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Soil acidification by intensified crop production in South Asia results in higher $N_2O/(N_2 + N_2O)$ product ratios of denitrification

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ABSTRACT

Agricultural soils emit significant amounts of N_2O to the atmosphere, and annual emissions are in some proportion to the input of reactive nitrogen to the system. Hence the ongoing intensification of cropping systems in South Asia will result in increased emissions of N_2O. The prospects are potentially worse than those predicted by the increasing doses of N-fertilizers, however. The reason for this is that intensive cropping systems may acidify the soils, which could increase the $N_2O/(N_2 + N_2O)$ product ratio of denitrification due to interference with the expression of the different enzymatic steps in this process (Liu et al., 2010 FEMS Microbiol Ecol 72 407–417). We investigated this phenomenon for agricultural soils in the central mid-hills of Nepal. We sampled soils from fields that had been under intensified cultivation for ≥20 years, and adjacent fields with more traditional cultivation, in areas with permanently drained soils as well as areas with frequent flooding. The characteristic kinetics of NO, N_2O and N_2 production by denitrification in these soils was measured by anoxic incubations after flooding and drainage of the soils with 2 mM NO_3^-, to secure similar NO_3-concentrations for all soils. The results demonstrate that intensification invariably lowered the soil pH and increased the $N_2O/(N_2 + N_2O)$ product ratios of denitrification. This effect of intensification was observed both for incubations with and without C-substrates (glutamic acid) added. The transient accumulation of NO varied grossly between sites, but was not affected by intensification. The results demonstrate convincingly that the intensification has resulted in higher intrinsic propensity of the soils to emit N_2O to the atmosphere, and the correlation with pH suggests that acidification is responsible. This causal relationship is underpinned by emerging evidence that low pH interferes with the assembly of the enzyme N_2O-reductase. We conclude that the ongoing intensification of agriculture in South Asia may result in severely increasing N_2O emissions unless acidification of the soil is counteracted.

1. Introduction

Anthropogenic activities have increased the availability of fixed nitrogen (N) in the biosphere (Galloway and Cowling, 2002) which alters major biogeochemical cycles of natural ecosystems (Elser et al., 2009) and enhances emissions of N_2O (Schlesinger, 2009). N_2O is a potent greenhouse gas and contributes significantly to the depletion of stratospheric ozone (Ravishankara et al., 2009). N_2O has a specific global warming potential (per mass) 298 times higher than that for CO_2 (calculated for a 100 years perspective, Solomon et al., 2007) As a result, N_2O emissions are a significant component of the global warming potential of agroecosystems (Crutzen et al., 2008). Globally, biogenic sources account for ~90% of emissions of N_2O, and agriculture accounts for around half of this biogenic N_2O as emissions from cultivated soils and as indirect emissions driven by deposition of N from agriculture (Fowler et al., 2009).

Dramatic changes in agricultural practice have taken place in large part of Asia, as a result of economic growth and increasing demand for food production. The subsistence-based traditional farming system (Rasul and Thapa, 2003) has gradually been replaced by severely intensified cropping systems (Paudel and Thapa, 2004; Brown and Kennedy, 2005; Tiwari et al., 2008). The traditional farming system is characterized by low fertilizer doses and maximum two crops per year. Intensification implies higher doses of fertilizers and generally three crops per year. This will most likely result in enhanced emissions of N_2O, but not necessarily in proportion to the fertilizer levels. One of the consequences of

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intensification of agriculture in Asia appears to be that the soil pH is significantly lowered since liming is not commonly practiced (Guo et al., 2010). This acidification could in theory aggravate the N_2O emission by interfering with the product stoichiometry of denitrification (see below).

Soils produce N_2O by microbial oxidation and reduction of N-species (Fowler et al., 2009). In nitrification, N_2O is a byproduct of the oxidation of ammonium to nitrite, primarily caused by decomposition of the intermediate hydroxylamine (Stein, 2011), and the N_2O/NO_2^- product ratio of the reaction suggest that nitrification is a relatively weak source of N_2O in soils (Mørkved et al., 2007; Jiang and Bakken, 1999). At low oxygen concentrations, however, nitrifying bacteria may contribute to N_2O emission via denitrification (Sutka et al., 2006). But the ultimate sources of electrons driving this nitrifier-denitrification are ammonium and nitrite, which is extremely limited compared to that driving heterotrophic denitrification. In denitrification (be it by autotrophic or heterotrophic organisms), N_2O is an intermediate product of the stepwise reduction of nitrate via NO_2^-, NO, N_2O to N_2. The four functional enzymes involved are not constitutively expressed; oxygen appears to be a master-repressor, and the four enzymes are expressed to a variable degree depending on the regulatory network of the organism (Bergaust et al., 2011) and external variables (Bergaust et al., 2010; Bakken et al., 2011). Unbalanced expression of the enzymes results in transient accumulation of the intermediates NO and N_2O, which may escape to the atmosphere.

The emission of N_2O from soil is extremely variable in time and space, hence annual emissions are uncertain unless excessive numbers of measurements are made. We need quantification of emissions as a function of soil management, however, both for estimating the total emissions from existing agricultural ecosystems, and to identify management options that will reduce emissions, i.e. mitigation options. The identification of mitigation options are important, but hard to accomplish by field emission measurements, for the reasons mentioned (spatial variability, hence low statistical power of comparisons). An approach complementary to *in situ* flux measurements is to study denitrification kinetics and their product stoichiometry ($NO/N_2O/N_2$) under standardized laboratory incubations. Such investigations hold a potential to unravel effects of management on the soils' propensity for NO and N_2O emissions, if such effects exist. In theory, management can affect this propensity for NO and N_2O emission via changes in the community composition (Holtan-Hartwig et al., 2000; Cuhel and Simek, 2011; Dörsch et al., 2012; Braker et al., 2012) or changes in soil properties that leads to consistent changes in the product stoichiometry of denitrification. The latter could be caused by changes in soil pH which recently has been found to systematically affect the ability of denitrifying bacteria to express N_2O reductase, hence affecting their $N_2O/(N_2O + N_2)$ product ratio (Bergaust et al., 2010; Liu et al., 2010). This phenomenon explains the previously observed negative relationship between soil pH and the $N_2O/(N_2O + N_2)$ product ratio of denitrification (Firestone et al., 1980; Simek and Cooper, 2002).

In Nepalese rural areas, there is a patchwork of fields which has been intensified and fields where traditional low input farming is still practiced. In a recent study of the past, present and future direction of farmers' practice in the Ansikhola watershed of Nepal (Raut et al., 2011), it was shown that the amounts of fertilizer N application has increased dramatically. The study, which included interviews with individual farmers, also revealed large contrasts between individual farmers' practice; some adopted intensified agricultural practice early (>20 years ago), whereas others have continued a more traditional, low input farming. The information gathered (Raut et al., 2011) allowed us to identify sites where fields with intensified and more traditional cultivation histories existed side by side. Liming is not commonly practiced in the Ansikhola watershed, despite the generally low pH of these soils compared to soils at lower altitudes in Nepal (Bajracharya and Sherchan, 2009). The study included sites with permanently drained soils (Bari upland soils) and sites with frequently flooded soils (Khet, lowland levelled soils).

We hypothesized that the increased fertilizer N input in the intensified agriculture would acidify the soil, and that this would result in higher N_2O/N_2 product ratios of denitrification. We selected these sites for studying the effects of intensification on pH and the characteristics of the denitrification product ratios. The latter was investigated by standardized anoxic incubations of soils as described by Liu et al. (2010). The results demonstrate that the intensification invariably reduced the pH and increased the $N_2O/(N_2O + N_2)$ product ratio of denitrification. We also found conspicuous contrast between well drained soils and soils frequently flooded (rice fields), with respect to the kinetics of denitrification and the transient accumulation of NO during denitrification. The results corroborate earlier findings that pH is a master variable controlling the product stoichiometry of denitrification and suggest that the ongoing intensification of plant production in Asia will lead to gradually increasing emissions of N_2O per kg fertilizer N, unless the acidification of the soils is counteracted.

2. Materials and methods

2.1. Soils

Seven sites were selected within the Ansikhola watershed of the central midhills of Nepal, each site harbouring adjacent plots with traditional versus intensified cropping history, thus allowing a pairwise comparison of soils from such contrasting plots to study the effects of intensification. The annual rainfall is 1389 mm and average maximum and minimum temperature are 25 °C and 17 °C, respectively (Dahal et al., 2007). Four sites were located in Bari, which is an area with rain-fed upland levelled or sloping terraces. The croprotations in Bari are maize–wheat, maize–mustard, maize–millet, maize–potato, maize–vegetables, maize–wheat–potato or maize–mustard–potato depending on household (farmers' choice). The three remaining sites were located in the lowlands (Khet), which is an area with bunded and levelled terraces, used to grow rice–rice, rice–potato, rice–vegetables, rice–mustard, rice–wheat, rice–potato–rice, rice–maize–rice or rice–rice–vegetables. Soil management in Khet has included frequent flooding of the soils (for rice) for over 40 years. The Khet soils are finer textured (more clay) than the Bari soils (Table 1). The plots within each site were 0.05–0.1 ha, depending on the farmer's landholding size and irrigation facility.

The Intensified plots had a history of >20 years of intensive cultivation, i.e. high inputs of fertilizers and three crops per year. The Traditional plots had a history of lower N inputs and only two crops per year. All farmers used compost based on farmyard manure, but average amounts of compost applied per year varied (Table 1). Each pair of adjacent Traditional and Intensified plots is considered as one contrast (C). Thus a total of 7 contrasts, 3 from Khet and 4 from Bari land were collected. From each plot 4 independent soil samples were taken at random positions (sampling depth 15 cm, which is the ploughing depth in the area). The Bari soil samples were sieved (5 mm) immediately after sampling, and stored at field moisture condition in plastic bags at 4 °C. Since the Khet soils were very wet, they were air dried to reduce the moisture by 30–35% w/w in order to make sieving possible. Soil sampling was carried out during May to June 2009. The Khet soils had recently been flooded at the time of sampling, resulting in high (but variable) soil moisture (Table 1). The

Table 1
Cropping system fertilizer practice, bulk density, soil moisture contents and air filled porosity (at sampling time), and soil texture.

Cultivated land type	Agricultural system	Cropping pattern	DAP[a] kg N ha^{-1}	Urea kg N ha^{-1}	Compost kg ha^{-1}	Bulk density (g cm^{-3})	Moisture (% of dry weight)	Air filled porosity[b] %	Texture[c]
Khet (C1)	Traditional	Rice–Rice	63	124	9823	1.38	38	10	Loam
	Intensified	Rice–Potato–Rice	66	362	14,558	1.49	35	18	Loam
Khet (C2)	Traditional	Rice–Rice	61	113	1965	1.20	61	27	Loam
	Intensified	Rice–Potato–Rice	79	366	19,232	0.90	70	18	Loam
Khet (C3)	Traditional	Rice–Rice	40	120	12,279	1.19	24	43	Clay loam
	Intensified	Rice–Maize–Rice	94	210	16,372	1.26	46	9	Clay loam
Bari (C1)	Traditional	Wheat–Maize	82	104	19,470	1.09	11	74	Sandy loam
	Intensified	Maize–Mustard –Potato	112	161	31,410	1.15	7	84	Loamy sand
Bari (C2)	Traditional	Wheat–Maize	16	52	6217	1.15	19	55	Sandy loam
	Intensified	Onion–Potato–Tomato	119	183	31,447	0.95	12	72	Sandy loam
Bari (C3)	Traditional	Wheat–Maize	26	69	14,735	0.96	7	84	Sandy loam
	Intensified	Maize–Wheat–Potato	40	125	10,730	1.07	7	84	Loamy sand
Bari (C4)	Traditional	Wheat–Maize	52	143	21,499	1.30	5	91	Sandy loam
	Intensified	Maize–Potato –Mustard	75	184	24,558	1.14	6	86	Sandy loam

[a] DAP = di ammonium phosphate.
[b] Air filled porosity was calculated from gravimetric soil moisture and bulk density assuming a particle density of 2.65 g cm^{-3}.
[c] Soil texture classification was done according to the USDA system.

incubation experiments were carried out over a period of 4–12 months after sampling. The soil pH was measured in suspension of soil in 0.01 M $CaCl_2$ (10 g air dried soil in 10 ml of 0.01 M $CaCl_2$, pH measured after shaking for 1 h). The cropping systems and soil physical properties are provided in Table 1.

2.2. Nitrate adjustment

In order to obtain a homogenous distribution and equal concentration of nitrate and soil moisture tension in all soil samples prior to incubation, the soil samples were flooded with 2 mM of KNO_3 solution in Buchner funnels with paper filters and then drained immediately by applying vacuum for 5 min. The flooding and drainage with NO_3^- solution was repeated three times with the vacuum left on for a final evacuation-drainage for 20 min (Liu et al., 2010), resulting in final moisture contents ranging from 0.25 to 0.33 mL g^{-1} soil dry weight for the Khet soils and 0.17–0.27 mL g^{-1} soil dry weight for the Bari soils (the differences depending on their water retention characteristics). The soils were then preincubated aerobically at 20 °C for 2 days prior to the incubation for measurement of aerobic respiration (for 24 h) and subsequent anoxic incubation for measuring denitrification.

2.3. Gas kinetics under oxic and anoxic conditions

The measurements of aerobic respiration under fully oxic condition and denitrification under subsequently anoxic conditions were carried out by means of a robotized incubation system described in detail by Molstad et al. (2007). The system allows quantification of O_2, CO_2, N_2 N_2O and NO in the headspace by frequent gas sampling (typically every 2–3 h) to a chemiluminescence analyzer for quantification of NO and a gas chromatograph for determining the remaining gases. The system replaces the sampled gas with He, thus sustaining ~1 bar gas pressure throughout an entire experiment. The net gas production/consumption by respiration and denitrification had negligible influence on the pressure, both during the oxic and anoxic incubations: the oxygen consumption rates during oxic incubations were largely compensated by equal amounts of CO_2 produced (and the absolute rates of O_2-consumption and CO_2 production were less than 0.05% of the headspace volume per hour). The net rate of gas production during anoxic incubation (= CO_2 + NO + N_2O and N_2) amounted to less than 0.03% of the headspace volume per hour. The routines for estimating net production or consumption of each gas taking account for the dilution of the headspace by replacing each sample taken with He and the solubility of the individual gases are described in detail by Molstad et al. (2007).

The robot can host 18 soil samples (+3 flasks with calibration gases) in each incubation experiment. Thus we decided to run 7 consecutive incubation experiments, one for each contrast (duplicates for each of the 4 soil samples from Traditional and Intensified plots = 16 flasks per run). After preincubation (see above) the soils (equivalent to 25 g soil dry weight per flask) were placed in 120 ml serum flasks which were then sealed with air-tight, butyl rubber septa (type 20-Machercy-Nagel GmbH & Co, London) and aluminium crimp caps. The flasks were immediately placed in the incubator water bath (temperature = 20 °C) for measurement of oxic respiration (as CO_2-evolution) during the first 24 h of incubation ("Phase OX"). Then the air was replaced with He by 3 cycles of evacuation and He-filling (through a needle piercing the septum), to induce denitrification by anoxic conditions ("Phase DEN"). The overpressure after the last He filling was released using a syringe without piston filled with some ml of distilled water. To one of the duplicate flasks for each soil, acetylene (C_2H_2) was injected to reach 10 vol% (10 kPa), and the overpressure was again released. After monitoring the headspace for 36 h (Phase DEN), we injected 2 ml of a solution containing 40 mM KNO_3 and 50 mM sodium glutamic acid (pH adjusted to 7). The solution was spread evenly on the soil surface by a syringe, and monitoring of the gas phase was continued for ~60 h. The purpose of this last phase of the experiment was to monitor substrate-(glutamic acid) induced denitrification, hence we termed this phase as "Phase SIDEN" (Substrate Induced Denitrification).

The acetylene treatments were included primarily to check for abiotic release of N_2 from the soil matrix. We were not sure whether the repeated evacuation and He-filling would effectively remove all N_2 from the soil matrix. If some N_2 remained, to be released gradually throughout the subsequent incubation, our estimates of N_2 production by denitrification would be erroneously high. The acetylene treatments allowed us to quantify abiotic N_2 release (since 10 kPa acetylene effectively inhibits N_2O-reductase).

2.4. Calculations and graphical presentations

Rates of oxygen consumption, CO_2 production and NO_x production/consumption were calculated from changes in headspace

concentrations for each time increment (between two samplings) and corrected for sampling loss (2.4% per sampling) as well as minor contamination of N_2 from the atmosphere due to leakage (Molstad et al., 2007). The cumulated consumption (of O_2) and production of CO_2, N_2, as well as N_2O were then calculated by integration over time.

The dilution by sampling was also included when estimating net rates of production or consumption of the intermediates NO and N_2O (in this case, leakage was negligible). These rates (together with those for N_2) were used to calculate $N_2O/(N_2 + N_2O)$ product ratios for Phase DEN (average for the entire Phase DEN). In response to substrate addition (glutamic acid + nitrate, initiating Phase SIDEN), the rate of denitrification increased immediately (for all three gaseous products, i.e. NO, N_2O and N_2). The denitrification rate increased further throughout Phase SIDEN reaching its maximum before all available nitrate (and nitrite) had been depleted, resulting in net-uptake of all N_2O and NO and a stable plateau for N_2 in the bottles. This is illustrated in Fig. 1. The $N_2O/(N_2O + N_2)$ ratios for the first 24 h of SIDEN (with increasing concentrations of both N_2O and N_2) were calculated (average values for each flask). In a previous study of similar type (Liu et al., 2010), the entire SIDEN phase was included by calculating an N_2O index (area under the N_2O curve, divided by the area of the $N_2 + N_2O$ curve), but this was not applicable here because some of the incubations were interrupted before all NO_3–N had been reduced to N_2.

Graphically, we present the cumulated production for $N_2O + N_2$, but the measured concentrations for the intermediates (NO and N_2O). The reasons for not plotting cumulated production/reduction of NO and N_2O is that such curves do not reach zero despite zero concentrations in the flasks (the bacteria cannot reduce NO and N_2O removed by sampling), which would be confusing. In the case of NO, the concentration in the liquid phase is of interest because of its potential toxic effect. We report measured NO as nmol NO g^{-1} soil, but these values can be transformed to nM in the liquid, assuming equilibrium between NO in headspace and in the liquid phase. The solubility of NO at 20 °C is 0.002119 mol L^{-1} bar^{-1} (Molstad et al., 2007). A measured NO-concentration of 1 μL L^{-1} in the headspace (partial pressure = 10^{-6} bar) would give 2.119 nM in the liquid (when in equilibrium). For the same NO concentration (1 μL L^{-1}), the total amount of NO is 4.42 nmol $flask^{-1}$, which is equivalent to 0.177 nmol NO g^{-1} soil dry weight (there was 25 g soil dry weight $flask^{-1}$). This means that our NO concentrations, which are reported as nmol NO g^{-1} soil dry weight can be converted to nM concentrations in the liquid by multiplying with 13 (1 nmol NO g^{-1} is equivalent to 13 nM in the liquid).

Statistical analyses were carried out using SAS (Institute Inc. Cary, NC, USA) and SPSS (version 16.0). The multiple comparisons of mean N_2 production rates and product ratios in the two cropping systems were carried out using Student-Newman-Keuls (SNK) test and *T*-test (LSD). ANOVA was used to analyse the effect of the two agricultural systems on N_2O product ratios. Paired *T* test was used to analyse differences in pH, denitrification rate, respiration rate and NO concentration at the $p < 0.05$ level. Multiple regression analysis was used to test for a combined effect of soil moisture and pH on product ratios.

3. Results

3.1. Kinetics of gas production

Aerobic respiration rates (CO_2-production) were practically constant for each soil throughout the 24 h of aerobic incubation (data not shown), with rates in the different soils ranging from 28 to 49 nmol CO_2 g^{-1} soil dry weight h^{-1} (Table 2). The rates of O_2 consumption (nmol O_2 g^{-1} h^{-1}) were of the same order of magnitude, but more variable due to the minor decrease in O_2 concentration compared to the initial amounts of O_2 present in the flasks (21 vol%) (the oxygen consumption rates per gram of soil were equivalent to 15–30 nL O_2 h^{-1}, thus consuming only 0.1–0.15% of the oxygen in the headspace per hour). Pairwise comparisons of traditional versus intensified plots showed large differences but no consistent effect of intensification, at least not in the Khet sites. For Bari there was a tendency of higher respiration rates in soils under traditional as compared with intensified cultivation, and for these sites, there was a significant positive correlation between respiration and pH (analysis of single flask values, $r^2 = 0.39$, $n = 16$). No such correlation with soil pH was found for respiration in the Khet soils.

After removal of oxygen, CO_2 production rates decreased instantly by 30–40%, and remained at this level throughout Phase DEN (i.e. anoxic incubation without substrate added). The rates increased immediately with a factor of 3–4 in response to glutamic acid addition, and increased gradually to reach a peak approximately at the same time as that for the rates of denitrification. An example of the CO_2 production rate throughout the entire incubation is shown in Fig. S1, and the average CO_2 production rates during Phase DEN are shown in Table S1. The responses of the CO_2 evolution to anoxic conditions, substrate addition and the depletion of NO_x were essentially the same for all soils and cultivation histories. The average reduction in CO_2 production rate by transition to anoxic conditions was 30% for Khet and 44% for Bari but this contrast was not statistically significant ($p = 0.075$).

The apparent rate of N_2 production (as estimated by measured N_2 accumulation in the headspace) varied between −0.6 and 6 nmol N_2–N g^{-1} h^{-1} during the first part of the anoxic incubation (Phase DEN), and between 2 and 40 nmol N_2–N g^{-1} h^{-1} after addition of glutamic acid. In theory, a fraction of this N_2 accumulation could be N_2 released from the soil matrix (i.e. release of N_2 adsorbed/trapped in the soil matrix). The measured N_2 in the flasks with acetylene can be used to evaluate this possible source of error. The gross average for all flasks with acetylene was −0.06 nmol N_2–N g^{-1} h^{-1}, which was not significantly different from zero (standard error of the mean = 0.11, confidence interval = [−0.29; 0.17], $n = 56$). Thus, there was no indication of a systematic bias of our estimated N_2 production rates, although slight variations in the leakage of N_2 for individual flasks resulted in uncertain estimates for N_2 production rates below ~1 nmol N_2–N g^{-1} h^{-1}. This experimental noise resulted in slightly negative numbers for a few individual flasks. In the analysis of the data, such negative N_2 production rates were taken to indicate zero N_2 production (i.e. not significantly different from zero).

A typical result of the O_2, NO, N_2O and N_2 gas kinetics is illustrated in Fig. 1, which shows average values for Khet C1. The partial pressure of oxygen declined gradually throughout the Phase OX, primarily due to the dilution by replacing sampled headspace with He. As oxygen was removed by evacuation and He-flushing, there was an immediate increase in NO concentration, which then either peaked or remained nearly constant throughout Phase DEN. In response to the subsequent addition of glutamic acid and nitrate, the NO concentration increased rapidly and reached very high peak values compared to those in Phase DEN. The maximum NO concentrations reached for the two phases of anoxic incubations showed consistent differences between individual sites, but the levels were not clearly affected by the cultivation practice (Traditional versus Intensified plots). The average maximum NO concentrations reached for each of the plots are shown in Table 3. In summary, the maximum amounts of NO reached for Khet were 3–63 nmol NO g^{-1} soil for Phase DEN and 162–632 nmol NO g^{-1} soil for Phase SIDEN. The equivalent ranges for Bari were 23–120 nmol

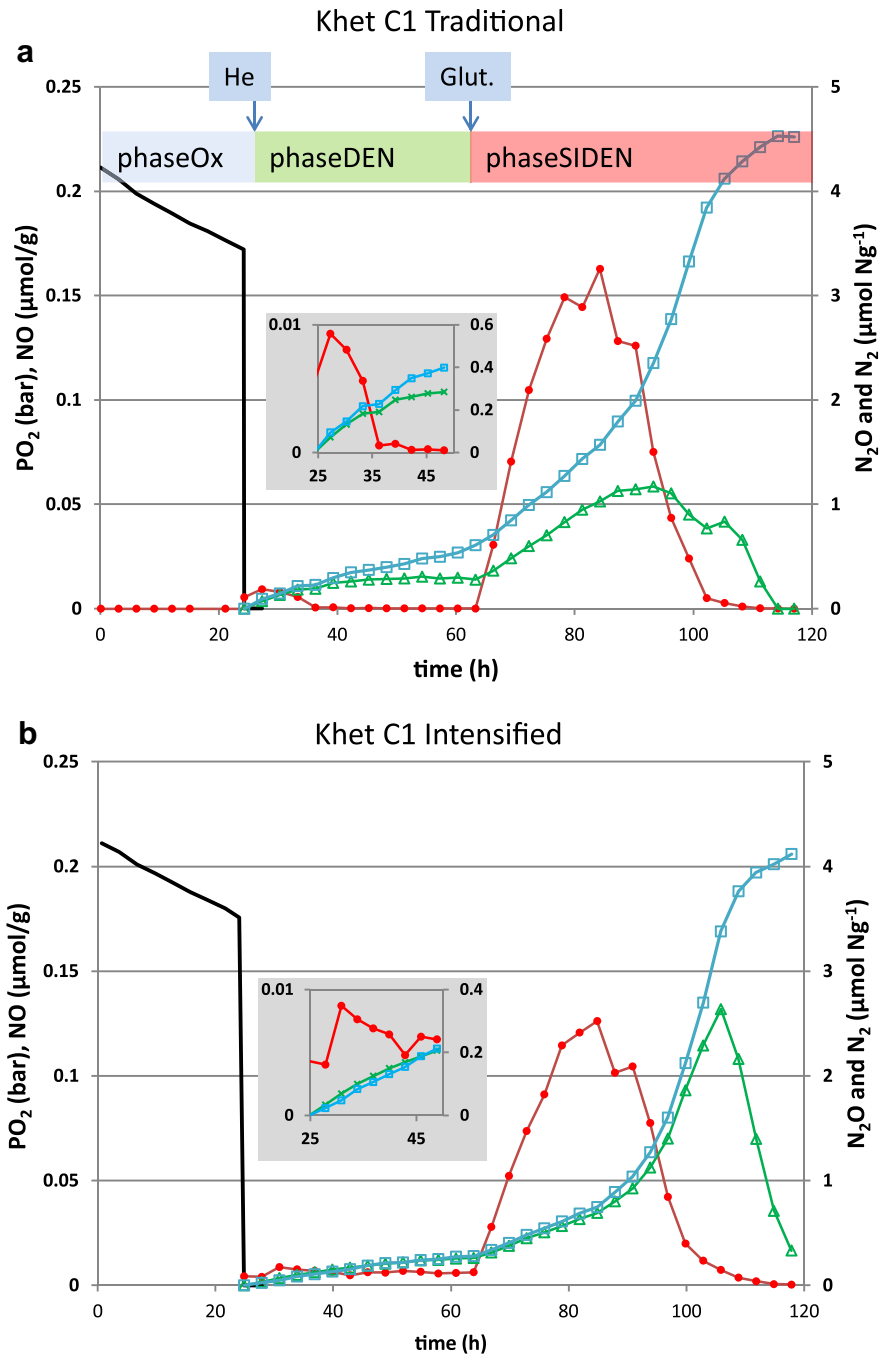

Fig. 1. Kinetics of NO, N_2O and N_2 during oxic and subsequent anoxic incubation without C-substrates added (Phase DEN) and after injection of glutamic acid (Phase SIDEN). The amounts of nitrogen gas species (NO, N_2O and $N_2 + N_2O$) are plotted as µmol N g^{-1} soil dry weight. The plotted NO (●) and N_2O (△) are the measured amounts, while $N_2 + N_2O$ (□) is the cumulated production (see text for explanation). The oxygen concentration (—) is shown as partial pressure (bar) in the headspace. The data shown are average values for the Traditional (panel a) and Intensified plot (panel b) in Khet C1. The inserted figure shows NO, N_2O and ($N_2 + N_2O$) in more detail for the first part of the anoxic incubation.

NO g^{-1} soil and 40–135 nmol NO g^{-1} soil, respectively. One nmol NO g^{-1} soil is equivalent to 13 nmol NO L^{-1} in the liquid (assuming equilibrium between headspace and the water in the soil), thus the maximum NO concentrations in the liquid for the different soils ranged from 38 nmol L^{-1} M to 7.7 µmol L^{-1}.

Accumulation of N_2O and N_2 started immediately in response to removal of oxygen, as illustrated in Fig. 1. The rates of denitrification (= sum of N_2- and N_2O-production) remained practically constant throughout the anoxic phase without substrate additions (Phase DEN) for all treatments (as illustrated for Khet C1 in Fig. 1). The ratio between N_2O- and N_2 production rates declined slightly throughout Phase DEN for some of the soils. This trend was stronger for traditional than for intensified soils (as illustrated for Khet C1 in Fig. 1), but was not statistically significant ($p > 0.05$). The rates of denitrification and N_2O production are summarized in Table 4 (average rates for each plot).

In response to the injection of glutamic acid (and nitrate), there was an almost immediate increase in the rate of N_2 and N_2O production, and the rates then increased further (gradually) until nitrate became limiting (nearly all nitrate reduced to N_2). The

Table 2
Oxic respiration rates (mean ± SD, $n = 4$) in nmol CO_2 g^{-1} h^{-1} in soils from Traditional and Intensified plots within the 7 contrasts.

	Traditional	Intensified	Traditional/Intensified
Khet (C1)	39.2 (2.3)	22.4 (2.6)	1.8
Khet (C2)	34.4 (1.9)	48.6 (10,4)	0.7
Khet (C3)	28.3 (6.6)	38.6 (7.0)	0.7
Bari (C1)	47.0 (3.4)	44.1 (5.7)	1.1
Bari (C2)	37.7 (8.4)	32.6 (9.8)	1.2
Bari (C3)	23.4 (7.9)	28.2 (4.9)	0.8
Bari (C4)	27.9 (1.4)	26.2 (2.7)	1.1

concentrations of N_2O peaked earlier than the depletion of nitrate + nitrite, however, reflecting an increasing relative rate of N_2O reduction. To characterize the N_2O and N_2 kinetics for the early phase of the substrate induced denitrification, we calculated the average rates for the first 24 h of the substrate induced denitrification for each flask. The average values for each plot are shown in Table 5.

The cumulated production of $N_2 + N_2O$ (Fig. 1) reached nearly stable levels around 4.5 μmol N g^{-1} soil after 115 h of incubation (single flask values were within ±13% of the average of all Khet C1 flasks). The cumulated N_2O production in the flasks with acetylene were within ±10% of the cumulated $N_2 + N_2O$ production in the flasks without acetylene (results not shown). The levels reached for Khet C2 were within ±10% of the average for Khet C1 (no data exist for Khet C3 because of instrument breakdown during Phase SIDEN). The cumulated $N_2 + N_2O$ production (4.5 μmol N g^{-1}) exceeds that added to the soil by flooding/drainage with 2 mM NO_3^--solution (~0.3 mL g^{-1} = 0.6 μmol NO_3^- g^{-1}) plus that added together with glutamic acid (3.2 μmol NO_3 g^{-1}) = 3.8 μmol NO_3 g^{-1}. The surplus N recovered as N-gas production (~0.7 μmol N g^{-1}) was tentatively ascribed to nitrification taking place during the three days incubation under oxic condition prior to the removal of oxygen (2 days pre-incubation and one day oxic incubation for measurements of oxic respiration). For all the Bari soils, the incubation was terminated before the cumulated N_2 reached a plateau, thus precluding a similar N mass balance calculation for these soils.

The denitrification rates in Phase DEN were strongly correlated with the measured oxic respiration rates, as illustrated in Fig. 2, and the results were very similar for Khet and Bari sites. The same regression analysis was done separately for all Intensified and all Traditional plots, and the results indicate essentially the same relationship between denitrification rate (D) and oxic respiration (R_{ox}): $D = 0.19^*R_{ox}$ for Traditional and 0.17^*R_{ox} for Intensified plots. We also investigated if the soil pH had any effect on the relationship between denitrification rates and oxic respiration. This was done by testing the correlation between soil pH and the R_{ox}/D-ratio. The result demonstrated that no such relationship exist ($r^2 = 0.02$, result not shown).

Table 3
Maximum NO concentrations (mean ± SD, $n = 4$) reached in the incubation flasks during the two phases of anoxic incubation; Phase DEN (without substrate added) and Phase SIDEN (after injection of glutamate). In nmol NO g^{-1} soil (in gas + liquid).

Land type	Phase DEN		Phase SIDEN	
	Traditional	Intensified	Traditional	Intensified
Khet (C1)	8 (3)	10 (5)	162 (23)	118 (23)
Khet (C2)	7 (4)	10 (3)	595 (80)	632 (93)
Khet (C3)	3 (4)	63 (55)	na[a]	na
Bari (C1)	119 (35)	120 (50)	271 (48)	135 (60)
Bari (C2)	24 (17)	24 (9)	60 (73)	97 (63)
Bari (C3)	90 (12)	86 (23)	184 (18)	97 (35)
Bari (C4)	38 (1)	32 (1)	40 (1)	38 (0.4)

[a] Khet C3 was terminated without substrate addition (Phase SIDEN is missing).

Table 4
Average rates (mean ± SD, $n = 4$) of denitrification ($N_2 + N_2O$) and N_2O production (nmol N g^{-1} h^{-1}) through the anoxic incubation without substrate added (first 24 h of Phase DEN).

Land type	Denitrification ($N_2 + N_2O$)		N_2O production	
	Traditional	Intensified	Traditional	Intensified
Khet (C1)	7.8 ± 1.2	4.3 ± 0.6	4.6 ± 1.3	3.9 ± 0.2
Khet (C2)	4.4 ± 0.3	8.7 ± 0.6	2.1 ± 1	5.7 ± 3.3
Khet (C3)	4.7 ± 1.6	6.4 ± 1.4	3.8 ± 1.1	6.4 ± 1.2
Bari (C1)	9.8 ± 1.0	7.1 ± 0.8	7.3 ± 0.9	6.4 ± 0.7
Bari (C2)	8.9 ± 0.9	7.7 ± 2.3	5.5 ± 0.5	4.8 ± 0.8
Bari (C3)	3.9 ± 0.9	4 ± 1.6	3.2 ± 1	3 ± 0.3
Bari (C4)	5.4 ± 2.8	4.1 ± 1.9	2.7 ± 0.4	2.6 ± 0.5

3.2. Effect of intensification on pH and the product ratio of denitrification

The results revealed that pH has been significantly lowered by intensive cultivation ($P < 0.05$) (Table 6). The average pH of soil under intensified cultivation was 4.49. In contrast, the average pH was 5.17 in traditional agricultural system.

The $N_2O/(N_2 + N_2O)$ product ratios for Phase DEN and SIDEN were calculated for each single flask, and used as independent observations for statistical analyses. The average values for each plot are shown in Table S2. Analysis of variance showed significant effect of cultivation history (Traditional versus Intensified) both for DEN ($p = 0.004$) and SIDEN ($p = 0.006$), and significant difference between sites for DEN ($p = 0.004$), but not for SIDEN ($p = 0.275$).

The average denitrification product ratios [$N_2O/(N_2O + N_2)$] are plotted against soil pH in Fig. 3, thus summarizing the effect of intensification on soil pH and the product ratio of denitrification at each paired site. With a few notable exceptions, the figure shows an increasing $N_2O/(N_2O + N_2)$ ratio with decreasing pH.

The relationship between the product ratio (R = the molar $N_2O/(N_2 + N_2O)$ ratio) and the soil pH was investigated in more detail across treatments and sites by regression analyses of the product ratio for single soil samples against their pH. By including all samples (both Bari and Khet; $n = 56$), we find that R for Phase DEN is negatively correlated with pH ($r^2 = 0.09$, $p = 0.019$). Separate analyses of Khet and Bari shows a strong relationship for Khet ($r^2 = 0.25$, $p = 0.008$) but not significant for Bari. Further, we investigated if R was in any way affected by the oxic respiration rates of the soils. The trend was that R decreased with increasing oxic respiration rate, but this was not statistically significant. Separate analysis of the Khet samples, however, shows a statistically significant effect of both pH and oxic respiration on R (the regression function $R_{KhetDEN} = 3 - 0.013^*R_{ox} - 0.37^*$pH, $r^2 = 0.58$; $R_{KhetDEN}$ = the molar product ratio for Phase DEN, R_{ox} = the oxic respiration rate).

Table 5
Substrate induced denitrification ($N_2 + N_2O$) and N_2O production. Rates (mean ± SD, $n = 4$) are given in nmol N g^{-1} h^{-1} for Traditional and Intensified plots within each contrast for the first 24 h after injection of glutamic acid and nitrate.[a]

Land type	Denitrification ($N_2 + N_2O$)		N_2O production	
	Traditional	Intensified	Traditional	Intensified
Khet (C1)	31.5 ± 4.1	20.6 ± 5.8	17.7 ± 7.4	17.2 ± 4.1
Khet (C2)	12.2 ± 4.6	21.3 ± 7.5	9.6 ± 2.4	17.7 ± 5.8
Bari (C1)	16 ± 2.3	16.1 ± 3.1	9.6 ± 1.8	13.2 ± 3.2
Bari (C2)	19.2 ± 5.2	12.2 ± 2.4	11.5 ± 1.4	9.4 ± 1.5
Bari (C3)	7.5 ± 1.7	7.5 ± 2.6	4.2 ± 1.4	5.4 ± 0.4
Bari (C4)	4.4 ± 0.7	4.4 ± 2.1	3.5 ± 0.0	2.9 ± 0.8

[a] Substrate induce respiration was not measured for Khet C3 due to instrument breakdown.

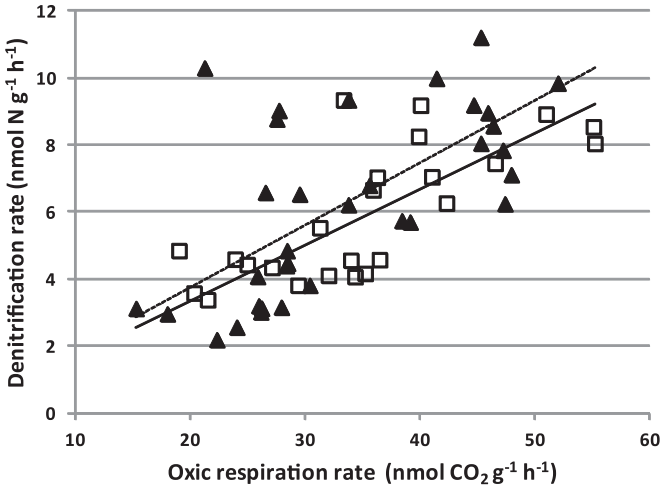

Fig. 2. Relationship between oxic respiration rates (Phase OX) and denitrification rates during anoxic incubation without substrates added (Phase DEN). Results for single flasks with soil from Khet (□) and Bari (▲) are shown. The proportionality between denitrification is illustrated by regression lines (forced through origo) for both areas (Bari: dotted line, Khet continuous line). The regression functions are: Bari: $D = 0.18^{*}R$ ($r^2 = 0.4$), Khet: $D = 0.17^{*}$R ($r^2 = 0.53$), where D is the denitrification rate (nmol (NO + N_2O + N_2)-N g^{-1} h^{-1}) and R is the oxic respiration rate (nmol CO_2 g^{-1} h^{-1}).

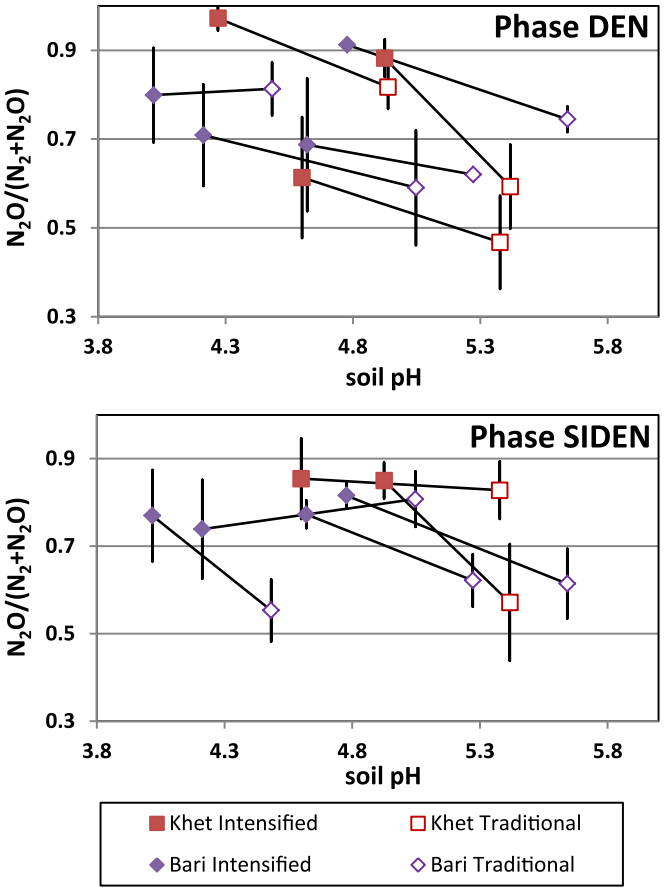

Fig. 3. Molar product ratio, $N_2O/(N_2 + N_2O)$, for all contrasts plotted against soil pH. Average product ratio for each cultivation history within Khet (□) and Bari (◇). The results for Traditional (open symbols) and Intensified (closed symbols) within each Contrast site are connected by lines, and the standard error of the mean indicated by vertical lines. Upper panel shows the result for Phase DEN (no C substrates added) and the lower panel shows the result for Phase SIDEN (substrate-induced denitrification).

4. Discussion

Oxic respiration rates were significantly different between some of the paired cultivation contrasts, and between plots belonging to the same cultivation contrast, but it is difficult to see any consistent effect of intensification. Neither did we find any correlation between oxic respiration (Table 2) and the annual amounts of compost applied to the different plots (Table 1). The oxic respiration rates are possibly reflecting recent inputs of plant residues prior to the sampling dates, which may vary arbitrarily between plots.

In response to anoxic conditions, denitrification started immediately and the rate remained practically constant throughout Phase DEN, suggesting severe carbon substrate limitation which is normal for prokaryotes in bulk soil. The constant rates of denitrification suggest that the organism use an existing denitrification proteome rather than synthesizing new enzymes (although a trend to increased activity of N_2O reductase was observed for some samples, as illustrated in Fig. 1). The significance of substrate limitation is illustrated by the immediate upshift in denitrification rates in response to injection of glutamic acid, and the subsequent gradually increasing denitrification rates. The observations corroborate results of similar incubation experiments by Liu et al. (2010), who were only able to detect transcription of the denitrification genes if carbon substrates were added to the soils. Thus, Phase DEN reflects the typical denitrification response of bulk soil, whereas the Phase SIDEN would represent denitrification in the vicinity of easily decomposable plant materials supporting microbial growth and *de novo* synthesis of denitrification enzymes.

On this background, it is not surprising that denitrification rates during the first anoxic incubation without substrates (Phase DEN) were correlated with oxic respiration rates, since both variables reflect the flux of C-substrates to support respiratory metabolism in the soils. The ratio between oxic respiration (R_{ox}, nmol CO_2 g^{-1} soil dry weight h^{-1}) and the rate of denitrification during Phase DEN (D, nmol N g^{-1} soil dry weight h^{-1}) was approximately 5:1 (regression function $D = 0.17 - 0.18^{*}R_{ox}$, Fig. 2). An R_{ox}/D-ratio of 5 would imply that the rate of electron flow to denitrification was around 20% of that to oxic respiration during the foregoing oxic incubation, based on the following: we can assume that the rate of O_2 consumption equals the rate of CO_2-production (mol/mol), thus the production of 1 CO_2-molecule is equivalent to the reduction of 1 O_2-molelule (to H_2O), which consumes 4 electrons. For denitrification, the reduction of 1 NO_3^--molecule to N_2O consumes 4 mol electrons, and if reduced further to N_2 the number of electrons is 5. N_2O amounts to 45–95% of the products for Phase DEN (Fig. 3), thus, the electron flow to denitrification is 4–4.5 mol electrons per mole NO_3 reduced to gaseous products.

This could be taken to indicate that around ~1/5 of the organisms with aerobic respiration in the soils were equipped with a denitrification proteome enabling them to switch instantaneously to anoxic

Table 6
Soil pH measured in 0.01 M $CaCl_2$ (mean ± SD, $n = 4$) in traditional and intensified cultivated sites. The last column shows the difference between Traditional and intensified plots for each contrast. All contrasts between Traditional and Intensified plots are statistically significant ($p < 0.05$).

Land type	Traditional	Intensified	Traditional–Intensified
Khet (C1)	5.42 ± 0.11	4.93 ± 0.16	0.49
Khet (C2)	5.38 ± 0.11	4.60 ± 0.04	0.78
Khet (C3)	4.94 ± 0.05	4.27 ± 0.06	0.67
Bari (C1)	5.64 ± 0.06	4.78 ± 0.09	0.87
Bari (C2)	5.27 ± 0.04	4.62 ± 0.08	0.65
Bari (C3)	4.48 ± 0.06	4.02 ± 0.05	0.47
Bari (C4)	5.05 ± 0.05	4.21 ± 0.06	0.84

respiration (denitrification) in response to oxygen removal. It is remarkable that the average R_{ox}/D-ratio was practically identical for Bari and Khet. The Khet soils have a history of periodic flooding each year (rice paddies), thus long periods with anoxic conditions prevail, whereas Bari soils are well drained most of the year. One would think that the long periods of flooding of the Khet soils would result in a high fraction of cells with denitrification proteome compared to the constantly drained Bari soils, but the experimental results suggest that this is not the case. We also investigated if intensification would stimulate a higher denitrification activity in relation to oxic respiration, but again, the data lend no support to this; R_{ox}/D was essentially unaffected by intensification. In conclusion, denitrification rates during Phase DEN were essentially proportional to the oxic respiration rate prior to anaerobization, and the relationship was unaffected by the hydrological regime (Khet versus Bari) or intensification.

The pairwise comparisons of plots with intensified cultivation history and adjacent plots with a more traditional low-input farming practice shows convincingly that the intensification (higher N inputs, three versus two crops per year) has lowered the pH of the soil substantially. In their analysis of soil acidification of Chinese croplands, Guo et al. (2010) underscored the direct and indirect effect of excessive amounts of nitrogen N as the main driver in acidification, in combination with enhanced extraction of base cations (through crop uptake and removal with harvests). In our case, the latter may be the most important factor, due to the growing of three versus two crops, although nitrogen levels were indeed very high for some of the intensified plots (Table 1). The ΔpH by intensification (net change in pH by intensification) ranged from −0.47 to −0.87 pH-units, and this variable showed no correlation at all with ΔN (= the net difference in annual fertilizer level between Intensified and Traditional). On the contrary, ΔH^+ (the net increase in the concentration of H^+ caused by intensification) declined with ΔN, although this was not statistically significant ($r^2 = 0.37$, $p = 0.08$). The mechanisms of soil acidification by agronomic management appear to be less trivial than commonly assumed. Perhaps it would be well worth to develop new approaches (as suggested for forest soils by Ross et al., 2008), considering the crucial role of pH for the product ratio of denitrification, and for the composition of the soil microbial communities in general (Rousk et al., 2010). Whatever mechanisms are involved in soil acidification, it appears quite clear that the widespread intensification of agriculture which is taking place in south Asian countries (Raut et al., 2011) has a potential to seriously acidify the soil unless counteracted by liming or other amendments that have the same effect.

The $N_2O/(N_2O + N_2)$ product ratio of denitrification during incubation without substrate (Phase DEN) was clearly higher for Intensified than Traditional plots (Fig. 3 and Table S2). The same trend was seen for substrate induced denitrification (Phase SIDEN), although somewhat weaker. The plot of the product ratios against soil pH (Fig. 3) illustrates the consistent effect of intensification both on pH and $N_2O/(N_2O + N_2)$. In isolation, the plot is of course no proof of a causal relationship, i.e. intensification causes higher product ratios due to its acidifying effect. However, there are strong reasons to believe that this is really the case. One is the wealth of empirical evidence that decreasing pH, within the range 7 to 4, directly increases the $N_2O/(N_2+N_2O)$ product ratio of denitrification (Simek and Cooper, 2002; Cuhel and Simek, 2011; Heuvel et al., 2011). Secondly, it has been shown that experimental manipulation of the soil pH in field experiments will give the same effect on the product ratio (Cuhel et al., 2010), as was also observed in long term liming experiments (Liu et al., 2010). Further, the mechanisms involved in the pH-control over the $N_2O/(N_2O + N_2)$ product ratio have been elucidated by experiments with the model organism, *Paracoccus denitrificans*: it appears that the relative activity of N_2O reductase (which determines the product ratio) decreases with pH, not so much because the enzyme is sensitive to low pH, but because low pH interferes with the assembly of the enzyme (Bergaust et al., 2010). Denitrification measurements in combination with quantification of gene transcription in soil incubation experiments suggest that this mechanism is also at work in soils (Liu et al., 2010). Finally, experiments with soil bacteria extracted from soils by density gradient centrifugation show essentially the same phenomena, although modulated by the composition of the denitrifying communities (Dörsch et al., 2012; Braker et al., 2012). In conclusion, there are strong reasons to conclude that soil acidification is the main reason for the observed effect of intensification on the $N_2O/(N_2O + N_2)$ product ratio of denitrification. This conclusion is further strengthened by recent experiments with the same soils, where we measured the immediate response of denitrification to pH adjustments. These experiments showed that the acidified soils performed like the others (in terms of N_2O/N_2 product ratios) when pH was equalized (manuscript in preparation).

For various reasons, the emission of N_2O from a soil is not necessarily proportional with the $N_2O/(N_2O + N_2)$ product ratio as determined in this study. As illustrated in Fig. 1, the accumulation of N_2O is normally a transient phenomenon, and the cumulated N_2O is reduced; at least after depletion of NO_3^- and NO_2^-. This is probably similar to what happens if soils are flooded for long periods: long lasting anoxic conditions and marginal transport of N_2O from the system due to water logging. This would explain why N_2O emissions are generally low from flooded rice fields (Tsuruta et al., 1997), and the main product of denitrification in such systems appear to be N_2 (Mosier et al., 1989). For drained soils, on the other hand, it appears more likely that the emissions of N_2O are in some proportion to the $N_2O/(N_2O + N_2)$ product ratio as measured using our setup, although the ratio may be substantially modulated by oxygen availability (Morley et al., 2008) and nitrate availability (Senbayram et al., 2012). Heuvel et al. (2011) found that soil pH and the product ratio of denitrification in anoxic incubations of soil slurries appeared to be a good predictor of N_2O emissions from riparian soils. We have similarly found that field N_2O emissions from long term liming experiments (pH ranging from 4.2 to 7.5) were essentially proportional to the $N_2O/(N_2O + N_2)$ product ratios as determined by anoxic incubations (Dörsch & Bakken, *unpublished*). Ongoing emission measurements within selected contrasts of Bari and Khet show similar proportionality (Raut et al., *in prep.*). It appears legitimate to claim that although N_2O emissions from soils are modulated by a number of factors, they are likely to be correlated with the product ratio of denitrification as measured here, and hence with soil pH.

In conclusion, the study provides compelling evidence that intensification has resulted in soil acidification, thereby increasing the propensity of the soils to emit N_2O, as predicted by the product stoichiometry of denitrification in standardized anoxic incubations. This implies that the intensification will greatly enhance N_2O emissions, more so than predicted by the IPCC Tier 1 emission factors (IPCC, 2006), which are based on a fixed proportionality between annual N fertilizer rate and N_2O emission. This needs rigorous testing by emission studies in field experiments, considering the potentially grave implications.

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Appendix A. Supplementary material

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.soilbio.2012.06.011.

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