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Advancement of the Acetylene Inhibition Technique Using Time Series Analysis on Air-Dried Floodplain Soils to Quantify Denitrification Potential

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Abstract: Denitrification in floodplain soils is one key process that determines the buffering capacity of riparian zones in terms of diffuse nitrate pollution. One widely used approach to measure the denitrification potential is the acetylene inhibition technique that requires fresh soil samples. We conducted experiments with air-dried soils using a time series analysis to determine the optimal rewetting period. Thus, air-dried soil samples from six different floodplain areas in Germany were rewetted for 1 to 13days to 100% water-filled pore space. We analyzed nitrogen accumulated as N_2O in the top of anaerobic flasks with and without acetylene by gas chromatography after four hours of incubation. We observed an overall optimal rewetting of at least seven days for complete denitrification. We also saw the strong influence of pH and field capacity on the denitrification product ratio; in soils with pH < 7, we hardly assumed complete denitrification, whereas the treatments with pH > 7 achieved stable values after seven days of rewetting. This advanced method provides the opportunity to carry out campaigns with large soil sample sizes on the landscape scale, as samples can be stored dry until measurements are taken.

Keywords: denitrification potential; acetylene inhibition technique; rewetting; floodplain soils; riparian zone; ecosystem services; nitrate pollution; N_2O; N_2

1. Introduction

Floodplains that link the aquatic and terrestrial environment range from low-water to high-water levels and are characterized by floods and changing groundwater levels [1]. They make a significant contribution to the biodiversity of landscapes and also serve as important nutrient traps [2]. Denitrification—a microbial process where nitrate is permanently removed to the atmosphere as N_2 helps to reduce the effects of mineralized nitrogen pollution [3,4]. It has long been considered as the most important process of removing nitrate permanently from soils. Newer studies show that dissimilatory nitrate reduction to ammonium (DNRA) also makes an important contribution to nitrogen removal, although the relationship between the two processes in floodplains remains partially unclear [5–7]. Further it is possible that ammonium produced via DNRA pathways is

reconverted to nitrate via nitrification or even assimilated into biomass [8]. However, the aim of this study is not to quantify all these different pathways but rather assessing the dominant process in floodplain systems. This is why focusing on denitrification in this context is nevertheless useful. Due to changes in the frequency and duration of floods and the pulsing water level, the water regime controls aerobic and anaerobic soil conditions and consequently microbial processes [9]. It is known that extensive drying and rewetting stimulates denitrification loss, which means that management leading to fewer fluctuations in soil water content, could ultimately result in reduced denitrification [10]. However, in the case of an incomplete reaction, nitrous oxide (N_2O), a highly efficient greenhouse gas, is emitted [11]. Various methods are used to measure denitrification, N_2O, and N_2 emissions in terrestrial and aquatic environments. Three of the most commonly used methods are denitrification enzyme activity (DEA) measurements through the acetylene-based inhibition of N_2O reduction with gas chromatography [12]; several methods use ^{15}N determined by isotope-ratio mass spectrometry [13] and the direct quantification of denitrification by measuring N_2 in flow or tight systems [14]. We recommend reading the reviews by Groffman et al. (2006) [15] and Saggar et al. (2013) [16], who presented the available methods for measuring and calculating denitrification, pointing out advantages but also potential problems and limitations in detail. In order to choose the appropriate method, it is important to consider it in the context of its targeted message and its scale and scope. The acetylene inhibition technique (AIT) has numerous limitations leading to bias that has not yet been sufficiently quantified [17–20]. Several reasons, e.g., the incomplete diffusion of acetylene in soil [17] or acetylene consumption by soil microorganisms [21] may be responsible for the incomplete inhibition of N_2O reduction as recently shown by Yu et al., 2010 [22], Qin et al., 2012 [18], and Yuan et al., 2019 [20]. In addition, an inhibition of nitrification, leading to the suppression of NO_3 supply [23,24] is receivable. Due to these limitations, an underestimation of actual soil denitrification potential (SDP) is likely [18,25,26]. Despite the possibility of overestimation, ^{15}N tracer methods with more reliable estimates of denitrification are considered to be more appropriate [16]. However, only the procedures for the direct determination of N_2 have the potential to quantify uncertainties in SDP based on AIT and should be used for correction [18,26]. Moreover, this direct method does not require the addition of an inhibitor or substrate (labeled or otherwise), i.e., no interference is expected with microbial soil processes [16]. However, it does require a complex incubation system and expensive instruments, as well as the ^{15}N tracer techniques, which are not affordable for everyone.

After careful consideration of our objectives in this study, we opted for the technique AIT. These parameters of AIT that are considered to be particularly critical, such as aerobic atmosphere [20], low acetylene concentrations [27], incomplete acetylene diffusion [17,28], and acetylene consumption [29,30] can be adjusted in such a way that underestimation and bias are probably present, but as low as possible: Our experiments were conducted in an anaerobic atmosphere, with 10 vol% acetylene, in shaken soil slurries to minimize diffusion effects and to experience a relatively short-time acetylene exposure to minimize consumption effects. It is also known that bias of AIT-derived SDP is higher in soils with low nutrient content and organic matter [19]. However, due to its simplicity and affordability, it has been proven very useful for comparing soils, ecosystems, and treatments [15,17,31,32], enabling SDP to be estimated at the landscape level. It needs to be mentioned here, that denitrification potentials derived from this method are not suitable for determining actual nitrate removing capacity [12,27]. Moreover, also other nitrate removing processes are not covered in this study. With the main goal to provide data respectively tendencies about N-removal in floodplains the focus of the denitrification as one major process responsible for it, is suitable at this stage since other approaches for quantifying this process at the landscape level are not available.

The common assay for measuring the DEA with the AIT developed by Smith and Tiedje (1979) [33] and modified by Groffman et al. (1999) [12], uses soil slurries in an anaerobic environment under non-limiting carbon and nitrate concentrations and is related to the denitrifier population in soils, reflecting the long-term variations of denitrification-controlling factors [34]. This method requires

"fresh" soil samples and many publications have described the use of this method [11,27,35–38] although many studies were conducted using rewetted "dried" soil samples [10,18,39,40].

Since drying and rewetting exert stress on the bacterial community [41], triggering biochemical processes that lead to pulsing N_2O emissions [42], it seems obvious that experiments with dried soils will influence soil processes such as denitrification [10]. For the comparison and categorization of AIT-derived SDP, it is therefore important to find a standardized procedure that generates stable N_2O values, even for air-dried soils. Preliminary experiments with 24-hour rewetting led us to select a broad and representative set of soil samples for our methodological DEA experiment, in terms of potential SDP. Factors affecting denitrification are divided into proximal and distal regulators: Proximal regulators directly affect the denitrifying community, leading to immediate changes in denitrification rates, while distal factors control the composition of these communities over larger spatial and temporal scales [16]. We opted for a selection based on the distal regulators field capacity (FC) and pH and the proximal regulator mineral nitrogen (Nmin) and applied different rewetting periods to 100% water-filled pore space (WFPS).

We aimed to further develop the AIT for DEA measurements with air-dried soils. In terms of the possible effects of drying and rewetting on the denitrification process, the main objectives were (i) to investigate the influence of different periods of rewetting for air-dried soils and (ii) to assess the influence of different soil characteristics that are known to strongly influence physicochemical properties in soils.

The advanced method offers comparable stable DEA results and the opportunity to carry out campaigns with large soil sample sizes on the landscape level, as samples can be stored dry until measurements are taken. Moreover, it enables the provision of data about denitrification as an ecosystem function in different floodplain areas. The delivery of such information is a key goal for the management of riparian zones [43]. With the help of AIT, we also obtain important information for improving the existing proxy-based approach for the quantification of N-retention through denitrification in the floodplains of Germany.

2. Materials and Methods

2.1. Field Sampling

During July and September 2017, 113 soil samples from 6 different study areas in 4 different floodplains along German rivers (the Rhine, the Main, the Weser, and the Elbe) were collected. Sampling points were selected in the study areas according to a randomized design. For each sampling plot (2 × 2 m), five repetitions (10 cm diameter × 20 cm depth) were carried out using a soil auger. The soil samples that were collected were combined into one mixed sample for further analysis. One part was stored at 4 °C until the soil physical and chemical parameters were measured, whereas the other was air-dried and sieved (2 mm) and stored at room temperature until the DEA measurements were taken. We also characterized the soil types of all plots with a 3 cm diameter and at a depth of 100 cm and the soil density using three repetitions of soil cutter cores (100 cm^3).

2.2. Measurements of Soil Physical and Chemical Parameters

The pH was measured with a glass electrode in a suspension of soil and water with a volume ratio of 1:5 [44]. The measurement of Nmin (the sum of nitrate and ammonium) was conducted by extracting ammonium and nitrate with a 0.0125 molar calcium chloride solution (ratio 1 + 4 (m + V)) and subsequent summation [45]. The particle size distribution of the soil material was determined by sieving and sedimentation [46]. Soil types were defined based on the amount of the fractions of clay, silt, and sand [47]. To calculate the FC from the soil type, soil bulk density and humus content (or total organic carbon (TOC)) were recorded. The soil bulk density was measured by drying and weighing 100 cm^3 samples at 105 °C until a constant weight [48]. TOC was measured by using a suspension

method with 0.22 molar hydrogen chloride (ratio 1:1 (m:V)) [49] and by subsequently multiplying by 1.7 to obtain the humus content [47].

2.3. Measurements of Potential Denitrification Rates and Net N_2O Emission Rates

We slightly modified the AIT standard procedure by Smith and Tiedje (1979) [33] that was modified by Groffman et al. (1999) [12] to determine the soil denitrification potential.

2.3.1. Preincubation

Soil samples were pre-incubated resp. rewetted for 1 day (d), 2d, 3 d, 4d, 5d, 6d, 7d, 9d, and 13d to 100% WFPS and stored at 21 °C in the dark.

2.3.2. Experiment

From the available 113 soil samples, 12 were selected by using 3 parameters that are known to be the most decisive for the development of the denitrifying bacterial community: pH, Nmin, and FC. For each soil sample and each pre-incubation period, six 130 mL flasks were filled with 5 to 8 g of rewetted soil (5 g equivalent of dry soil). For 0 days of rewetting, dry soil was added, after which 5 mL of nutrient solution (final concentrations: 6.8 g/L $C_2H_3NaO_2$and 2.9 g/L KNO_3 equal to 2 mg C g^{-1} dry soil and 0.4 mg N g^{-1} dry soil) were added. By adding NO_3-N and soluble C any temporal changes were eliminated due to differences in sampling time [50] and an upper-bound estimate of in situ denitrification potential was provided [27]. The flasks were sealed with caps (air-tight from butyl rubber septa) that have two hoses with one valve each, followed by 3 minutes of flushing with N_2 while shaking the flask gently to induce anaerobic conditions. Subsequently, in three of the six bottles, 13 mL (10 vol%) of N_2 was removed and replaced by acetylene (C_2H_2) using a syringe. Without the addition of acetylene, it was possible to transform N_2O into N_2, enabling the net N_2O emission rate to be determined, as the presence of acetylene would inhibit the reduction of N_2O to N_2 [51]. After four hours of incubation in the dark at 21 °C, gas samples (20 mL) were taken and transferred into empty vials with butyl septa and crimped caps and stored upside down in water until measurements were taken. Due to this short incubation period, we decided on a one-time rather than hourly sampling, supported by several studies which analyzed a linear increasing N_2O production over periods of up to 8 hours (e.g., [12,18–20,33,39]).

2.3.3. Gas Chromatography

Nitrogen that accumulated as N_2O in the top of all flasks was analyzed by gas chromatography (GC-14B, Shimadzu, Duisburg, Germany) connected to an automatic sample-injection system (APS 96/20-K, ESWE, Gera, Germany). The gas chromatograph was equipped with an electron capture detector and a packed column (1/8" Hayesep-Q, 80/100 mesh, length 4m; Sigma-Aldrich, Darmstadt, Germany). For the N_2O emission rates, the final N_2O concentrations (ppm) were corrected for N_2O dissolved in water (Bunsen coefficient = 0.65 (21 °C)), divided by the incubation time (4 h) and expressed on the basis of dry soil matter (ng N g^{-1} h^{-1}). The potential denitrification rates were calculated as the difference between the net N_2O emission rates in the two different treatments (with and without acetylene).

3. Results

3.1. Selection of Soil Samples for the Time Series Analysis

From all of the 113 samples pH varied between 5.92 and 9.04, whereas Nmin ranged from 0.21 mg/100 g dry matter (DM) to 6.72 mg/100 g DM with a median of 1.72 mg/100 g DM. Both were divided into two classes: pH below and above 7; Nmin below and above 1.72. The subset of the 12 soil samples ranged from a pH of 5.15 to 8.66, whereas Nmin ranged from 0.21 mg/100 g DM to 3.02 mg/100 g DM. Field capacity varied from 20 vol% to 60 vol% and was subdivided into three classes

on the basis of set threshold values of 39 vol% and 48 vol%. As a result, we created a matrix of the 12 selected soil samples based on the 12 possible combinations of these 3 parameters (see Table 1). None of the 113 samples fit into group 11, which is why we investigated two samples from group 12, which was the most highly represented group.

Table 1. Selection of soil sample subset based on the combination of the defined classes and on the parameters: pH, mineral nitrogen (Nmin), field capacity (FC) (pH value into 2 classes: low (pH < 7) and high (pH > 7); Nmin into 2 classes: low (Nmin < 1mg/100g DM) and high (Nmin > 1mg/100g DM); FC into 3 classes: class I—very low/low/medium (FC = 21 vol% < FC ≤ 39 vol%), class II—high (FC = 39 vol% < FC < 48 vol%), class III—very high (FC > 48 vol%). Since there was no soil sample in the subset corresponding to combination 10, two soils from combination 11 were analyzed.

Combination #	Group	Symbol (Figure 1)	pH Value		Mineral Nitrogen Content mg/100 g DM		Field Capacity vol%		River
			Class	Value	Class	Value	Class	Value	
1	I	■	low (pH < 7)	6.53	low (Nmin < 1)	0.206	I	34	Elbe
2		•		5.93		0.451	II	43	Elbe
3		▲		5.50		0.342	III	57	Elbe
4	II	■	Low (pH < 7)	5.15	high (Nmin > 1)	1.974	I	34	Elbe
5		•		5.90		3.024	II	43	Elbe
6		▲		5.86		1.870	III	57	Elbe
7	III	■	high (pH > 7)	8.66	low (Nmin < 1)	0.693	I	20	Rhein
8		•		8.25		0.556	II	43	Rhein
9		▲		8.30		0.648	III	51	Rhein
10	IV	na	high (pH > 7)	na	high (Nmin > 1)	na	I	na	na
11		•		7.92		2.129	II	46	Main
12.1		▲		7.80		2.323	III	49	Weser
12.2		△		7.95		2.636		57	Rhein

3.2. Time Series Analysis—The Effects of Rewetting on DEA (N_2O Emissions in Treatments with Acetylene)

N_2O emission rates in treatments with acetylene (DEA) from soils rewetted directly before incubation (0 days of rewetting) were generally very low, with a mean net N_2O flux rate ranging from 1 to 32 ng N_2O-N g^{-1} h^{-1} in the groups I and II compared to 0–99 ng N_2O-N g^{-1} h^{-1} in the high pH class (groups III and IV). With rewetting we measured increasing N_2O rates in the soils of all groups, but with differently shaped curves over time. In group I and II N_2O emissions continued to rise over the rewetting period in some cases (e.g., #1 and #2), whereas in others they decreased after an initial increase (#3) and in others, we hardly found any N_2O emissions over the entire rewetting period (e.g., #4: mean N_2O emission rate 1.6 ± 0.9 ng N_2O-N g^{-1} h^{-1}). In general, we measured the highest N_2O emission rates of up to 304 ng N_2O-N g^{-1} h^{-1} in those soils with pH < 7, with the highest Nmin and the highest FC (#6).

In group III N_2O emissions were of a similar magnitude to those in groups I and II, but more stable over time.

The three soils from group IV showed obvious peaks after 1 d of rewetting, which were more than three times greater than the highest N_2O emission rates in all other groups (up to 1584 ng N_2O-N g^{-1} h^{-1}), resulting, after a major drop, in stable rates ranging from 701 to 722 ng N_2O-N g^{-1} h^{-1}).

The effects of different periods of rewetting (preincubation) on the emissions of N_2O from the differently treated soils and on the denitrification product ratios calculated from these are shown in Figure 1.

3.3. Time Series Analysis—The Effects of Rewetting on N_2O Emissions in Comparison Between Treatments with and without Acetylene—Denitrification Product Ratio $N_2O/(N_2 + N_2O)$

All soils from the groups I and II showed the same trend: curve progressions with acetylene followed the curves without acetylene, resulting in ratios fluctuating around one (Figure 1).

In contrast, the soils in group III showed relatively constant ratios below one after day 1. The stable mean ratios between rewetting on day 1 and day 13 range from 0.85 ± 0.05 (#8) over 0.48 ± 0.08 (#9) to 0.30 ± 0.08 (#7).

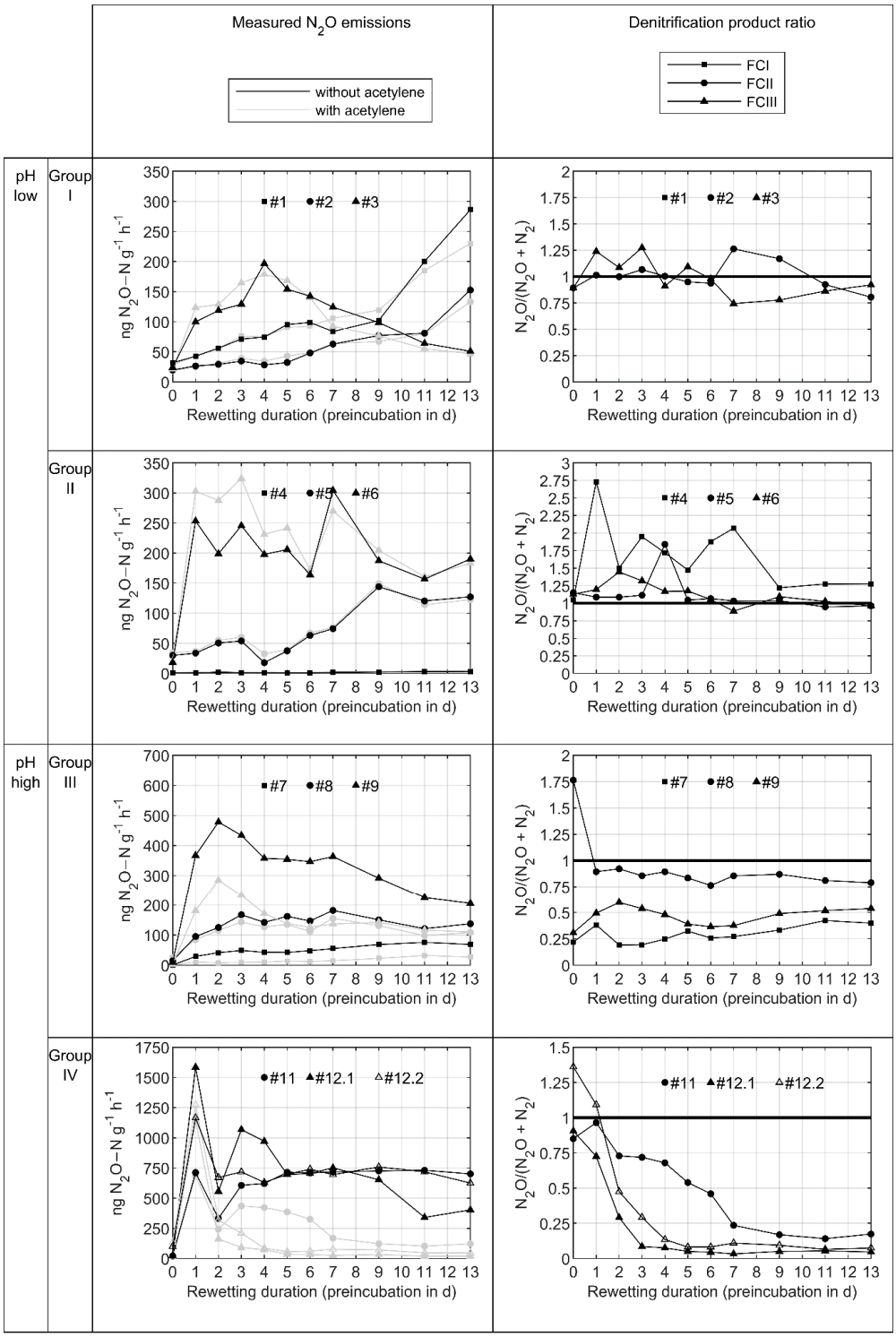

Figure 1. Measured N_2O emissions in treatments with and without acetylene (left) and the denitrification product ratio ($N_2O/(N_2O + N_2)$) calculated from them (right). For a detailed legend see Table 1.

The three soil groups from group IV also showed stable mean ratios after different durations of preincubation ranging from 0.18 ± 0.04 (rewetting day 7 to day 13, #11) over 0.09 ± 0.02 (rewetting day 4 to day 13, #12.2) to 0.05 ± 0.02 (rewetting day 3 to day 13, #12.1).

Figure 2 shows that the median of the $N_2O/(N_2 + N_2O)$-ratio for the entire subset varies between 0.99 (day 1) and 0.80 (day 7) with no clear trend. When we only considered the median of the $N_2O/(N_2 + N_2O)$-ratio of the soil samples from groups III and IV we observed a clear decrease in rates with an increasing rewetting period (up to day 7 = minimum of 0.25), and further on constant medians up to day 13 (day 7–13: mean $N_2O/(N_2 + N_2O)$ of 0.27 ± 0.02).

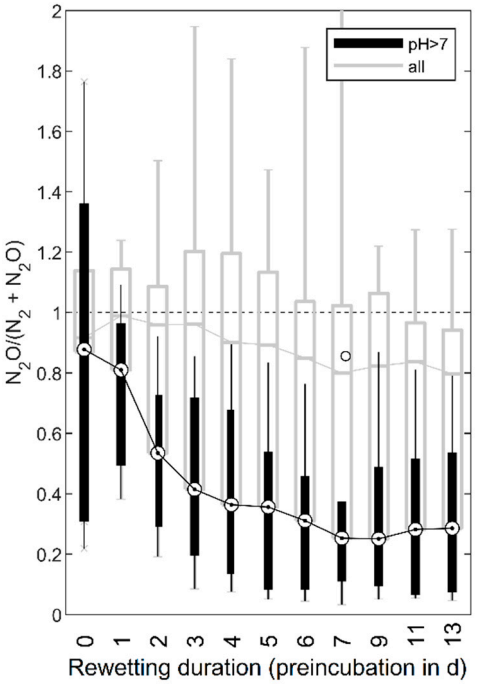

Figure 2. Boxplots of denitrification product ratios ($N_2O/(N_2O + N_2)$) after different periods of rewetting for the entire soil sample subset (grey) compared with the soils from pH class > 7 (black). The boxplots show the inter-quartile-range (box = 25th to 75th percentile with median (50th percentile)), 90th and 10th percentile (upper and lower whisker), and outliers (circle).

3.4. Influence of the Soil Parameters pH, Nmin and FC

The results of the time series reveal that there is a significant influence of pH and FC on the denitrification product ratio $N_2O/(N_2 + N_2O)$ even when all rewetting periods were considered. We found significant differences between the pH class > 7 and < 7 and between the FC class I and III as well as between the FC class II and III. The Nmin-content of the soils had no significant influence on the denitrification product ratio (Table 2).

Table 2. Comparison of the denitrification product ratios ($N_2O/(N_2O + N_2)$) for the rewetting days 0 to 13 of all subdivided classes (pH class (n = 66), Nmin class (n = 66), FC class (n = 66)). Method used: Mann–Whitney U-Test (U). (significance levels $p > 0.05$ (-) = not significant, $p \leq 0.01$ (**) = very significant, $p \leq 0.001$ (***) = highly significant result).

Class		$N_2O/(N_2O + N_2)$ Ratio
pH	pH low/pH high	$U = 4123$, $p < 2.2 \times 10^{-16}$ (***)
mineral nitrogen content	N_{min} high/N_{min} low	$U = 2042$, $p < 0.5374$ (-)
field capacity	FC I/FC II	$U = 798$, $p < 0.4648$ (-)
	FC I/FC III	$U = 1208$, $p < 0.009702$ (**)
	FC II/FC III	$U = 1583$, $p < 0.008785$ (**)

4. Discussion

4.1. Denitrification Product Ratio: $N_2O/(N_2O + N_2)$

The applied method to measure denitrification is based on the ability of acetylene (C_2H_2) to inhibit the reduction of N_2O to N_2 [15]. However, higher denitrification rates do not necessarily lead to higher N_2O emissions, since increased denitrification can also change the proportion of N_2O between the two gaseous products N_2O and N_2 [52]. The ratio between N_2O emissions in treatments without acetylene and N_2O emissions in treatments with acetylene (DEA) is interpreted as the denitrification product ratio $N_2O/(N_2 + N_2O)$ respectively DEA_{N2O}/DEA [53]. If this product ratio is used as a measure for the optimal rewetting period for denitrification, the lowest ratio must be considered, as it covers the potential N_2 emissions from the ratio between N_2O and $(N_2 + N_2O)$: the lower the ratio, the greater the proportion of N_2 in the denitrification product $N_2 + N_2O$. A ratio above 1 is theoretically impossible. However, ratios above one can be found if measurements are inaccurate or analytical replicates are averaged or measured denitrification values are very low, and thus the calculation shows artifacts. In this case, instead of calculating the ratio, it would be reasonable to describe N_2 as the difference between N_2O emissions with and without the acetylene treatment [17,54,55]. However, in our study, this case of very low values only occurred once (#4). Since elevated N_2O fluxes were observed in all other situations, it makes sense to include a scaling factor by calculation of the ratio [17].

Because N_2O can be produced both in the autotrophic nitrification and the heterotrophic denitrification process [56], additional ratios above one can be found if the environment is not completely oxygen-free during incubation. The amount of N_2O produced through nitrification increases with decreasing soil oxygen concentrations [57]. In addition to the O_2 partial pressure in the gas phase, the availability of O_2 in soil is controlled by moisture content [58,59]. Studies have shown an increased denitrification rate through restricted soil aeration linked with high soil water content (e.g., Hefting et al. (2004) [9]). For this reason, we pre-incubated the soil sample subset to 100% WFPS over different periods of duration to obtain anaerobic conditions and to favor N_2O release through denitrification [59]. in addition to these, other processes such as nitrifier denitrification, heterotrophic nitrification (fungi and bacteria), and DNRA can also produce N_2O [60]. Especially nitrifier denitrification could be an undetected contributor to measured N_2O emissions, since Wrage et al., (2004) [21] showed that C_2H_2 inhibited N_2O production by *Nitrosomonas europaea* but it did not affect the N_2O production in *Nitrosomonas briensis.* Kool et al., (2010) [60] conjecture that lower pH might favor nitrifier denitrification, which could be the explanation of ratios above one. To verify the importance of this process dual-isotope measurements with ^{15}N and ^{18}O should be done because only this could prove the presence of nitrifier denitrification in soils [60]. Following studies should examine the relationship between N_2O and changing nitrate levels during incubation for better interpretation of the results.

4.2. The Influence of Rewetting

In addition to the described effect of missing O_2 availability through saturation, rewetting causes enhanced organic matter mineralization [61,62]. Many studies have shown pulses of N_2O emission following the rewetting of dried soils [10,42,63,64]. As expected, in both treatments we found an increase in N_2O emission after rewetting with short time (1d) N_2O emission peaks in the entire subset, but especially for group 4 (Figure 1). However, to establish a standardized procedure, we required a time series analysis to find the optimal duration of rewetting for measuring potential denitrification. To achieve this, we therefore searched for the lowest (< 1) and most stable denitrification product ratios.

Based on the results obtained showing that soils of both acetylene treatments in the pH class < 7 have very similar N_2O emission curve progressions (Figure 1), we assume that in this instance the complete denitrification process (up to N_2) rarely takes place, resulting in high $N_2O/(N_2 + N_2O)$ ratios of around one. This means that for this group we were not able to analyze the effect of different rewetting durations on denitrification. Furthermore, we found that soils in the pH class > 7 showed

constant ratios, even if they occurred in different groups after different periods of rewetting (Figure 1). The analysis of those constant ratios showed that one should rewet at least seven days to obtain the most stable denitrification product ratios. These findings are in line with, e.g., Qin et al. (2013) [19] and D'Haene et al. (2003) [39]. In both studies air-dried soil samples were pre-incubated for seven days before conducting denitrification experiments, although fresh field samples are a prerequisite for the AIT.

In addition to the achieved goal of finding an optimal rewetting duration, this also allows assumptions to be made about the influence of potential flooding dynamics in the field. For example, the observed decrease in the $N_2O/(N_2O + N_2)$ ratios for the class pH > 7 suggests that floodplain soils must be flooded for at least seven days after a dry period in order to achieve a maximum N_2 emission through denitrification and to enable a more complete denitrification process. However, for the general activation of denitrifying bacteria, a short flood of one day would already be sufficient, as shown by the measured DEA peaks (see N_2O emission with acetylene in Figure 1).

Furthermore, Ruser et al. (2006) [64] and Yanai et al. (2010) [63] described the stimulating effects of rewetting. Nevertheless, a general decrease in the activity of microorganisms through dried storage is also likely [41]. This was not the aim of this study, however, because AIT-derived SDP results (even with fresh soils) can only be poorly related to actual denitrification activity [12].

4.3. Influence of the Soil Parameters pH, Nmin, and FC

Despite the ambiguous relationship between pH and DEA [65,66], it is generally accepted that lower soil pH results in higher denitrification product ratios [67]. We were able to prove that denitrification in acidic soils resulted in more N_2O, leading to increased $N_2O/(N_2 + N_2O)$ ratios when compared with neutral or slightly alkaline soils [66,68,69]. A synopsis of various studies across different soil types shows that there may be a negative exponential correlation between ratio and pH [70]. The control mechanisms behind this are not fully understood. However, the following can be considered: sensitive bacterial denitrification enzymes (Nos) [67], pH-based fungi dominance resulting in a lack of Nos, favored N_2O production by nitrifiers in acidic soils, and enhanced abiotic transformations of NO_3 [16].

Moreover, we assumed the proximal regulator Nmin (especially in the form of NO_3) as one of the factors limiting denitrification, to be essential for the formation of a denitrifying bacterial community. Many studies found this positive relationship between the denitrification rate and NO_3 concentrations (e.g., Zhong et al., 2010 [71]). However, this only applies up to a certain value: more than 50 µg NO_3-N per g DM may be harmful to denitrification activity [72]. Due to the suppression of Nos, higher NO_3 concentration usually results in higher denitrification product ratios [73], because reducing NO_3 is a priority in terms of energy compared to reducing NO_2 [16]. We were not able to confirm this with our experiment as we did not find any significant differences between the high and low Nmin class in the $N_2O/(N_2O + N_2)$ ratios. It is more likely to be a question of the nitrate availability during the reaction. Nmin as an influencing variable would only become apparent if it had previously affected the soil since NO_3 was added in excess during the experiment. N was added at 0.4 mg/g DM while the original Nmin soil content was between 0.002 and 0.067 mg/g DM, resulting in more or less equal N content in all samples and thus an overlay of the effect.

It is also known that denitrification increases with increasing fineness of the soil texture [39]. Therefore, FC, which depends on grain size distribution, soil structure, and soil organic matter content, was used as the third parameter for selection. Soils with a finer texture and thus a higher FC have a greater and longer water storage capacity, which in turn influences nutrient cycling within soil microsites [74]. Changes found with increased WFPS in denitrification product ratios also depend on the ability of the denitrifiers [75]. It can therefore be assumed that these kinds of soils are prone to high denitrification rates compared to coarser substrates. We were also able to partially observe this relationship in our experiment. We found significant differences between the $N_2O/(N_2 + N_2O)$ ratio of soils with a very high FC (FC class III) and the other two classes (FC class I and II). It should be noted, however, that the well-known relationship of increasing denitrification rates with increasing water content [76,77] is not fully represented here because all soils were rewetted to 100% WFPS.

Nevertheless, we can still confirm the findings of D'Haene et al. (2003) [39], namely that despite the same water content while rewetting, the denitrification potential is still strongly dependent on the soil texture.

In this context carbon availability, temperature, and the availability of trace metals, to name but a few, also affect denitrification. In short, we can confirm the known influences of the known parameters on the SDP. The aim of this study, however, was not to identify factors that influence the SDP. Known factors influencing the SDP were used to select a broad and representative set of soil samples despite the diversity of soils in order to determine a standard rewetting period that could be applied to the overall data set in the future. Although this was achieved, the limitations imposed by the relationships that were investigated between soil properties and distortions of the method must be taken into account: Essentially, the applicability of the AIT is given in soils with high or moderately nutrient content, since bias is low there [19]. Furthermore, high silt, clay, and low sand contents reduce these errors [19]. In our total data set soils will consist mainly of clays, silts, and loams, and nutrients are added before the four-hour incubation, relatively small amounts of distortion can be expected. Additionally, they are also more likely to occur in the sense of underestimation, as studies comparing the AIT with N_2:Ar ratio methods with membrane inlet mass spectrometry (MIMS) and direct N_2 measurements have shown [18,26]. To achieve better reliability, AIT's SDPs, whether from fresh or rewetted samples, should undergo the application of a correction coefficient derived from other mentioned methods [18,26].

5. Conclusions

When determining the denitrification potential of air-dried soil samples using AIT, we can make the following recommendations. In order to assess nitrate reduction by denitrification in general, it is sufficient to consider the N_2O emissions alone. For this purpose, we recommend a rewetting time of one day or a further time series analysis over 24 hours after rewetting. For the latter, as our results showed a high microbial activity in the early phase of rewetting, a higher resolution of the data is required for accurate statements.

However, this study aimed in finding an optimal time period for the rewetting of air-dried soils, which ideally leads to complete denitrification in laboratory experiments. Thus, the largest possible proportion of N_2 was of main interest here and we can recommend at least seven days of rewetting as the lowest stable calculated denitrification product ratios occurred after this period. This also means that active soils with high N_2O emissions would be completely ignored in terms of their denitrification potential, even though they can contribute to nitrate reduction, even if it is only up to N_2O. This should be taken into account when using the ratio of N_2O emissions with and without acetylene as a measure of the denitrification potential, especially for soils with low pH values.

The influence of the previous drying could not be determined and should be investigated in further experiments. However, even with soil samples that are fresh from the field, it is only the denitrification potential and not the actual denitrification rates that can be measured using this method.

Differences due to soil parameters such as pH and FC were also clearly visible in the results, and therefore this method is very promising for the comparison concerning denitrification of soils and ecosystems and for use in the next step to studying all 113 soil samples. When applying the method, in the context of N-retention in floodplains, it remains to be noted that other nitrate-removing processes, such as DNRA, are not quantified. The proportion of these processes can be very high under certain circumstances [5]. Whether or not these are given for our study is difficult to predict, as freshwater ecosystem DNRA hotspots reported so far described sediments of riparian zones [5].

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