N$_2$O-reducing activity of soil amended with organic and inorganic enrichments under flooded conditions

Alicja Księżopolska[1]*, Teresa Włodarczyk[2], Małgorzata Brzezińska[2], Paweł Szarlip[2], Marek Pazur[2]

[1]The John Paul II Catholic University of Lublin/Off-Campus Faculty of Social Sciences, ul. Ofiar Katynia 6a – 37-450 – Stalowa Wola – Poland.

[2]Institute of Agrophysics, Polish Academy of Sciences – Dept. of Natural Environment Biogeochemistry, ul. Doświadczalna 4 – 20-290 – Lublin – Poland.

*Corresponding author <ala.ksiezopolska@gmail.com>

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ABSTRACT: Changes, apparent after investigation, in the physical and chemical properties in soil, as a result of organic and inorganic enrichments under flooded conditions, influence the growth of denitrifiers. The aim of this study was to determine the effect of the addition of manure (8 kg m^{-2}) (M), clay (50 kg m^{-2}) (CL) and lime (1.12 kg m^{-2}) (Ca) on the N$_2$O-reducing activity (N$_2$O-RA) of sandy loam soil (clay content - 24 % in 0-20 cm), during NO$_3$ reduction under flooding. The soil samples were taken from field plots after 3 years of enrichment with grass cultivation. The enrichments had a distinct effect on the N$_2$O-RA and N$_2$O-released, due to the change in pH, the porosity, and the sorptive properties of the soil. The pH had the greatest impact on the N$_2$O-RA of the soil and ranged from 4.9 to 7.6. For actual denitrification to N$_2$O-realized (aD-N$_2$O), the maximum N$_2$O-releasing (mcN$_2$O-releasing) followed the order: 1.36 for the M-treatment, 6.39 for the M+CL+Ca-treatment, 7.79 for the c-soil and 8.69 N$_2$O-N mg kg^{-1} for the M+CL-treatment. For actual denitrification (aD), the mcN$_2$O-releasing was followed the order: 10.37 for the M-treatment, 10.49 for the control soil, 14.60 for the M+CL+Ca-treatment and 20.00 N$_2$O-N mg kg^{-1} for the M+CL-treatment. The N$_2$O-RA of the soil samples increased as pH increased. The average N$_2$O/N$_2$+N$_2$O ratio and the N$_2$O-RA of the soil samples increased in the following order: M+CL, control soil, M+CL+Ca, M-enrichments. The addition of enrichments did not pose a threat to the environment due to increased N$_2$O emissions, but as regards conserving NO$_3^-$ in the soil, the addition of clay distinctly increased the complete denitrification process.

Keywords: N$_2$O/N$_2$+N$_2$O ratio, clay and Ca-enrichments, manure, actual denitrification

Introduction

The rate of denitrification and the relative proportions for NO, N$_2$O, and N$_2$ produced depend on the complex interactions between soil properties, soil microorganisms, climatic factors and management practices (Bieganowski et al., 2013; Franklin et al., 2015; Liu et al., 2010; Stępniewski and Stępniewska, 2009; Włodarczyk et al., 2014). From an environmental perspective, a very important result is the N$_2$O/N$_2$+N$_2$O ratio, which shows the N$_2$O-reducing activity of soil (Yanai et al., 2008). The NO$_3$ concentration is one of the key factors that influence the N$_2$O/N$_2$ ratio of denitrification (Saggar et al., 2013; Weier et al., 1993; Zaman et al., 2007). Increasing the availability of C is considered to decrease the ratio of N$_2$O/N$_2$ (Dendooven et al., 1998; Saggar et al., 2013; Smith and Tiedje, 1979). In anaerobic zones of fertilized soil, the NO$_3$ concentration controls the denitrification product ratio (N$_2$O/N$_2$). Vallejo et al. (2006) noticed a decrease in N$_2$O/N$_2$, with increasing concentrations of dissolved organic carbon (DOC), in various sources of effluents. The denitrification can also alter the way in which N$_2$O is shared among the two gaseous products, N$_2$O and N$_2$ product ratio (Senbayram et al., 2012). The next environmental factor that has an impact on the ratio of denitrification products (N$_2$O and N$_2$) is the pH. Šimek and Cooper (2002) found that, in soil samples with higher pH values, the activity and synthesis of N$_2$O reductase is supported – more N$_2$ is produced and the N$_2$O/N$_2$+N$_2$O

ratio decreases. Cavigelli and Robertson (2000) showed that denitrification reductases and consequently the N$_2$O-reducing activity of two soil samples differed in their sensitivity to pH and that the communities responded differently to changes in pH. The destructive function of N$_2$O and the positive influence of manure (M), clay (CL) and lime (Ca) on soil mechanical properties of light textured soils, has led us to study the relationships between the N$_2$O-reducing activity (N$_2$O-RA) of soil and the added treatments. In terms of periodic flooding of cultivated fields, little is known about the relationship between N$_2$O-RA and soil in the presence of M, CL and Ca treatments. We hypothesized that, M, CL and Ca change the physical and chemical properties of soil and affect the N$_2$O-RA in flooded soils. The main aim was to determine the effect of M, CL and Ca on the ability of soil to reduce N$_2$O to N$_2$, during NO$_3$ reduction under flooding. The specific aim was to determine both the complete actual denitrification and the incomplete actual denitrification.

Abbreviations

mcN$_2$O-releasing: maximum cumulative N$_2$O released.
aD-N$_2$O: incomplete actual denitrification, leading to N$_2$O formation.
aD: complete actual denitrification.
N$_2$O-RA: N$_2$O-reducing activity of soil.
c: control soil.
M-treatment: soil with manure addition.

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M+CL-treatment: soil with manure and clay addition.
M+CL+Ca-treatment: soil with manure, clay and lime addition.
C$_{org}$: organic carbon.

Materials and Methods

Field experiment with manure, clay and Ca enrichments

The field plots were located in Boniewo (18°54′ E; 52°28′ N; 211 m asl) near Lublin (Poland). They were established on sandy loam texture (Haplic Luvisol), with a 47 % sand content, and a pH in 1 mol L^{-1} KCl 4.46 and 4.7 g kg^{-1} of organic C (C$_{org}$) (Table 1). Haplic Luvisols (according to the FAO, 1998) make up the majority of field cultures in Poland. Enrichments and their doses on 2 m^2 field plots were as follows: (i) No treatments (untreated soil as control). (ii) Soil + manure (8 kg m^{-2}). (iii) Soil + manure (8 kg m^{-2}) + clay (50 kg m^{-2}). (iv) Soil + manure (8 kg m^{-2}) + clay (50 kg m^{-2}) + post flotation lime (1.12 kg m^{-2}).

The enrichments were mixed with soil to a depth of 20 cm. All field plots were covered with the grass, *Dactylis glomerata* (L.).

Cattle manure (M) was taken from an agricultural farm and added to three field plots, except the control. In dry mass, the M contained: 0.2-0.25 g kg^{-1} N, 0.06 g kg^{-1} P, 0.28 g kg^{-1} K and 0.08 g kg^{-1} Mg. The clay (CL) came from the "Jeziórko" sulfur mine near Tarnobrzeg (21°41′ E; 50°35′ N), where it was being used for improving the soil around the area of the mine. The final clay content increased from 24 to about 30 % after the enrichment.

Post flotation lime contained 2.6 to 3 g kg^{-1} Ca, and was a waste product of the processing of sulfur. All of the enrichments were applied at the beginning of the experiment.

The soil samples were taken 3 years after adding enrichments to the soil. From the upper horizons (0-20 cm) of the four field plots (control, M, M+CL and M+CL+Ca) the soil was taken from five sites to the experiment's laboratory and thoroughly mixed.

Determination of the chemical and physical attributes of the soil

The particle size distribution was determined by the sedimentation method and the distribution of particle sizes was expressed as a percentage (Öhlinger, 1995). The determination of the rest of the soil properties included C$_{org}$ (Tiurin's method) and the pH was determined in an aqueous suspension of soil (v:v = 1:1) using a pH-meter. The initial NO$_3^-$ and NH$_4^+$ content in the soil solution were determined in 5 g of air-dried soil, suspended in 105 mL of 0.0125 mol L^{-1} CaCl$_2$. The suspension was shaken for 2 h and the filtered solution of NO$_3$ and NH$_4^+$ ions were determined using a flow spectrophotometer (Table 2). After analyzing the gases, the final NO$_3^-$ and NH$_4^+$ content were measured in the soil suspensions. The soil suspensions were quantitatively transferred from glass bottles into plastic flasks, shaken for 2 h with 105 mL of 0.0125 mol L^{-1} CaCl$_2$ and then filtered through filter paper. After this, the extracts were analyzed for NO$_3^-$-N and NH$_4^+$-N content using the same analyzer (Włodarczyk et al., 2011). The sorption capacity of soil (T) was determined by Kappen's method. Sorption capacity of soils was calculated as follows: T = S + H$_h$ mmol$_{c+}$ kg^{-1} of soil, where S - the sum of metal cations of an alkaline; H$_h$ - hydrogen ions contained in the sorption complex (hydrolytic acidity) (Turski, 2008).

Denitrification measurements

The 5 g of each soil portion (in three replications) was placed into (60 mL) glass bottles with deionized water. Taking into account the starting soil moisture, the soil/water ratio was 1:1 (w:w) with 0.5 cm of stagnant water on top of the soil surface. The vessels containing the soil and water were tightly sealed with rubber stoppers and incubated. The incubation was performed with 10 % v:v C$_2$H$_2$ block and without C$_2$H$_2$ block. C$_2$H$_2$ can inhibit the reduction of N$_2$O to N$_2$. When C$_2$H$_2$ is not added, the N$_2$O gas is free to transform into N$_2$, allowing for the determination of the net N$_2$O production rate (Xu et al., 2008). Paraffin films were used on the stoppers to ensure hermetic seals. The headspace gas was sampled

Table 1 – The initial characteristic of soil.

Soil depth	Topography	Soli type (texture)	Particle size distribution (%)			pH		Corg	N	C/N	NO$_3^-$	NH$_4^+$
			> 0.05	0.05-0.002	< 0.002	H$_2$O	KCl					
cm				mm				——— g kg^{-1} ———			——— mg kg^{-1} ———	
0-20	Pain	sandy loam	47	29	24	5.19	4.46	4.7	0.56	8.3	36.9	17.7

Table 2 – The initial (i) and final (f) characteristics of soil.

Enrichments	Corg (f)	Corg (f)		pH (f)		NO$_3^-$(i)	NO$_3^-$(f)	NO$_3^-$(f)	NH$_4^+$(i)	NH$_4^+$(f)	NH$_4^+$(f)
		Oxidable carbon	Non oxidable carbon	H$_2$O	KCl		without C$_2$H$_2$	with C$_2$H$_2$		without C$_2$H$_2$	with C$_2$H$_2$
	g kg^{-1}	——— % of Corg content ———				——————————————— mg kg^{-1} ———————————————					
control	5.7	44.7	55.3	4.90	4.00	36.9	33.9	16.1	17.7	20.7	34.0
Manure	6.7	40.5	59.5	5.50	4.56	27.8	27.1	8.0	15.8	19.0	31.2
Manure+Clay	5.9	42.9	57.1	7.10	6.50	21.0	8.2	0.0	17.9	21.6	36.5
Manure+Clay+Ca	5.2	40.7	59.3	7.60	7.20	15.8	2.9	0.0	21.2	20.0	33.7

through the stopper with a gas tight syringe and the soil samples in three replications were incubated at 20 °C for 7 days (Włodarczyk et al., 2005).

After one, two, three and seven days of incubation, the concentration of N_2O in the headspace gas was determined using a gas chromatograph. This was fitted with an electron capture detector (ECD) at 300 °C. The gas components were separated on a 2 m long column, which was packed with porous polymer composed of ethylvynilbenzene and divynilbenzene (Porapak Q) at 80 °C. The carrier gas was He, at a flow rate of 40 mL min⁻¹. The temperature of the injector was 120 °C. The concentration of N_2O-N was corrected for the gas dissolved in water, values taken from the literature for the Bunsen absorption coefficients (Gliński and Stępniewski, 1985). The results were calculated in terms of kg of dry soil. The amount of N_2O released (incomplete denitrification - aD-N_2O) and the sum of N_2O and N_2 (complete denitrification - aD) was determined during the seven days of incubation, using a cumulative N_2O release curve. In principle, the processes of N_2O production and consumption occur simultaneously, with a prevalence of production before the maximum N_2O value and a prevalence of consumption after the maximum of production. In our study, the predominant process of N_2O production was expressed as the maximum cumulative amount of N_2O-N mg kg⁻¹ soil over the seven days of incubation (mcN₂O-releasing) or as the amount on the day before the consumption of N_2O. The average mcN₂O-releasing was calculated from the maximum cumulative amount released in the subsequent days of incubation and all of the enrichments were taken together.

For each day of incubation, the N_2O/N_2+N_2O ratio was calculated as follows: maximum cumulative N_2O release (treatment without C_2H_2) divided by the cumulative amount of N_2+N_2O (treatment with C_2H_2 addition). The mean value for N_2O/N_2+N_2O ratio was calculated as follows: the mean value of cumulative N_2O release from each day of incubation for all of the enrichments taken together (treatment without C_2H_2), divided by the amount of N_2+N_2O (treatment with C_2H_2 addition).

The treatments were divided into two groups in order to determine the incomplete actual denitrification, leading to N_2O formation and the complete actual denitrification. The soil samples were prepared according to the following scheme:

I – soil without the addition of C_2H_2 – corresponding to actual denitrification, leading to N_2O formation (aD-N_2O) – incomplete denitrification.

II – soil with the addition of C_2H_2 – corresponding to actual denitrification (aD) – complete denitrification.

The aD-N_2O and aD was analyzed in the c soil: (treatments I-II). This was done by an analogy of the soil with M-treatment, M+CL-treatment and M+CL+Ca-treatment. The additives used in the different treatments

(I–II) determined the type of denitrification (incomplete and complete).

Therefore, the study included the following measurements: (i) aD-N_2O, (ii) aD and (iii) the content of native nitrate (NO_3^-) and ammonium (NH_4^+).

Statistical data analysis

The results were subjected to statistical analysis. The linear (y = a + bx) and logarithmic (y = alnx + b) models were used in the regression analysis and, in each case, the model with the highest R^2 was selected as the best fit for the experimental data. A one-way analysis of variance (LSD test) was used to test the significance of the effect of the soil enrichments (M, CL and Ca), the treatment (C_2H_2) and the days of incubation on N_2O release. The mean values for cumulative N_2O release were calculated based on twelve replicates for each of the enrichments and one treatment (treatments I–II separately) from each day. The least significant differences (LSD) at $p < 0.05$ were calculated following an analysis of variance (ANOVA).

Results

Chemical and physical attributes

The basic soil characteristics are presented in Table 1. The Haplic Luvisol was formed from sandy loam silt. The soil particles were 47 % sand, 29 % silt and 24 % clay. The final clay content was about 30 % by addition of clay. The control soil used for the experiment had an acidic pH in H_2O (5.19), a relatively high content of NO_3^- (36.9 mg kg⁻¹) and NH_4^+ (17.7 mg kg⁻¹) and a low C_{org} content (4.7 g kg⁻¹). The basic initial (i) and final (f) soil characteristics are presented in Table 2. Generally, C_{org} content increased during the final stage of the field experiment, compared to the initial values (4.7 g kg⁻¹) and ranged from 5.2 g kg⁻¹ (M+CL+Ca-treatment; $p < 0.01$) to 6.7 g kg⁻¹ (M-treatment; $p < 0.001$). The pH slightly decreased in the c-soil (from 5.19 to 4.9; $p > 0.05$), increased in the M-treatment (from 5.19 to 5.50; $p < 0.05$) and clearly increased in the M+CL-treatment (from 5.19 to 7.10; $p < 0.001$) and the M+CL+Ca-treatment from acid reaction (5.19) to alkaline reaction (7.60; $p < 0.001$). The initial content of NO_3 in the enriched soil was lower, compared to the control soil and ranged from 27.8 to 15.8 mg kg⁻¹ of soil (p value ranged from 0.05 to 0.001). The initial content of NH_4^+ in the enriched soil ranged from 15.8 to 21.2 mg kg⁻¹ of soil (p value ranged from 0.05 to 0.01) and was close to the value of the control soil except that of the M+CL+Ca-treatment (Table 2). In the case of M+CL-treatment there were no significant differences ($p > 0.05$).

The sorption capacity of the soil ranged from 32.0 to 33.8 mmol$_C^+$ kg⁻¹ of soil (for lower pH), and from 49.8 to 67.3 mmol$_{C+}$ kg⁻¹ of soil (for higher pH). The sorption capacity was significantly higher in soils with higher pH as compared to soil with lower pH ($p < 0.001$). The total porosity of soil for a lower and higher pH ranged from

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39 to 40 and 42 to 45 % (v:v), respectively which was significantly higher in soils with higher pH as compared to soil with lower pH ($p < 0.01$).

Incomplete denitrification (aD-N₂O) under flooding soil conditions

The release of N_2O increased on the third day of incubation. It then decreased in three cases of the enriched soil samples, except in the c-soil (Figure 1A). This was due to the consumption of N_2O produced and then released into the headspace.

The incomplete mcN_2O-releasing for the c-soil was 7.79 N_2O-N mg kg^{-1}, which was 21 % of the natural NO_3^- (Table 3).

The addition of M resulted in a distinct decrease ($p < 0.001$) in aD-N_2O, compared to the c-soil. The mcN_2O-releasing for the M-treatment was 1.36 N_2O-N mg kg^{-1} and represented 17 % of the c-soil (Figure 2). The percent of denitrified NO_3^- leading to the formation of N_2O, was 5 % of the natural content (Table 3).

Compared to the c-soil, the addition of M+CL to the soil slightly increased aD-N_2O ($p > 0.05$). The mcN_2O-releasing for the M+CL-treatment was 8.69 N_2O-N mg kg^{-1}, which was 41 % of the natural NO_3 (Table 3). Furthermore, it was 1.1 times higher than the c-soil and 6.4-fold higher than the M-treatment ($p < 0.001$) soil (Figure 2).

The addition of M+CL+Ca to the soil decreased aD-N_2O ($p < 0.05$), compared to the c-soil. The mcN_2O-releasing for the M+CL+Ca-treatment soil was 6.39 N_2O-N mg kg^{-1}, which was 41 % of the natural NO_3 (Table 3). This was 1.2 times lower than the c-soil, 4.8-fold higher than the M-treatment soil ($p < 0.001$) and 1.4 times lower than the M+CL-treatment ($p < 0.01$) soil (Figure 2).

The highest consumption of N_2O was in the M+CL+Ca-treatment soil (28 % released N_2O). The lowest consumption was in the M-treatment soil (17 % released N_2O). Slightly higher consumption was found in the M+CL-treatment soil (19 % released N_2O) (Table 3).

At the beginning of incubation, the natural content of NO_3^- ranging from 15.8 to 36.9 mg kg^{-1} (Table 3), depending on the enrichments. The natural content of NO_3^- decreased as a function of the increased enrichment of the soil. The highest content was in the c-soil and the lowest in the M+CL+Ca-treatment soil. There was no correlation between the natural NO_3^- content and the mcN_2O-releasing for aD-N_2O and aD.

Complete denitrification (aD) under flooding soil conditions

The release of N_2O increased on the seventh day of incubation with different activity (Figure 1B). There was no consumption by reason of the C_2H_2 addition.
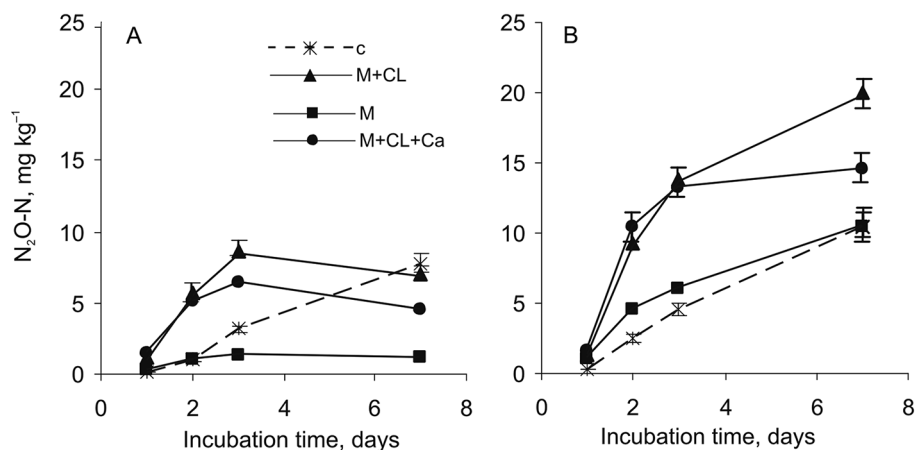

Figure 1 – Dynamic of actual denitrification leading to N₂O release (aD-N₂O) (A) and actual denitrification (aD) (B) as a function of time of incubation depending on the type of enrichments: c = control soil; M = manure enrichment; M+CL = manure and clay enrichments; M+CL+Ca = manure, clay and lime enrichments.

Table 3 – Natural NO₃⁻ content, maximum N₂O release, percent of NO₃⁻ denitrified and N₂O consumption depending on type of enrichment.

Enrichments	NO₃⁻ content	Maximum N₂O release		NO₃⁻ denitrified		N₂O consumed
		Without C₂H₂	With C₂H₂	Without C₂H₂	With C₂H₂	Without C₂H₂
		mg kg⁻¹		——————————— % ———————————		
control	36.9	7.79	10.49	21.1	28.5	0.0
Manure	27.8	1.36	10.37	4.9	37.3	16.6
Manure+Clay	21.0	8.69	20.00	41.4	95.1	18.6
Manure+Clay+Ca	15.8	6.39	14.60	40.5	92.6	28.4

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The complete mcN$_2$O-releasing for the c-soil was 10.5 N$_2$O-N mg kg^{-1}, which was 29 % of the natural NO$_3^-$ (Table 3).

The complete mcN$_2$O-releasing for the M-treatment soil was nearly the same as the c-soil (Figure 1B) and was 10.4 N$_2$O-N mg kg^{-1} ($p > 0.05$). The percentage of the denitrified NO$_3$, leading to complete denitrification, was 37 % of the natural content (Table 3).

Much higher mcN$_2$O-releasing values for the complete aD were found in the M+CL-treatment soil (20.0 mg N$_2$O-N kg^{-1}), which was 95 % of the natural NO$_3^-$ (Table 3) and higher (about two times higher) than the c-soil and M-treatment ($p < 0.001$) soil (Figure 2).

The addition of M+CL+Ca to the soil increased (1.4 times) the mcN$_2$O-releasing aD, compared to the c-soil and M-treatment ($p < 0.01$) soil (Figure 2). Furthermore, it decreased (1.4 times) compared to the M+CL-treatment ($p < 0.01$) soil (Figure 2). The mcN$_2$O-releasing for the M+CL+Ca-treatment soil was 14.6 N$_2$O-N mg kg^{-1}. The percentage of denitrified NO$_3$, leading to complete denitrification, was 93 % of the natural content (Table 3).

The regression analysis for aD-N$_2$O and aD in the c-soil showed a positive linear relationship after seven days of incubation ($p < 0.001$). The statistical analysis for aD-N$_2$O in the enriched soil, showed a positive non-linear relationship with the first three days of incubation (when the production and release of N$_2$O enabled consumption) ($p < 0.001$), and after seven days of incubation ($p < 0.001$) for aD.

Incomplete denitrification (aD-N$_2$O), complete denitrification (aD) and N$_2$O-reducing activity of soil as a function of soil pH

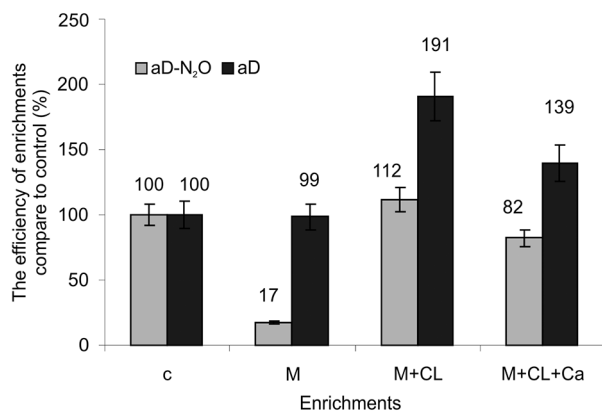In the current study, the addition of M-, M+CL- and M+CL+Ca-treatments had increased pH by 0.31, 1.91 and 2.41 units, respectively. The aD-N$_2$O in the soil with a lower pH in H$_2$O ranged from 4.9 to 5.5 (pH in the range of acid soil) for the c-soil and M-treatment, respectively, characterized by lower denitrification activity. Furthermore, the average mcN$_2$O-releasing was 4.58 N$_2$O-N mg kg^{-1}, compared to the higher pH in H$_2$O, which ranged from 7.10 to 7.60 (pH in the range of neutral or alkaline soil) for the M+CL- and M+CL+Ca-treatments, respectively, characterized by the higher denitrification activity. Here, the average mcN$_2$O-releasing was 7.54 N$_2$O-N mg kg^{-1}. A similar phenomenon was observed during aD. The average mcN$_2$O-releasing was 10.43 and 17.3 N$_2$O-N mg kg^{-1} for the lower and higher pH, respectively.

The lowest N$_2$O-RA was found in the c-soil with the lowest pH (the highest N$_2$O/N$_2$+N$_2$O ratio – 0.74; pH 4.9). However, the highest N$_2$O-RA was found in the M-treated soil (the lowest N$_2$O/N$_2$+N$_2$O ratio – 0.22; pH 5.5). The N$_2$O/N$_2$+N$_2$O ratios for the other two treatments were 0.63 and 0.48, respectively for M+CL (pH 7.1) and M+CL+Ca (pH 7.6).

N$_2$O-reducing activity of soil (N$_2$O/N$_2$+N$_2$O ratio) under flooding

The ratio of N$_2$O/N$_2$+N$_2$O described the N$_2$O-reducing activity (N$_2$O-RA) of the soil samples. The N$_2$O-RA of the soil varied (Figure 3). At the time of incubation, the N$_2$O-RA showed an upward or downward trend, depending on the enrichments. In the c-soil, the N$_2$O-RA of the soil decreased with the incubation time and the ratio ranged from 0.40 to 0.74. The average percentage of N$_2$O formation in the products of denitrification in the c-soil was 56 %.

In the case of the remainder of the treatments the N$_2$O/N$_2$+N$_2$O ratio showed a downward trend at the time of incubation compared to the c-soil and ranged from 0.34 to 0.22 (M-treatment), from 0.91 to 0.63 (M+CL-treatment) and from 0.86 to 0.48 (M+CL+Ca-treatment). The average percentage of N$_2$O formation in the products of denitrification in the M-treatment soil was 23 %, in the M+CL-enriched soil 62 % and in the M+CL+Ca-treatment soil 54 %.
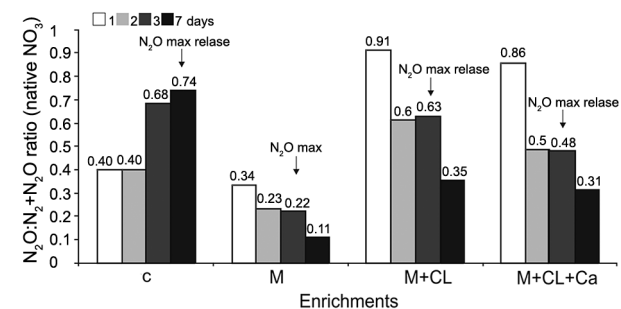

Figure 2 – The efficiency of organic and inorganic treatments compared to control soil for the actual denitrification leading to N$_2$O release (aD-N$_2$O) and actual denitrification (aD); c = control soil; M = manure enrichment; M+CL = manure and clay enrichments; M+CL+Ca = manure, clay and lime enrichments

Figure 3 – N$_2$O-reducing activity of soil depending on the type of enrichments: c = control soil; M = manure enrichment; M+CL = manure and clay enrichments; M+CL+Ca = manure, clay and lime enrichments.

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The decline in the value of $N_2O/N_2 + N_2O$ ratio to 0.11, 0.35 and 0.31 for the M-, M+CL-, and M+CL+Ca-treatment, respectively (Figure 3), was due to the consumption of N_2O after the third day of incubation in aD-N_2O.

Based on the average $N_2O/N_2 + N_2O$ ratio, the N_2O-RA of the soil increased in the following order: M+CL < c < M+CL+Ca < M.

Discussion

Incomplete denitrification (aD-N₂O) and complete denitrification (aD) under flooding

The rate of denitrification depends on the manure's capability, and both labile and complex forms of C. (Zhang et al., 2015). The use of manure in farming practice inspired us to investigate aD-N_2O and aD and consequently N_2O-RA in soil enriched with manure. This assumption allowed us to find out which combination of soil enrichments had a greater impact on the denitrification process under flooding conditions, i.e., hypoxia. The native organic matter introduced into the soil by manure and other enrichments, i.e., clay and lime, as well as products of microbial transformation, have a positive effect on the physical, chemical and biological properties of soil (Czyż and Dexter, 2016; Cima et al., 2015; Haruna and Nkongolo, 2015; Księżopolska and Joniec, 2014). The application of manure caused an increase in the number of bacteria in the activity of soil dehydrogenases, and in the intensity of the process of soil respiration. Clay minerals generally reduce decomposition rates due to lower accessibility of the organic matter and increased adsorption of metabolites to surfaces. Liming is used in acid agricultural soils, as it increases pH up to values where the availability of nutrients is higher (Księżopolska and Joniec, 2014). The tested soil had very different aD-N_2O levels, shown as mcN$_2$O-releasing, which took the following order: 1.36 (M-treatment), 6.39 (M+CL+Ca-treatment), 7.79 (c-soil) and 8.69 N_2O-N mg kg⁻¹ (M+CL-treatment). The results of complete aD, compared to aD-N_2O, were different and mcN$_2$O-releasing took the following order: 10.37 (M-treatment), 10.49 (c-soil) 14.60 (M+CL+Ca-treatment) and 20.00 N_2O-N mg kg⁻¹ (M+CL-treatment). This implies that, in different combinations, the addition of M, CL and Ca to the soil strongly influenced the denitrification process and its N_2O-RA in the soil. This was particularly the case for the M-treatment. After the application of the enrichments, the changes included levels of nutrient content and its availability, the pH, the sorptive properties and the porosity of soil.

Conservation of soil organic matter is widely recognized as a strategy used for improving soil quality and reduce, amongst others, N_2O emissions into the atmosphere. The addition of enrichments differentiated the availability of organic C and influenced especially the aD-N_2O, where the lowest N_2O release (M-treatment) was connected with the lowest oxidable C content.

Księżopolska et al. (2011) investigated the C_{org} transformation in sandy soil (Haplic Luvisol), enriched in field experiments with organic and inorganic materials and found that treatments stimulated or inhibited C_{org} mineralization and stimulated their enzymatic activity (Księżopolska and Joniec, 2014), which varies with aeration status (water-filled pore-space, WFPS) (Weymann et al., 2010).

We noted that, in the c-soil (aD-N_2O), the linear function of the cumulative increased N_2O in the headspace was similar to the 0-order of the reaction rate and the growth of denitrifiers was adapted to the natural NO_3^- and C_{org} where production outweighed N_2O consumption. Undoubtedly, this balanced activity of denitrification can determine the C:N ratio (which amounted to 8.3:1 in the control soil) and approach the value defined by the microclimate conditions e.g., the C:N ratio 10:1 or 8:1 (Schulten and Schnitzer, 1997). The addition of the fermented manure in the stabilized soil C:N ratio (7.9:1) to the rest of the treatments (with and without the CL and Ca-additions) modified the denitrification rate and the N_2O-releasing indicated a similar response as the 1-order reaction and was non-linear (logarithmic increase of N_2O at the time). Pastorelli et al. (2011) found that the influx of C sources and energy into the oligotrophic soil system was a major driving force in biogeochemical cycles.

Furthermore, the pH was a very important factor that influenced the microbial processes. In the current study, the addition of M-, M+CL- and M+CL+Ca-treatments had an increased pH of 0.31, 1.91 and 2.41 units, respectively. This is reflected in the mcN$_2$O-releasing both aD-N_2O and aD, but especially in the case of aD-N_2O and lower pH value. The average mcN$_2$O-releasing during aD-N_2O and aD showed that N_2O release was less in acidic soil than in neutral or slightly alkaline soil. Šimek and Cooper (2002) found that the total gaseous emissions into the atmosphere (N_2O, NO and N_2) have repeatedly been shown to be less in acidic soil than in neutral or slightly alkaline soil. uhel and Šimek (2011) found that denitrifying enzyme activity was highest at 8.4 pH, regardless of the native soil pH. First, our study did not confirm the highest denitrification activity when the pH in the soil was alkaline. In our case, the N_2O releasing was highest at a neutral pH for both the incomplete (N_2O) and complete ($N_2O + N_2$) denitrification and dropped in an alkaline pH. It cannot be unequivocally stated that denitrification activity increased linearly, pH values increased. This is because the optimum pH for denitrification depends on many factors, which directly or indirectly affect the environment of microbial growth. Secondly, under the conditions of our experiments, it was not so much that the C availability had a greater influence on the denitrification activity, as the pH and the sorptive properties of the soil, which were highly modified by the enrichments. As confirmation of this thesis, the highest C availability was at the lowest pH (4.9) and accounted for 45 % of the C_{org}.

The reasons for the higher activity of aD-N$_2$O and aD in soil with a higher pH also lie in the sorption capacity (T mmol$_{c+}$ kg^{-1}), and the higher degree of saturation of the sorption complex with basic cations (V%) in the soil and the addition of clay in combination with manure under field conditions. The sorption capacity of the soil and the degree of saturation of the sorption complex with basic cations increased as the pH increased.

And finally, the highest aD-N$_2$O and aD were accompanied by the highest contents of two macro-components of soil: phosphorus and potassium. Thus, the higher abundance of nutrients in the soil affected the higher denitrification activity in the neutral and alkaline soil. In this case, the addition of clay was essential.

In conclusion, the addition of enrichments does not pose a threat to the environment due to increased N$_2$O emissions and, whatsmore, in the case of M-treatment, decreased. However, in the case of aD, the addition of clay proved to be disadvantageous with regard to conserving NO$_3^-$ in the soil. This is because it increased the complete denitrification, compared to the control soil. From the point of view of protecting the environment, the results led to the conclusion that the addition of manure, in particular, should be recommended.

N$_2$O-reducing activity (N$_2$O/N$_2$ + N$_2$O ratio) under flooding

From an environmental perspective, a very important result is the N$_2$O/N$_2$ + N$_2$O ratio, which shows the N$_2$O-RA of soil (Yanai et al., 2008). This directly resulted, amongst other effects, in the diversity of soil pH and the availability of substrates, and indirectly resulted in the type of microbial community and the biochemical reaction. Pastorelli et al. (2010, 2011) have shown that the extent and degree of active bacterial diversity and the microbial communities in soil are clearly affected by the type of management and agriculture practices. The results indicated that the tested soil showed the highest N$_2$O-RA in a weakly acidic reaction (M-treatment) but much lower in an acid reaction (c-soil). The cause of different denitrification activity of the soil was the impact of the sensitivity of the nitrate reductases participating in the denitrification on soil acidity and N$_2$O reductase. Šimek and Cooper (2002) found that under an acidic pH, the activity of N$_2$O reductase is lowered and the synthesis of new N$_2$O reductases is inhibited. This results in increased accumulation of N$_2$O. In soil with higher pH values, the activity and synthesis of N$_2$O reductase is supported. Our results confirm the significant effect of pH on the N$_2$O-RA of soil with additions that are often used in farming practices, such as manure and Ca where the N$_2$O-RA of the soil increases with the increasing pH. Čuhel and Šimek (2011) stated that the ratio of denitrification products (N$_2$O and N$_2$) is pH-specific rather than soil-specific. Laboratory and field studies have demonstrated that the denitrification reductases and the N$_2$O/N$_2$ ratio differed in their sensitivity to pH and increased when the pH of soil was reduced (Cavigelli

and Robertson, 2000; Šimek and Cooper, 2002). This increase is non-linear. In our opinion, in the case of the M-treatment, other factors (described below) affect its activity. Generally, the N$_2$O-RA was highest in the M-treated soil. Undoubtedly, this can be associated with a higher content of readily available organic carbon added with manure, compared to the c-soil. Following a large input of labile C, Senbayram et al. (2009) observed low N$_2$O emissions without significant NO$_3^-$ input in the soil amended with organic matter. The nature and availability of soil C affects the denitrification enzyme activity (DEA), microbial community composition and the N$_2$O/N$_2$ ratio. This is due to the ability of manure to provide both labile and complex forms of C for soil organisms.

In the c-soil, the N$_2$O-RA of the soil with natural NO$_3^-$ content decreased with the incubation time caused by a gradual depletion of easily available carbon. In various sources of effluents, Vallejo et al. (2006) noticed a decrease in N$_2$O/N$_2$ with increasing concentrations of DOC.

In the case of the treated soil samples, there was a sharp decline in the value of the N$_2$O/N$_2$ + N$_2$O ratio after the first day of incubation, compared to the c-soil. The sharp decline could have been due to the various durations needed to activate reductases in the denitrification pathway after flooding. Dendooven and Anderson (1995) found that NO$_3$ reductase was formed within 2 to 3 h, NO$_2$ reductase between two and 12 h and N$_2$O reductase between 24 and 42 h.

The high declining value of N$_2$O/N$_2$ + N$_2$O ratio requires a separate discussion. There is a high probability that in all the soils with the addition of manure, the nitrification process occurred very intensively on the first day of incubation and the N$_2$O, like a byproduct of the nitrification, was an additional source of N$_2$O (Ciarlo et al., 2008; Martins et al., 2015; Włodarczyk et al., 2011; Zhong et al., 2015) when N$_2$O reductase was still inactive. In flooded soil, there is a thin oxygenated layer at the interface between air and water, which may occur at the same time as the processes of nitrification and denitrification (Yu et al., 2006). In the soil with manure, there was an intense mineralization of organic matter, with a release in the form of ammonium (NH$_4^+$). The confirmation of our hypothesis is the accumulation of NH$_4^+$ after seven days of incubation in the soil with the addition of acetylene (C$_2$H$_2$) (Table 3), which is an inhibitor of nitrification (Liu et al., 2015). The decrease in the N$_2$O/N$_2$ + N$_2$O ratio can be explained by lower nitrification, caused by the decline in the substrate and the lowered oxygen concentration in the course of incubation for nitrifiers, as obligatory aerobic. Indirect evidence for the activity of nitrifiers in aD-N$_2$O was a much lower percentage of denitrified NO$_3^-$ (M + CL – 41 % and M + CL + Ca – 40 % for aD-N$_2$O) and the very high percentage of denitrified NO$_3^-$ in aD with a higher pH (M + CL – 95 % and M + CL + Ca – 93 % for aD), where the nitrification process was inhibited by the addition of C$_2$H$_2$. In this case, denitrification only occurred with the use of native NO$_3^-$.

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In the soil with the addition of manure, there was a significantly lower ratio of N$_2$O/N$_2$ + N$_2$O, compared to M + CL and M + CL + Ca. This could have been caused by the significant reduction of nitrification due to the lower soil pH (acidic soil - pH 5.5). The pH affects nitrification. A hydrogen ion toxicity at pH values of 5.7 and below has also been proposed as a mechanism for nitrification inhibition (Szwerinski et al., 1986). This explains the reduction observed in the nitrification rate when the pH suddenly changed from 7.2 to 5.8 but not when it changed from 7.2 to 6.4 in an activated sludge system. The optimal pH for the growth of nitrifying bacteria varies widely (Ma et al., 2014). Chen et al. (2006) found that the optimum pH for nitrification ranges from 7.0 to 9.0, with the optimum pH ranging from 7.2 to 8.8 for Nitrosomonas and 7.2 to 9.0 for Nitrobacter.

Secondly, a significantly higher soil porosity with the addition of M, in combination with CL (45 %) and CL + Ca (42 %), could have stimulated the development of nitrifiers in the field, resulting in their active development under the conditions of a laboratory experiment.

The N$_2$O-RA of soil allows us to determine the capability of soil to release N$_2$ as the final product. With regard to protecting the natural environment and predicting nitrous oxide (N$_2$O) emissions, this information is extremely important to understanding and modeling field fluxes of N$_2$O from soil under conditions of hypoxia..

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