term emission of nitrous oxide from oxic denitrification in soil

2.1. Introduction

Denitrification is the reduction of nitrate to gaseous products, primarily nitrous oxide (N_2O) and dinitrogen (N_2), but also including nitric oxide (NO). Denitrification represents a loss of available N for plant growth, a means of reducing nitrate contamination of ground and surface waters, and a source of greenhouse gaseous. This process occurs via anaerobic respiration when bacteria and other microorganisms substitute nitrate for oxygen as a terminal electron acceptor. It is commonly assumed that when oxygen is present, a superior terminal electron acceptor, is readily available that nitrate respiration will not occur, and that oxygen represses or inhibits the synthesis of denitrifying enzymes and their activities[15–17].

The production N_2O is relevant since it is an important greenhouse gas with a global warming potential 298 times than carbon dioxide for a 100-years' time frame. Besides global warming, N_2O is also one of the important ozone-depleting agents in the stratosphere. Soil emits 6.6 Tg N yr^{-1} annually as N_2O, accounting for about 60% of natural N_2O emission and about 37% of total N_2O emission[1]. N_2O is produced by a wide range of microbiological processes, with autotrophic nitrification and anoxic denitrification as the main pathways in soils[2]. Other microbial processes include nitrifier denitrification[3–5], co-denitrification, dissimilatory nitrate reduction to ammonia (DNRA) and heterotrophic nitrification. Nitrous oxide production

pathways have been reviewed extensively, first by Wrage et al. (2001), then by Baggs (2011), Braker and Conrad (2011), and Butterbach-Bahl et al. (2013)[3,9,10,147]. However, the quantification N_2O production pathways among the variety of soil N cycling processes are difficult to separate and assign significance. Source partitioning of N_2O production is further complicated by abiotic reactions such as N_2O production from hydroxylamine, a nitrification intermediate[13].

Aerobic or oxic denitrification (ODn) has been reported since 1984[177]. The great majority of the studies that later investigated this process have used pure cultures of denitrifying organisms[84,177]. The organisms in question are isolated and their activity measured under optimal growth conditions with minimal confounding factors. Recently, ODn as a novel biological nitrogen removal technology has motivated researchers to screen and isolate new aerobic denitrifiers and to understand the expression characteristics of key functional genes. This advancement has been reviewed by Ji et al. (2015)[31] and more recently, with a focus on intracellular electron transfer, by Yang (2020)[88]. In contrast to pure culture studies which are designed to specifically favor and observe ODn, denitrification in general is considered essentially an anaerobic process in terrestrial and marine ecosystems, as substantial rates of ODn have not been postulated or observed. Recently, through a combination of advancement in stable isotope techniques and enhanced temporal resolution, Gao et al. (2010)[89], challenged the role of oxygen as a primary control of N_2 production from denitrification, a position supported by Marchant (2017)[90] who reported ODn as an important sink for anthropogenic N inputs. However, the mechanism of ODn remains unclear[89]. The ability of denitrifiers to emit N_2O from soil under aerobic conditions has not been well studied; evidence for this process has been reported in only a few studies[5,91,93,94,178,179].

The intent of our work is to evaluate the potential significance of oxic denitrification in soil. We use a $^{15}NO_3^-$ tracer technique to estimate the existence and extent of the aerobic denitrification process in whole, intact soils, with all their inherent complexity and naturally occurring factors that may influence this process. ODn was measured under natural conditions, that is, at typical concentrations of nitrate and carbon; therefore we did not alter any of these pre-existing conditions apart from a small addition of nitrate. We focused specifically on emission of N_2O.

2.2. Methods

2.2.1. Soil selection and characterization

To represent a variety of managed and unmanaged ecosystems found in California, nineteen soils were collected from the top 5 cm and were air-dried following collection, sieved to 2 mm, and stored for 1-2 months before use. Air dried is the natural state of these soils during long time periods in this Mediterranean climate. Soil pH was measured in deionized water (DI) (1:1 w:v). Ammonium and nitrate was extracted by 1M KCl and determined colorimetrically[180]. Dissolved organic carbon (DOC) was determined in water extracts by high-temperature combustion (Multi N/C 3100, Analytik-Jena, United States). Pyrophosphate-extractable iron (FeP) was determined following the method in Zhu et al. (2013)[181] and determined colorimetrically. Soil properties are reported in Supplemental Table S1.

2.2.2. Experimental set-up

2.2.2.1. Preparation of oxic slurry

All experiments were performed using soil slurries to produce a homogenous environment amenable to continuous aeration and avoid persistent anaerobic microsites that can form in soil samples not actively aerated. In addition to ensuring continuous aeration, this also ensures maximum substrate (i.e. nitrate) availability, especially important during short-term measurements. Slurries were established in quart-size Mason jars with gas-tight lids fitted with a butyl rubber septum for gas sampling. Soil (50 gram) was weighed into each jar and water was added to form a slurry. The amount of water added was empirically determined as the minimum amount necessary so that the slurry was well mixed during stirring; moreover, avoiding excess water would facilitate aeration and minimize dissolved N_2O. Stirring was maintained throughout the experiment to favor oxygen diffusion into the slurry. Dissolved oxygen was monitored during the sampling periods with an optical sensor (Fibox 4, PreSens, Germany) affixed to the wall of each jar just above the bottom (the location most likely to suffer oxygen depletion).

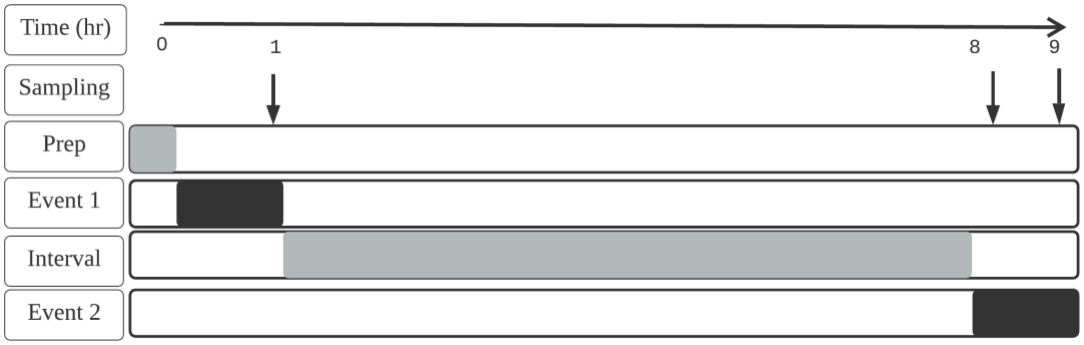
2.2.2.2. Measurement of N_2O derived from nitrate

To determine if ODn activity leading to N_2O emission is present in soil, ^{15}N-labeled nitrate was added to soils immediately before beginning an experiment. The amount added was 10% of the existing nitrate pool; this amount was chosen to avoid a large increase in nitrate concentration while still providing sufficient ^{15}N enrichment to be able to detect any N_2O derived from nitrate. Each experiment consisted of two measurement periods, denoted Event 1 and Event 2. The experimental scheme was as follows: ^{15}N-labeled nitrate plus the pre-determined amount of water were added to 50 grams dry soil in a mason jar. Stirring was begun, and the jars were capped. After 1 hour, the headspace was sampled by inserting a syringe through the septum,

mixing several times with the plunger, and transferring to evacuated gas sampling vials; this is "Event 1". The jar was then uncapped and left, with continuous stirring, for 6-8 hours; during this time any water lost through evaporation was replaced. The jars were then capped a second time, and a gas sample was taken to mark the beginning of Event 2. One hour later, at the end of Event 2, a second sample was taken. Dissolved oxygen concentration remained above 6.8 mg/L during the entire experiment in all soils, confirming that oxic conditions were maintained for the duration of measurements. To account for any changes in nitrate concentration during the 8-hour interval, nitrate concentration at the beginning of Event 2 was measured by extracting the contents (entire jar) of a parallel sample that had undergone the same sampling scheme (Figure 2.1.).

The concentration and ^{15}N enrichment of N_2O in the gas samples were determined by GC-IRMS at the University of California-Davis Stable Isotope Facility. The limit of quantitation of N_2O is approximately 150 picomoles. The long-term standard deviation of ^{15}N is 0.1‰. The reference. N_2O is converted to N_2 via thermal decomposition in a heated gold tube (800°C). The resulting N_2 is calibrated against an Oztech N_2 standard (Oztech Trading Co., $\delta^{15}N$ vs air = -0.61 ‰).

Figure 2.1. Headspace sampling scheme. The headspace was sampled by inserting a syringe at the end of Event 1 and at the beginning and end of Event 2 (black arrows).



2.2.2.3. Initial screening and further analysis of soils

A preliminary evaluation was made by conducting the above experiment on all 19 soils collected (Table S2). The atom%^{15}N of N_2O was compared to the background value for N_2O in ambient air. Any soil for which the atom%^{15}N of N_2O in either Event 1 or Event 2 was more than 0.003 higher than background was considered to have ODn activity leading to N_2O emission, and was set aside for more rigorous, replicated evaluation. Five soils were met these criteria; their characteristics are given in Table 2.1. The experiment was repeated twice for these five soils, for a total of three replicates (for each of event 1 and event 2).

2.2.2.4. Calculations

N_2O produced during ODn was calculated and expressed as N_2O-N derived from nitrate (that is, recovery of nitrate-N as N_2O-N). To determine if there was significant conversion of nitrate to N_2O, a one-sample t-test was used to compare the atom%^{15}N of N_2O from the three replicates of a given sample with the atom% of N_2O in ambient air. Any sample (Event 1 and/or Event 2) for which this difference was significant at $P < 0.1$ was considered to have significant conversion of nitrate to N_2O, and the amount of this conversion was calculated as follows.

Event 1

The amount (moles) of N_2O-N in the headspace at the end of Event 1 was calculated from the measured concentration of N_2O (ppm), using the ideal gas law. This was multiplied by the atom%^{15}N of N_2O to give the moles ^{15}N as N_2O. The same was done for the beginning of the Event, using the ppm N_2O and atom%^{15}N of ambient air. The difference between the beginning and the end of Event 1 is the amount (moles) of ^{15}N as N_2O accumulated during this time.

The initial enrichment of soil nitrate was calculated using the concentration of nitrate present in soil (measured just prior to the start of the experiment) and the amount of the addition of ^{15}N-labeled nitrate (added as 99 atom% KNO_3). The amount of N_2O derived from nitrate was then calculated:

Moles N_2O derived from nitrate = moles ^{15}N as N_2O accumulated / (atom%^{15}N excess of soil nitrate/100)

where atom%^{15}N excess of soil nitrate is atom% - 0.367.

The conversion of nitrate-N into N_2O-N (or recovery of nitrate-N as N_2O -N) is then calculated:

Conversion of nitrate-N into N_2O-N = moles N_2O-N derived from nitrate / moles nitrate-N initially present.

This is then divided by the length of Event 1 (one hour) to give a rate of N_2O production from ODn.

Event 2

The calculation is analogous to that of Event 1, except that measured values of gas samples (ppm N_2O and atom%^{15}N) taken at the beginning as well as the end of the Event were

used. The amount of nitrate at the start of Event 2 was determined (see above, Section 2.2.2) and used to calculate the atom%^{15}N of nitrate at this time.

The portion of total N_2O present in solution was calculated using Henry's Law, given that the slurries were continuously mixed. The dissolved N_2O in slurry is combined with emitted N_2O-N in headspace, as calculated above, to represent total N_2O production during each event. Due to the large ratio of headspace to liquid, N_2O (aq) was very low (less than 3% of total N_2O). Step-to-step equations for the ODn calculation can be found in supplemental material.

2.2.2.5. Predictive power of soil properties

Partial least squares (PLS) multivariate analysis was performed to identify the soil properties that most strongly explained ODn (i.e., conversion of NO_3^- to N_2O), following the method in Zhu et al. (2013)[181]. Prior to data analysis, predicting variables (soil properties) and the observed response (ODn) were standardized by centering and scaling the data to have a mean of zero and a standard deviation of one to ensure that all variables have the same weight. PLS is particularly suitable when the number of predicting variables is greater than the number of observed variables, and when multicollinearity is expected among predicting variables. PLS ranks the predicting variables by importance based on linear regression models that project the predicting variables and the observed variables to a new, multivariate space. The variable importance in the projection (VIP) value is calculated to indicate the relative strength of each predicting variable in explaining the observed variable. Following the exploratory PLS analysis, linear regressions (Table 2.4) between ODn and soil properties were calculated using unweighted, untransformed data. The correlations among variables do not change the

interpretation given by PLS. Nonetheless, a correlation matrix is presented (Table 2.3) to assess the relationship between the soil properties used in this study. All statistical analyses were performed using R Studio (version 1.4.1717). PLS analysis is performed using R packages *pls* and *plsVarSel*.

Table 2.1. Basic properties of selected soils (see supplement for all 19 soils and location of sampling)

Soil	pH	DOC (µg/g)	$N\text{-}NO_3^-$ (µg/g)	$N\text{-}NH_4^+$ (µg /g)	FeP (µg/g)
Forest	6.1	15.6	24.3	7.4	577
Grassland	7.3	13.1	14.8	5.0	73
Vineyard	6.3	9.7	43.5	2.4	684
Ag 1	6.2	7.2	17.3	3.7	2150
Ag 2	5.7	22.4	23.2	26.1	5600

Table 2.2a. Conversion of NO_3^- to N_2O under oxic conditions (mean +/- standard error); n.s. = not significant (p> 0.1). For explanation of events see Section 2.2.2.2.

Soil	Event 1	Event 2
	µmol N_2O produced per mol NO_3-N per hour	
Forest	1.1 (± 0.3)	3.7 (± 2.7)
Grassland	n.s.	2.8 (± 1.7)
Vineyard	2.8 (± 0.6)	0.4 (± 0.1)
Ag1	4.6 (± 0.4)	2.3 (± 1.0)
Ag2	48.9 (± 11)	43.0 (± 12)

Table 2.2b. Conversion of NO_3^- to N_2O under oxic conditions, expressed as mass N_2O-N on a soil basis; n.s. = not significant (p> 0.1).

Soil	Event 1	Event 2
	ng N_2O-N per gram soil per hour	
Forest	0.03	0.09
Grassland	n.s.	0.41
Vineyard	0.12	0.02
Ag 1	0.08	0.04
Ag 2	1.14	1.00

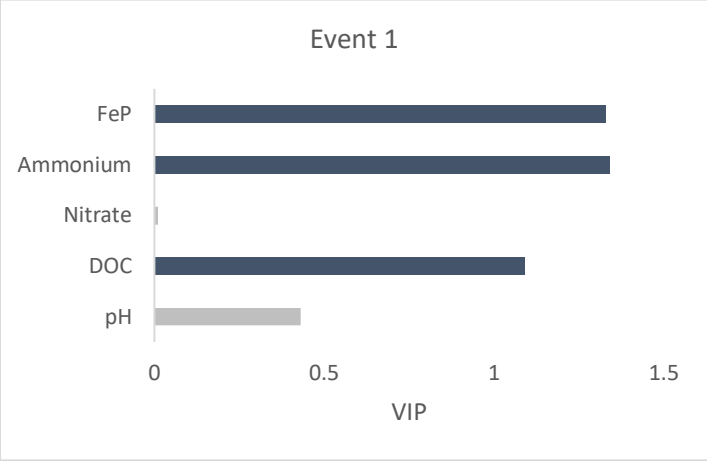
Table 2.3. Correlation matrix of the soil properties evaluated in this study. Bolded values are significance (p< 0.1).

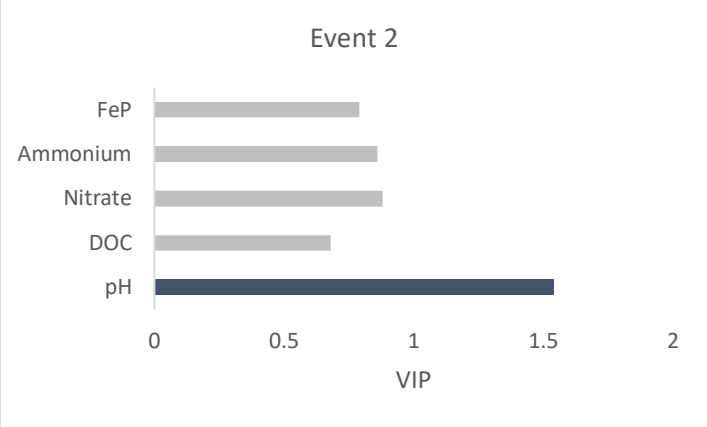
	pH	DOC	Nitrate	Ammonium	FeP
pH	1.00	-0.13	0.08	-0.30	-0.30
DOC	-0.13	1.00	0.12	0.86	0.72
Nitrate	0.08	0.12	1.00	0.03	0.08
Ammonium	-0.30	**0.86**	0.03	1.00	0.93
FeP	-0.30	**0.72**	0.08	0.93	1.00

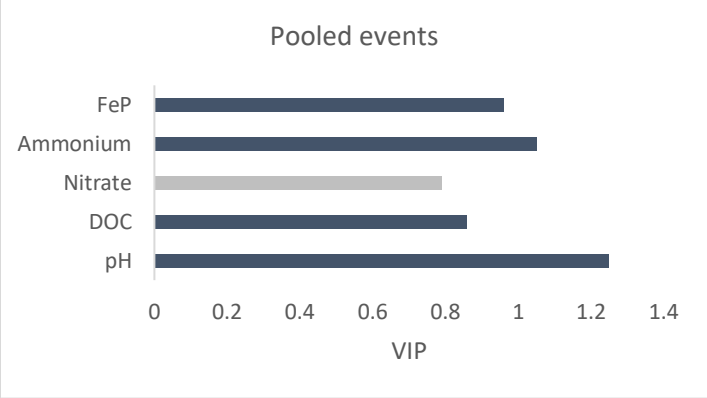
Table 2.4. Results of simple linear regression of ODn (conversion of NO_3^- to N_2O under oxic conditions) against soil properties, for all 19 soils except missing values. The first value given is that of the slope of the regression, the second value given is that of r^2, and the third is that of significance at the 0.05, 0.01, and 0.001 probability level (*, **, ***), respectively.

Event 1			
FeP	0.008	0.92	***
Ammonium	1.9	0.93	***
Nitrate	-0.008	0	n.s.
DOC	1.9	0.62	**
pH	-11	0.09	n.s.
Event 2			
FeP	0.005	0.09	n.s.
Ammonium	1.5	0.15	n.s.
Nitrate	-0.7	0.17	n.s.
DOC	1.3	0.09	n.s.
pH	-35	0.59	***
Event1&2			
FeP	0.006	0.23	*
Ammonium	1.7	0.29	*
Nitrate	-0.49	0.1	n.s.
DOC	1.5	0.18	*
pH	-28	0.38	***

Figure 2.2. Relative importance of soil properties in explaining oxic denitrification. The size of each bar in each figure is given by the variable importance in the projection (VIP) value and indicates the relative strength of each variable in explaining ODn in that analysis. DOC= dissolved organic carbon; FeP = pyrophosphate-extractable iron. Significant results from linear regression between ODn and each soil properties at $p<0.05$ are highlighted (dark color).







2.3.**Results and Discussion**

2.3.1. General N_2O production from ODn in the present study

Five of the 19 soils tested showed the ability to convert $^{15}NO_3^-$ to N_2O under oxic conditions (Table 2.2a). Except for the grassland soil, there was significant conversion both following rewetting (Event 1) and after 8 hours (Event 2). On a soil basis, the rate of N_2O produced during ODn observed in our soils (ranging from approximately 0.02-1.14 ng N_2O -N / g soil /hr; Table 2b) are similar to the range reported previously[91,93]. Our values for the amount of nitrate converted to N_2O (Table 2.2a) are 1-2 orders of magnitude lower than what has been reported in other studies conducted in soil[93,182]. While previous studies speculated on anaerobic microsites to explain N_2O production via denitrification under presumably aerobic conditions[94], we used a soil slurry with consistent stirring in our study to avoid this possibility. Locally higher concentrations of organic carbon and nitrate may also favor greater conversion to N_2O by either process independent of aerobicity. The use of soil slurry maintains homogenous conditions to minimize the effects of substrate availability.

2.3.2. Previous observations of N_2O production from ODn

N_2O production from nitrate in soil under aerobic conditions was observed in early investigations of soil denitrification. For example, Parkin and Tiedje (1984)[91] reported 0.4-10.29 ng N_2O -N/g/hr from a sandy loam. Using a ^{13}N technique, Speir et al. (1995)[93] found 0.1-183 ng N_2O -N/g /g/ hr. These observations of N_2O production from ODn are 1-3 orders of magnitude lower than the rate of N_2O generation under anaerobic conditions, which might explain the subsequent lack of research interest to follow up on this topic. Nonetheless, since then more evidence has appeared in support of N_2O production from ODn. In an effort to determine the

contribution of nitrification and denitrification to N_2O emission at different values of water-filled pore space (WFPS), Batemen and Baggs (2005)[94] found that 50% of N_2O was derived from denitrification under 20% WFPS (0.2 ng N_2O -N /g soil / hr) in a silt loam soil. Zhu et al. (2013)[5] found that 45% of N_2O emitted from a clay loam soil was derived from heterotrophic denitrification in an atmosphere of 20% oxygen (0.3 ng N_2O -N /g soil / hr). Based on N_2O site preference (SP) methods, Congreves et al. (2019)[178] found denitrification contributed 40% of the N_2O production from soils under dry conditions (20-40% WFPS), consistent with Thilakarathna and Hernandez-Ramirez (2021)[179], who reported that 60% of N_2O came from denitrification pathways at water content less than 31% WFPS. In instances such as these, ODn may indeed be occurring or it is also possible that denitrification could proceed in anaerobic microsites[86]. Site preference (SP) is becoming a powerful tool for N_2O source partitioning, however, at this moment, it cannot distinguish between denitrification and nitrifier-denitrification[183]. Despite the scarcity of studies of ODn, there are indications that this process may in fact be more significant and widespread than previously thought. For example, periplasmic nitrate reductase is involved in initial reduction of nitrate, allowing for the possibility of denitrification under aerobic conditions[86]. Moreover, recent work by Roco et al., (2016)[99] found that aerobic nitrate reduction is a common trait present in bacteria across a wide range of soils and ecosystems. The five soils in which we observed N_2O production from ODn indeed span a fairly diverse range of natural and cultivated environments.

2.3.3. Differences between Event 1 and Event 2

Soils were air-dried before use. This was done to create the same initial condition in all soils before slurry preparation, in accordance with the intent of Event 1, which was to observe

any pre-existing enzymes and the ability to perform ODn immediately following rewetting. This method represented soils that switch from an inactive to an active state, as is typical of rewetting soils following the dry season in a Mediterranean climate. Because short-term emission is of particular interest of this study, we chose not to preincubate the soils as the pre-existing enzyme effect would most likely be masked under preincubation. Event 2 was set up to observe any *de novo* synthesis within 6-8 hours.

As described above, soils were tested for N_2O produced from ODn at two points: Event 1, immediately after rewetting, and Event 2, 6-8 hours later, allowing a short time for the stimulation of microbial activity relevant to ODn. In particular, data for Event 1 showed that the microorganisms and/or enzymes responsible for this process remain viable in dry soil for at least 1-2 months, and can generate N_2O shortly after rewetting, indicating the pre-existing enzyme capacity[182,184] and the persistence of denitrifying enzymes under aerobic conditions. A pulse of N_2O after rewetting is a common phenomenon, although the relative importance of specific microbial processes to changes in N_2O fluxes remains poorly understood [68,185,186]. The significant enrichment of ^{15}N in N_2O just one hour after the $^{15}N\text{-}NO_3^-$ additions in our study implies rapid nitrate reduction to nitrite and subsequent reduction to N_2O. Typically, *de novo* synthesis of denitrifying enzymes requires 4-8 hours[187]; if this is true for ODn as well, this means that the relevant enzymes were present and active in the soil at the start of Event 1. Only in the grassland soil was there no significant for N_2O production during Event 1. In the forest and grassland soils, there was greater nitrate-derived N_2O in Event 2 compared to Event 1, while in the other three soils, the reverse was true. This might relate to different induction times required for ODn activity.

2.3.4. Differences among soils

The five soils with significant N_2O production from ODn are not particularly notable with respect to the five basic properties measured (pH, DOC, NO_3-N, NH_4-N and FeP), that is, these properties are not particularly indicative of the potential for ODn in the five active soils. Although organic carbon is a strong determinant of (anaerobic) denitrifier activity[46], bulk DOC may not indicate a soil's ODn activity if certain carbon sources are preferred over others. Other soil attributes are likely more important than these, the most obvious being the composition of the microbial community and the presence or absence of organisms capable of ODn. A soil's natural environment may also play a role. For example, the sample with the highest values in Tables 2.2a and 2.2b (Ag 2) is a soil under rice production; the frequent aerobic-anaerobic cycles characteristic of such soils may favor the abundance or activity of oxic denitrifiers.

Treatment manipulations of nitrate and carbon content, along with soil moisture (or oxygen) and pH have been used to demonstrate that these variables regulate denitrification rates and end products[45]. The amount of ^{15}N and NO_3^- added to the soils was relatively small. This approach may neglect to identify soils that do have inherent ODn activity, but may simply have such small native concentrations of nitrate that ODn is not detected or induced, especially under the short-term experiment used here. On the other hand, the amount of NO_3^- added would not, have stimulated subsequent processing rates and thus reflects the occurrence and extent of ODn under natural conditions. Enhanced N_2O production might be triggered by addition of nitrogen or carbon substrates, through events such as N deposition in forest systems and fertilization in managed systems.

2.3.5. Ranking of soil properties from PLS analysis

To further elucidate the relative importance of each soil property in explaining ODn, the PLS analysis was performed on all 19 soils. Each soil property was ranked based on its ability to explain ODn (i.e., conversion of NO_3^- to N_2O under oxic conditions). This analysis was first performed for event 1 and event 2 separately; then, results from both events were pooled to aid the interpretation (Figure 2.2). For event 1, NH_4-N ranked highest and explained 93% of variability. Like NH_4-N, a significant predictive ability was also found for FeP and ODn. It is worth noting that ammonium and FeP were highly correlated (Table 2.3). DOC explained 62% of the variability in conversion of NO_3^- to N_2O. NO_3^- and pH had lowest rankings with no significant connection with ODn. In contrast to Event 1, pH ranked highest for Event 2, while all other properties were insignificant in predicting ODn. When data for both events were pooled, pH ranked highest and nitrate concentration ranked lowest.

The negative values of slopes from linear regressions between pH and ODn agreed with previous studies of aerobic denitrification. Ji et al. (2015)[31] reviewed pure culture studies and reported pH of 7-7.5 as the optimal range for aerobic denitrification. Denitrification activity in general decreases below pH 6, and below pH 5 denitrification gene expression is significantly reduced[188]. The relationship between pH and ODn is intriguing as pH is often viewed as a distal, not a proximal, control that directly affects instantaneous denitrification rate and its gaseous emission. In our study, occurrence of the highest rate of conversion of NO_3^- to N_2O coincided with a lower pH range (5 to 6.1). At this pH, chemodenitrification is possible, by which Fe(II) can react with nitrite to form N_2O. However, chemodenitrification is induced under anoxic

conditions that favor accumulation of Fe(II) and is thus unlikely to occur in our slurry incubation when oxic conditions were maintained. In environments with oxic-anoxic fluctuations, chemodenitrification may be coupled with denitrification to fuel rapid N_2O production. The relative contribution of N_2O from chemodenitrification and ODn might be affected by the availability of reduced form of iron and carbon availabilities.

The non-significant relationship between NO_3^- and ODn indicates that under aerobic conditions, denitrification rate may not be directly controlled by nitrate availability. The ranking score of nitrate in event 2 was higher than that of event 1, suggesting nitrification occurred over the course of the experiment. In a preliminary experiment of 6 soils, both ammonium and nitrate concentrations were found to slightly increase over the course of incubation (data not shown). Unlike anaerobic denitrifiers, obligately aerobic heterotrophs make up the bulk of the microbial biomass and compete for carbon in the presence of oxygen.

2.4. **Ecological importance of ODn**

The tight coupling of denitrification enzymes is not necessarily universal in soils[30] and the expression of each enzyme may have different sensitivity to oxygen[31]. Despite the relative lack of understanding of ODn compared to classical (anaerobic) denitrification, our study and several others indicate that this process is active in a variety of environments. In particular, the ability to carry out ODn can persist over short periods (months) in dry soils, and emission of N_2O from reduction of nitrate can occur readily as soon as the soil is rewetted. We agree with Lloyd (1987, 1993)[84,189] that, in view of survivability, the capability for both aerobic and anaerobic

denitrification would presumably enhance the resistance of an organism, or community of denitrifiers, to environmental changes. Heterogeneity in soils may provide considerable ecological niches for aerobic as well as anaerobic denitrifiers, and the periods during which denitrification can occur may in fact be far greater than originally thought given that both aerobic as well as anaerobic pathways appear to be important in soil.

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Supplement Materials

Part 1. Tables and figures

Table S1. Basic properties of all soils and sampling locations. Bolded soils are examine in triplicates.

Soil ID	Vegetation	Location	pH	DOC (µg/g)	NO_3-N (µg/g)	NH_4-N (µg/g)	FeP (µg/g)
1	Forest	Pollock Pines	5.9	5.57	1.57	9.35	354.6
2	**Forest**	**Pollock Pines**	**6.1**	**15.6**	**24.36**	**7.45**	**577.3**
3	Forest	Pollock Pines	5	12.54	0.16	5.79	705.8
4	**Grassland**	**Yolo**	**7.3**	**13.12**	**14.8**	**5.04**	**73**
5	Grassland	Cache Creek	6.5	11.94	20.25	4.39	473.8
6	Grassland	Cache Creek	6.2	4.74	43.71	1.43	299.7
7	**Vineyard(L)**	**Kearney**	**6.3**	**9.72**	**43.5**	**2.48**	**684.8**
8	Vineyard (S)	Kearney	6.5	4.41	19.69	1.53	245.9
9	**AG 1**	**Twitchell Island**	**6.2**	**7.27**	**17.3**	**3.74**	**2149.3**
10	**AG 2**	**Twitchell Island**	**5.7**	**22.44**	**23.28**	**26.16**	**5600**
11	Riparian	Pollock Pines	6	2.45	0.28	1.38	455.4
12	Grassland	Cache Creek	5.9	5.84	21.7	3.33	392.2
13	Forest	Cache Creek	5.7	6.09	11.97	3.18	287.5
14	Forest, subsurface	Pollock Pines	5.9	5.97	6.13	2.33	390.7
15	Forest, subsurface	Pollock Pines	5.2	8.66	0	3.18	258.9
16	Bare soil, unmanaged	Cache Creek	6.8	7.76	8.79	2.23	251.1
17	Bare soil, unmanaged	Cache Creek	6.4	0.6	0.7	0.83	127.9
18	Grassland	Cache Creek	6.2	1.74	0.96	2.13	196.2
19	AG 3	Capay Valley	5.6	3.15	39	1.43	290.3

Table S2. Conversion of NO_3^- to N_2O under oxic conditions and expressed as mass N_2O-N on a soil basis; n.d. = not detectable or zero value. Bolded soils are examined in triplicates.

Soil ID	Event 1	Event 2	Event 1	Event 2
	µmol-N/mol N-NO_3/hr	µmol-N/mol N-NO_3/hr	ng N-N_2O/g/hr	ng N-N_2O/g/hr

1	n.d.	n.d.	n.d.	n.d.
2	**1.1**	**3.73**	**0.03**	**0.09**
3	n.d.	81.47	n.d.	0.01
4	**n.d.**	**2.84**	**n.d.**	**0.04**
5	1.42	0.39	0.03	0.01
6	0.22	0.12	0.01	0.01
7	**2.8**	**0.43**	**0.12**	**0.02**
8	0.23	0.44	0	0.01
9	**4.6**	**2.35**	**0.08**	**0.04**
10	**48.9**	**43.05**	**1.14**	**1**
11	n.d.	n.d.	n.d.	n.d.
12	0.58	15.43	0.01	0.33
13	0.1	45.23	0	0.54
14	0.49	0.05	0	0
15	n.d.	n.d.	n.d.	n.d.
16	3.6	n.d.	0.03	n.d.
17	n.d.	15.65	n.d.	0.01
18	n.d.	9.26	n.d.	0.01
19	1.23	n.d.	0.05	n.d.

Table S3. Correlation matrix (r values) among the measured soil properties for the conversion of NO_3^- to N_2O under oxic conditions (ODn) for all soils

	Event 1	Event 2
	ODn	ODn
pH	-0.82*	-0.96**
DOC	0.95**	0.57
NO_3^--N	-0.40	-0.70
NH_4^+-N	0.99***	0.67
FeP	0.99***	0.61

*, **, *** Significant at the 0.05, 0.01, and 0.001 probability level, respectively.

Part 2. Calculations

We calculate OD% as follows.

$$R_{sam} = ((\delta N_{sam} /100) + 1) * R_{std}$$

$$F_{sam} = R_{sam} / (R_{sam} + 1)$$

Where R_{sam} =isotope ratio of gas sample, δN_{sam} = the ^{15}N enrichment of a gas sample, and R_{std} = $^{15}N/^{14}N$ ratio of standard (atmospheric N_2)

$$Mol\text{-}^{15}N = Mol\text{-}N * F_{sam}$$

Where mol^{15}N = moles of ^{15}N-N_2O in the sample, mol-N = moles of total N-N_2O in the headspace, and F_{sam} = the fraction of N in the sample as ^{15}N

$$Net\ Mol\text{-}^{15}N = mol\text{-}^{15}N\ before\ event - mol\text{-}^{15}N\ after\ event$$

Where net mol-^{15}N = moles of ^{15}N-N_2O generated in headspace during one sample event when the jar is closed, for event one, lab room air values are substituted as before event values

$$Total\ Mol\text{-}^{15}N = Net\ Mol\text{-}^{15}N + aq\ Mol\text{-}^{15}N$$

$$aq\ Mol\text{-}^{15}N = K_H * P_{N2O}$$

Where aq Mol-^{15}N = dissolved N_2O proportion to headspace N_2O, calculated based on Henry's Law, K_H for N_2O at 25 °C is 0.024 $\frac{mol}{L*atm}$

$$Mol\text{-}Ni = Total\ Mol\text{-}^{15}N / F_{Ni}$$

Where Mol-Ni = equivalent moles of N-NO_3^- of total ^{15}N-N_2O produced during one sample event, and F_{Ni} is the enrichment of nitrate pool expressed as at-%

$$OD\text{-}N_2O\% = Mol\text{-}Ni / Mol\text{-}total$$

Where Mol-total = mole of nitrate present at the start of a sampling event