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Sequence and preference in the use of electron acceptors in flooded agricultural soils**

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Abstract. Specifically, it was tested whether the presence of O_2 in the headspace modified the sequence and preference of electron acceptor use under hypoxic conditions after prolonged drought in arable soils. This laboratory study was conducted in order to examine the use of electron acceptors: oxygen (O_2), nitrate (NO_3^-) and nitrous oxide (N_2O), during aerobic and anaerobic respiration (denitrification). Agricultural soils (Typic Dystrudepts) classified as sandy, silty and loamy soils from arable top soils (0-30 cm) were used in the study. The change of oxidation states of different chemical species in the soil affected the use of electron acceptors during denitrification. The use of O_2, NO_3^- and net N_2O use was gradual and differed greatly among the soils. Furthermore, microorganisms were shown to be able to use all three investigated electron acceptors simultaneously, but with clearly visible preferences. The rate of electron acceptor use per day differentiated the investigated soils into a few different groups. Overall, the results of this study indicated that N_2O was a more preferable electron acceptor than NO_3^- when O_2 was present in the headspace for the most investigated soils. Moreover, a correlation existed between the final electron acceptor use and particle-size distribution and the native organic C content (C_{org}). The rate of electron acceptor use per day calculated for O_2, NO_3^- and N_2O may provide very important information for distinguishing the preference of electron acceptor use during aerobic and nitrate respiration in agroecosystems under hypoxic conditions after prolonged drought for different kinds of electron acceptor.

Keywords: electron acceptor use, N_2O net use, O_2 consumption, NO_3^- reduction, flooded soils after drought

INTRODUCTION

The reduction of O_2 and other oxidized inorganic redox components that serve as electron acceptors is generally sequential, with O_2 being reduced first, followed by NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2 (Turn and Patrick, 1968). The content of soil oxidants in the soil which function as electron acceptors for organic matter degradation contributes significantly to these processes. The reduction of various oxidants in homogeneous soil suspensions occurs sequentially (Ponnamporuma, 1972). Nitrate is a key node in the network of the assimilatory and respiratory nitrogen pathways. For bacteria, it is both a nitrogen source and an electron acceptor. In agriculture, nitrate respiration by microorganisms is an important issue with respect to greenhouse gas emission (Kraft *et al.*, 2011).

The highest N_2O emissions primarily occur under hypoxic conditions characterized by a moderate O_2 deficiency. The sequence of the reduction process that occurs under completely anoxic conditions (N_2 atmosphere) is consistent with thermodynamic theory, with soil NO_3^- being reduced first, followed by Mn^{4+} and Fe^{3+}, and finally CO_2 to CH_4. For as long as O_2 is present in an adequate amount, other electron acceptors are relatively inactive, especially those that are more difficult to reduce (Vor *et al.*, 2003). Dhamole *et al.* (2007) found that NO_3^- was preferred as an

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electron acceptor when both NO_3^- and nitrite were available. Moreover, reduction processes have been reported to have a sequential nature, with one oxidized component not beginning to be reduced until all other oxidized components were completely reduced (Patrick and Jugsujinda, 1992). In many of the studies cited above, the authors concluded that the use of electron acceptors occurs: (i) in a strict sequence (O_2 being reduced first, followed by NO_3^-, Mn^{4+}, Fe^{3+}, SO_4^{2-}, and CO_2) and in accordance with thermodynamic theory, (ii) during the respiratory reduction chain of denitrification, which is typically NO_3^- → nitrite (NO_2^-) → nitric oxide (NO) → N_2O → N_2, (iii) under completely anoxic conditions soil NO_3^- is reduced first and (iv) under a specific redox potential (Patrick and Jugsujinda, 1992; Vor *et al.*, 2003). Several microbial processes compete for the available nitrate in the soil: denitrification, dissimilatory nitrate reduction to ammonium and anaerobic ammonium oxidation (Kraft *et al.*, 2011). Kraft *et al.* (2011) demonstrated that knowledge concerning the mechanism of nitrate reduction in natural ecosystems is still lacking for the most part. Additionally, there is a lack of information pertaining to the sequence and preference of use when O_2, NO_3^- and N_2O are available as electron acceptors in the soil during respiration under hypoxic conditions after prolonged drought. Therefore, it was decided to fill this research gap. We hypothesized that the presence of O_2 in the headspace and the particle-size distribution in the soil would modify the sequence and affect the preference of electron acceptor use (EAu). To accomplish this, O_2, NO_3^-, and N_2O were analysed in agricultural soil samples after a prolonged drought, amended with NO_3^- and incubated under hypoxic conditions.

MATERIALS AND METHODS

Typic Dystrudepts (according to Soil Taxonomy) are classified as sandy soils (soil Nos 39, 224, 434, 772), silty soils (soil Nos. 922, 947, 951, 984) and loamy soils (soil Nos 328, 342, 351, 543) from cultivated top soils (0-30 cm) were selected from the Soil Bank and the database of the Institute of Agrophysics, Polish Academy of Sciences in Lublin and used in the study (Bieganowski *et al.*, 2013). The particle-size distribution was determined by using the sedimentation method and the distribution of particle sizes was expressed as a percentage (Öhlinger, 1995).

The soils tested showed wide variations in texture, pH, organic C content, and native NO_3^- content (Table 1). Soil samples were collected from various agricultural Polish regions. Their basic properties and topography are presented in Table 1.

The bulk samples (5 g portions of air-dried soils) were sieved (1 mm sieve), placed in 38-cm^3 cylindrical glass vessels and enriched with KNO_3 at a rate of 100 mg NO_3^--N kg^{-1} of dry soil, which corresponded approximately to 300 NO_3^--N kg ha^{-1} (calculated on a 20 cm layer basis). The soil:water ratio was approximately 1:1 (w:w) with 0.5 cm of stagnant water on top of the soil surface. The vessels with soil suspensions were tightly sealed with rubber stoppers secured with metal caps which were fitted for gas sampling and incubated in an atmosphere of reduced O_2 in relation to the air. Soil flooding and reduced O_2 content in the headspace created hypoxia conditions in the investigated soils. The initial concentration of O_2 in the gas headspace at the beginning of the incubation period was 10% v:v ± 0.5 (half of the air in the vessels was replaced with N_2). Because the

Table 1. Basic properties, particle size distribution and topography of the Typic Dystrudepts investigation

Parent material	Texture (Soil No.)	Topography	% of particle size fraction >0.05 mm	0.05-0.002 mm	<0.002 mm	$C_{org.}$ g kg^{-1}	NO_3^--N_0^a mg kg^{-1}	pH_0^a in H_2O
Sand	Sand (39)[b]	Glacial plain	95	5	0	6.7	4.53	4.69
	Sand (224)	Plain	85	12	3	3.2	6.47	7.35
	Sand (434)	Glacial plain	81	13	6	7.4	18.49	4.45
	Sand (772)	Glacial plain	84	15	1	4.9	13.66	4.5
Silt	Silty sand (922)	Mountainous terrain	55	41	4	18.9	5.37	3.83
	Silty sand (947)	Mountainous terrain	52	43	5	23.1	32.93	5.37
	Sandy silt (951)	Back of mountainous terrain	32	60	8	28.5	65.76	3.94
	Sandy silt (984)	Hilly terrain	27	67	6	12.4	7.87	4.71
Loam	Loamy sand (328)	Light undulating terrain	63	23	14	7.7	9.46	6.85
	Loamy sand (342)	Glacial plain	74	21	5	4.4	29.94	7.38
	Loamy sand (351)	Glacial plain	71	21	8	5.7	17.88	7.61
	Loamy sand (543)	Glacial plain	69	28	3	8.8	9.78	5.56

[a]Values from the start of incubation, [b]soil number according to the Bank of Soil numeration and database of the Institute of Agrophysics, Polish Academy of Science, Lublin, Poland.

stoppers were punctured several times, after each analysis, paraffin films were placed on the stoppers to ensure hermetic seals. Soil samples were incubated in triplicate at 20°C from 21 to 34 days depending on the type of soil.

Gas samples of the headspace atmosphere from each vessel were directly injected into a gas chromatograph (Shimadzu GC-14, Japan) and analysed for N_2O and O_2 after 1, 2, 3, 7, 10, 14, 21, 28, 32 and 34 days of incubation. The N_2O concentrations were determined using an electron capture detector (ECD) at an operating temperature of 300°C and a column oven temperature of 80°C. The column packing material used for N_2O detection was Porapak Q. The O_2 concentrations were determined using a thermal conductivity detector (TCD) at an operating temperature of 60°C and a column oven temperature of 40°C. The column packing material used for O_2 detection was Molecular Sieve 5A. Helium was used as a carrier gas for both detectors. The contents of N_2O-N and O_2 were corrected for gas dissolved in water using Bunsen absorption coefficients (Gliński and Stepniewski, 1985). Additionally, the content of O_2 was corrected for Ar by the subtraction of its content in the headspace.

A set of 360 incubation vessels was prepared for the soils (12 types of soils with 3 replicates and 10 sampling periods). One set of incubation vessels (36 vessels) was designed for gas analysis, while other sets were opened simultaneously for the analysis of their gas contents, to measure the NO_3^- content using an FIA Star 5000 autoanalyser – FOSS Tecator. Soil suspensions were quantitatively transferred into plastic flasks, shaken for 1 h with 250 ml of 0.0125 mol L^{-1} $CaCl_2$, and then filtered through a filter paper, after which the extracts were analysed for NO_3^--N content. The organic C content was estimated based on the reduction of $Cr_2O_7^{2-}$ by organic matter, through the titration of unreduced $Cr_2O_7^{2-}$ (Gajda *et al.*, 2016). The pH of the H_2O was determined using a pH-meter (PHM82 STANDARD pH METER Radiometer Copenhagen).

The results were presented as: (i) O_2, NO_3^- and N_2O content, (mmol kg^{-1}) as a function of the incubation time for the soils developed from sand, silt and loam, (ii) and the rate of O_2 consumption, NO_3^- reduction and N_2O net use, presented as a percentage of the EAu value calculated in terms of mmol day^{-1} (the graphics compare the percentage part of each value to all categories).

Taking into consideration the oxydo-reductive conditions of the studied soils from the beginning to the end of the incubation period (from hypoxia to conditions close to anoxia), the N_2O sources in the headspace could be due to the following processes: denitrification, nitrification, nitrifier denitrification and nitrate denitrification (Baggs, 2008). A clear loss of nitrates would indicate the predominance of denitrification. An additional source of nitrates in the initial phase of incubation could be nitrification (Baggs, 2008). The discussion of results below concerns the total pool of available NO_3^- and N_2O as electron acceptors, they are both utilized in the denitrification process.

Net N_2O use by the soil will be observed if N_2O reduction exceeds N_2O production (Chapuis-Lardy *et al.*, 2007).

The results were subjected to a regression analysis using linear ($y = a + bx$), and logarithmic ($y = a\ln x + b$) models. In each case, the model with the highest R^2 value was selected as the best fit for the experimental data. All analyses were conducted using Microsoft Office Excel 2007. A one-way analysis of variance (LSD test) was used to test the significance of the effect of soil texture on the NO_3^-:N_2O ratio calculated for the day of the beginning of net N_2O use from the headspace. The statistical analysis was performed using Statgraphics Centurion XVI.

RESULTS

The percentage of the EAu value produced by the process of microbial respiration under the conditions of hypoxia and anoxia were calculated in terms of mmol day^{-1}. In order to illustrate the dynamics of EAu expressed in terms of mmol kg^{-1} as a function of the incubation time for the investigated soils (Figs 1-6).

An evaluation of the O_2 and NO_3^- ratio revealed that O_2 rather than NO_3^- was predominantly used on day 1 of the incubation with a varied efficiency in all of the soils used (Figs 1-6). Over the time period of the next day of incubation, O_2 was used with a higher degree of efficiency than NO_3^- in the three sandy and silty soils with the exception of soil Nos 772 and 947 (Figs 1 and 3), while in loamy soils NO_3^- was used which had a higher degree of efficiency than O_2 (Fig. 5). After two days of incubation in all investigated soils, the dominance of NO_3^- utilization was observed although the O_2 concentration in the headspace of some soils was still relatively high, especially in some sandy and loamy soils, for example, on the second day of incubation the concentration of O_2 was 16.6 mmol kg^{-1} which corresponded to 6.4% vol., and the NO_3^- content was 7.7 mmol kg^{-1} for soil No. 772. The O_2 concentration was 16.5 mmol kg^{-1} corresponding to 6.3% vol., and the NO_3^- content was 2.6 mmol kg^{-1} for soil No. 328 (Figs 1, 2, 5, and 6). Between day 3 and 14, all of the available NO_3^- was used in all silty soils (Figs 3 and 4) and in the 2 loamy soils this occurred between day 7 and 28 (Figs 5 and 6) at that point O_2 use increased in some soils. The dominance of NO_3^- utilization was maintained for a few days, especially in some silty and loamy soils at various stages of incubation (Figs 3 and 6). During the following days of incubations the EAu values for the O_2 and NO_3^- ratio showed the value decreasing and increasing, depending on the type of soil being tested. During the first 3 days of incubation, the sandy, silty and loamy soils used an average of 53, 79 and 72% of the available O_2, while an average of 7, 36 and 62%, respectively of the available NO_3^- was consumed.

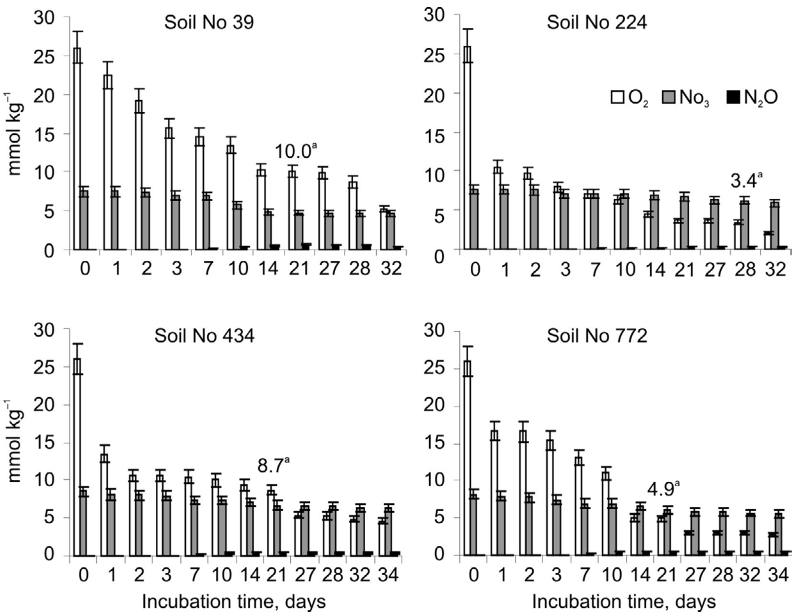

Fig. 1. Oxygen, nitrate and nitrous oxide content as a function of incubation time (mmol kg^{-1}); $^{a}O_2$ concentration at which N_2O consumption took place.

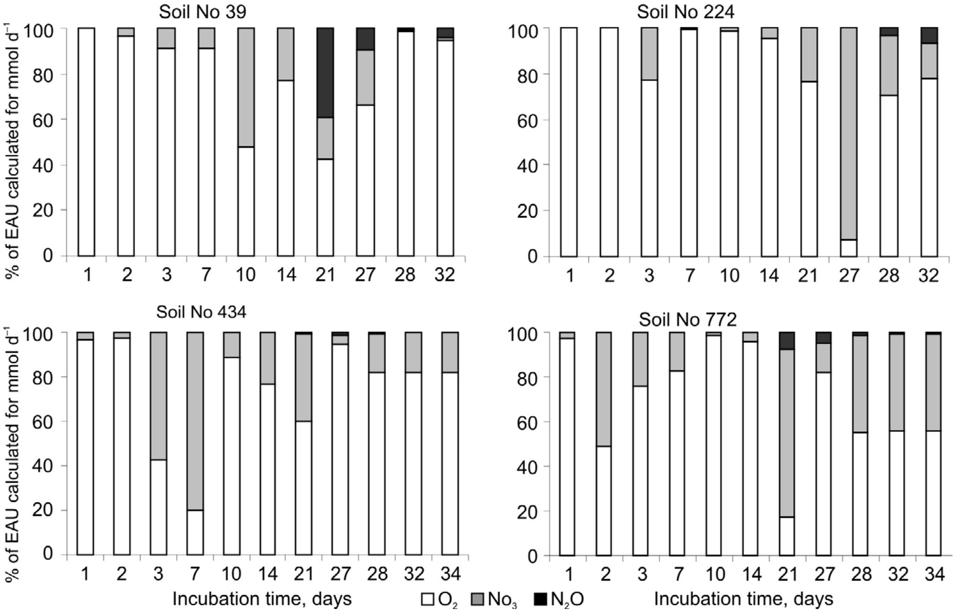

Fig. 2. Percentage of EAu in the process of microbial respiration in anoxia conditions calculated in terms of mmol day^{-1} in the sandy soils.

The net N_2O use began between day 2 and day 28 of the incubation period, depending on the type of soil. The N_2O content decreased in parallel with O_2 presence in the headspace and differed greatly between the soils. The beginning of net N_2O use which occurred in the presence of O_2 in the headspace ranged from 3.4 to 10.0 mmol kg^{-1} for sandy soil corresponding to 1.3 to 3.9% vol., 1.0 to 6.9 mmol kg^{-1} for silty soils corresponding to 0.4 to 2.7% vol., and 3.1 to 16.5 mmol kg^{-1} and for loamy soils corresponding to 1.2 to 6.3% vol. (Figs 1-6). The EAu value for the O_2 and N_2O ratio, at the first day of net N_2O use, showed that the amount of N_2O used was similar to the amount of O_2 used in one of the sandy soils (No. 39), although the O_2 concentration in the headspace was 10 mmol kg^{-1} corresponding to a 3.9% vol. and N_2O was 0.6 mmol kg^{-1}. In the rest of the soils, O_2 was used by denitrifiers with a higher efficiency than N_2O. Over the following days of incubation, N_2O was used with a higher efficiency than O_2 in three silty soils Nos 922, 951 and 984 on day 14th, 21st and 14th day of incubation, respectively and in one loamy soils (No. 351) on day 7 of

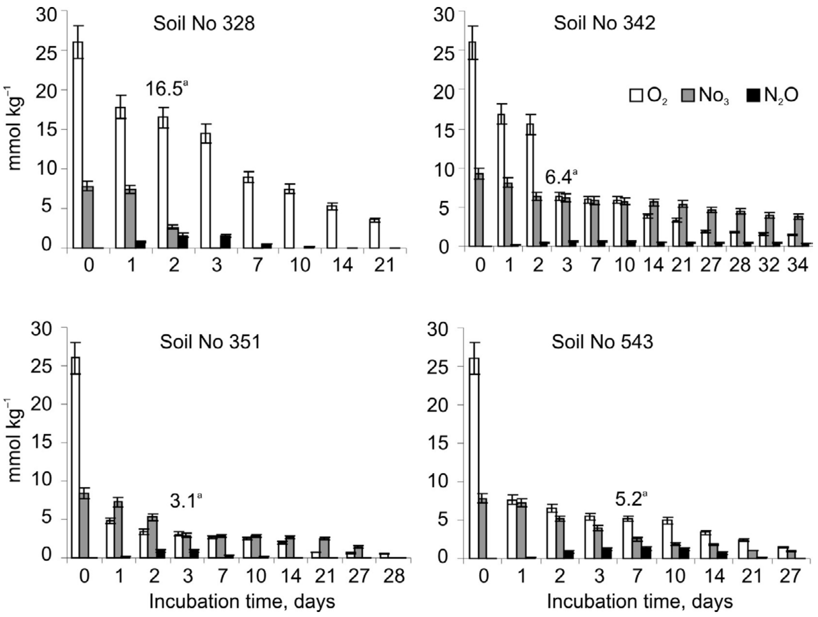

Fig. 3. Oxygen, nitrate and nitrous oxide content for silty soils as a function of incubation time (mmol kg^{-1}); $^{a}O_2$ concentration at which N_2O consumption took place.

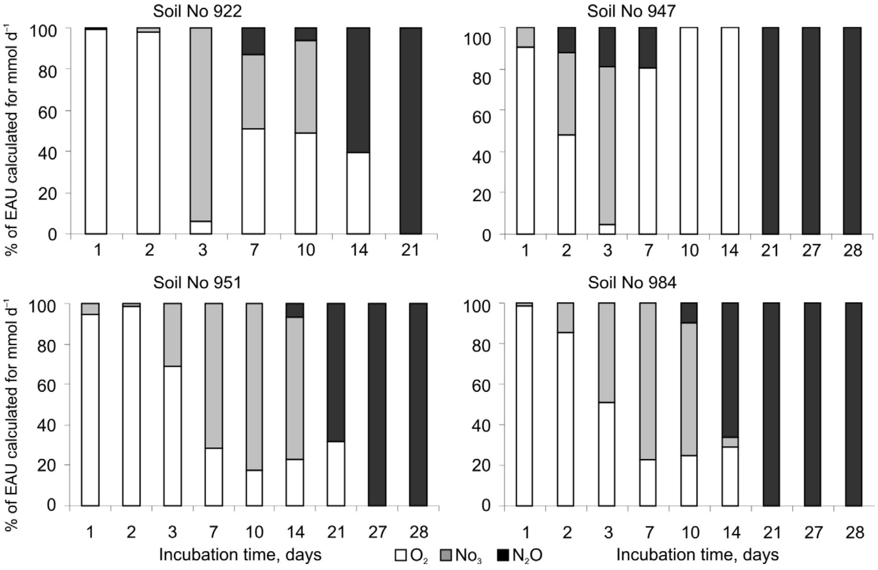

Fig. 4. Percentage of EAu in the process of microbial respiration in anoxia conditions calculated in terms of mmol day^{-1} in the silty soils.

incubation, where the O_2 concentration in the headspace was 2.7 mmol kg^{-1} and N_2O was 0.9 mmol kg^{-1} (Figs 1, 3, and 5).

Given the significant decrease in oxygen concentration after the first day of incubation and the low pH value of some soils, it was assumed with a high degree of probability that nitrification was not an additional source of N_2O (Dinçer and Kargi, 2001) except for the first day of incubation.

The N_2O-net use began when the NO_3^- in the soil solution ranged from: 4.7 to 6.7 mmol NO_3^--N kg^{-1}, while the N_2O content ranged from 0.4 to 0.6 mmol N_2O -N kg^{-1}, for the sandy soils. It ranged from 0.0 to 3.6 mmol NO_3^--N kg^{-1}, and 1.4 to 2.4 mmol N_2O-N kg^{-1} for the silty soils, and from 2.5 to 6.2 mmol NO_3^--N kg^{-1} and 0.7 to 1.6 mmol N_2O-N kg^{-1} for the loamy soils (Figs 1-6). On the first day of net N_2O use, N_2O was used faster than NO_3^- in one of the sandy soils (No. 39) (Fig. 1), and in one of the loamy soils (No. 351) (Fig. 5), which implies that N_2O was preferred as an electron acceptor in these soils. While in all silty soil net N_2O use was more intensive after the exhaustion of the NO_3^- content in the soil solution (Fig. 3).

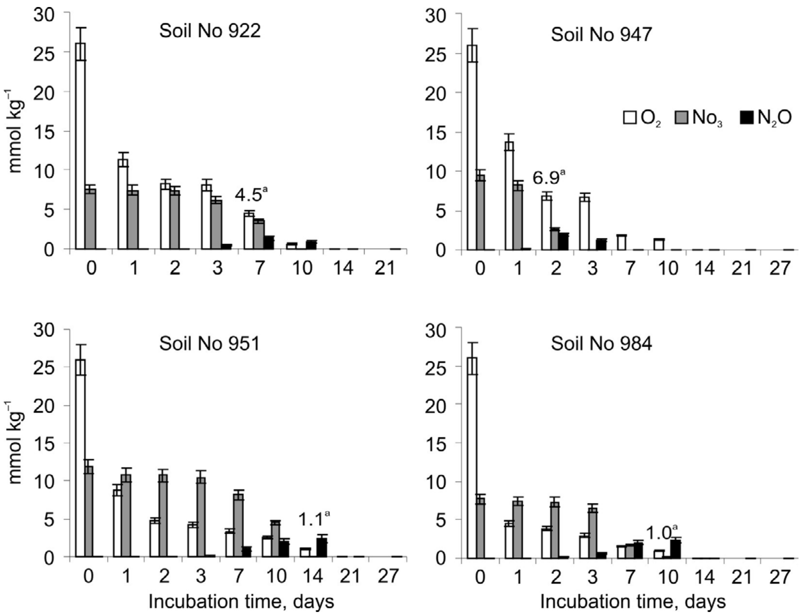

Fig. 5. Oxygen, nitrate and nitrous oxide content for loamy soils as a function of the incubation time (mmol kg^{-1}); $^{a}O_2$ concentration at which N_2O consumption took place.

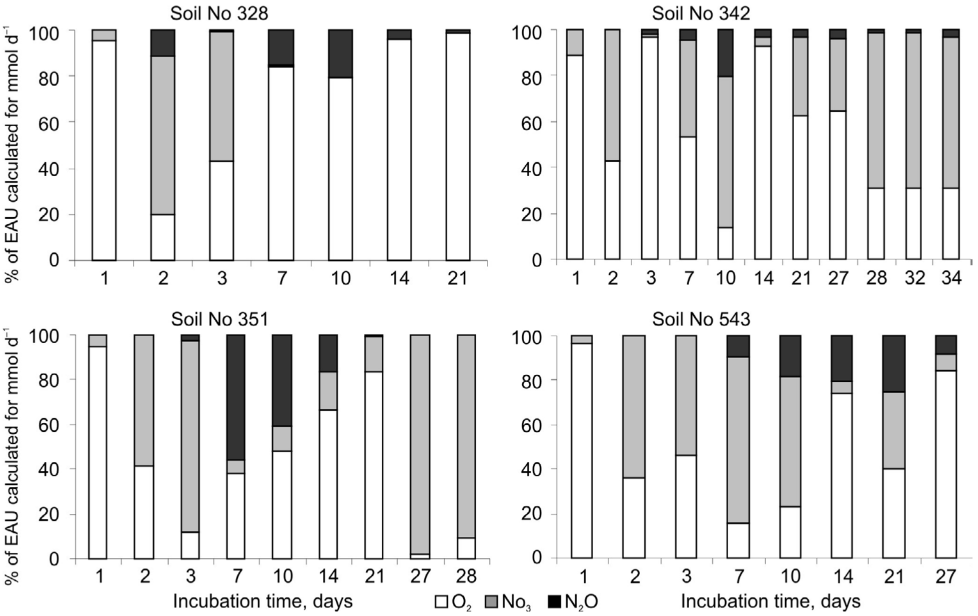

Fig. 6. Percentage of EAu in the process of microbial respiration in anoxia conditions calculated in terms of mmol day^{-1} in the loamy soils.

In summary, the average use of electron acceptors during the period under consideration depending on the particle-size distribution was as follows: O_2 – 86, 100 and 93% for the sandy, silty and loamy soils, respectively, NO_3^- - 29, 100 and 87% for the sandy, silty and loamy soils, respectively, and N_2O – 28, 100 and 85% for the sandy, silty and loamy soils, respectively.

A correlation between the EAu and particle-size distribution (sand, silt and clay) of the soils was found. A negative correlation was found between all EAu values (O_2, NO_3^- and N_2O) expressed as a percentage of the cumulative EAu values and the sand fraction (p value ranging from < 0.01 to <0.001) and a positive correlation for the silt ($p < 0.001$) and clay fraction except for N_2O use ($p < 0.05$ and <0.001) when all of the soils were compared to each other. These findings indicate that the use of O_2 and NO_3^- as electron acceptors under hypoxic conditions were correlated with sand, silt and clay fractions in the sandy, silty and loamy soils, and N_2O in sandy and silty soils.

A slightly inferior relationship was observed between NO_3^- and N_2O which was expressed as a percentage of the cumulative EAu value and the C_{org} for the soils analysed together ($p < 0.01$), but no relationship was found between the percentage of O_2 consumption and the content of the soil C_{org}.

DISCUSSION

The rates of O_2, NO_3^- and net N_2O use may be controlled by the characteristics of the supply of electron acceptors, aeration states, particle-size distribution, organic C content and other conditions. The microorganisms used the three main available electron acceptors simultaneously *e.g.* O_2, NO_3^- and N_2O showing some degree of sequentiality and preference.

On the first day of incubation, the type and state of oxidation of electron acceptors appeared to be the most important factor affecting the sequence of their use during this experiment. All of the microorganisms O_2 over NO_3^- as an electron acceptor. In general, the energy efficiency of aerobic respiration is much higher than that of anaerobic respiration (Gliński and Stępniewski, 1985). All sandy and loamy soils had more than one day when the microorganisms showed a preference for O_2 over NO_3^- as an electron acceptor during different incubation stages. This means that a change in the preference of EAu over time was observed. Patrick *et al.* (1985) found that the composition of gases varies over time after flooding and is dependent on the soil and other environmental conditions. There are several possible explanations for the different preferences of EAu.

Firstly, O_2 is a more energetically effective acceptor of electrons than NO_3^-. It is assumed that the denitrification process is less energetically favourable than the reduction of dissolved oxygen. In a system that contains oxygen, nitrate and organic C, oxygen will normally be the preferred electron acceptor (Rivett *et al.*, 2008).

Furthermore, the 5 mm of stagnant water above the soil did not limit the diffusion of O_2 from the headspace to the soil suspension, and some of the O_2 could be utilized by the organisms living at the border between them. The rate of disappearance of O_2 reached a peak on the first day of incubation. The gradual decrease in the O_2 use rate may be caused by the gradual decrease in O_2 diffusion into the soil suspension with time and its lower availability. Cho (1982) investigated O_2 consumption, as well as N_2O, and N_2 production and found that the rates of O_2 consumption changed over time.

Secondly, the results indicate the importance of particle-size distribution in the preference of EAu. According to the average EAu value for O_2 and NO_3^-, the highest O_2 use was observed during the first 3 days of incubation in sandy soils (53% O_2:7% NO_3^-= 7.6), while it was used to a far lower extent in silty soils (79% O_2:36% NO_3^-= 2.2), and the value observed for loamy soils was close to one (72% O_2:62% NO_3^-= 1.2). These findings imply that the degree of O_2 and NO_3^- use was only similar in loamy soils and was closely associated with particle-size distribution. In general, over the following days of incubation NO_3^- was utilized with a higher degree of activity by microorganisms than O_2 and when almost all of the NO_3^- was used, O_2 use increased sharply. Particle-size distribution (especially the finer fraction) appeared to be one of the most important factors affecting the sequence of electron acceptors used. The results suggest that the particle-size distribution and organic C content rather than respiratory substrate availability appeared to be more important to the rate of O_2 and NO_3^- use during the following days of incubation. Johnson *et al.* (2012) suggested that the coupling between C and N in agricultural streams involves potentially complex interactions with sediment texture and organic matter. However, the relatively high organic C content (12.4-28.5 g kg^{-1}) in silty soils appeared to play one of the main roles in determining the preference of NO_3^- use after day 2 of the incubation. Peterson *et al.* (2013) suggested that, with the input of the DOC substrate, alluvial gravel materials could generate enough enzyme during a 1-2 day anaerobic period to denitrify significant quantities of nitrate. Harrison-Kirk *et al.* (2013) stated that the soil organic matter (SOM) content is one of the important factors affecting carbon (C) and nitrogen (N) mineralization under constant soil moisture. The intensive utilization of O_2 at the beginning of the incubation period, when an average of 68% of the available O_2 was used, stimulated the consumption of NO_3^- during denitrification under more suitable conditions for its reduction.

Among the soils with a lower C_{org} content, a second period of intensive O_2 use, which was not related to decreasing NO_3^- use caused by a lower NO_3^- availability, was noted. One exception was loamy soil No. 328, where the highest content of the finest fraction (particle size <0.002-14%), rather than O_2 availability and organic C content (7.7 g kg^{-1}), may be the cause of the very intensive NO_3^- use and subsequent O_2 consumption after the exhaustion of NO_3^-. Soil No. 328 had the lowest starting value of Eh (+229 mV), indicating that it was likely to be more susceptible to reduction under field conditions, resulting in a greater degree of adaptation to the respiration of NO_3^- compared to other soils. Włodarczyk *et al.* (2011) stated that the percentage of clay in the soil is one of the most significant determining factors for the rate of NO_3^- reduction.

Finally, for silty soils with a higher C_{org} content, there was an absence of any second period of predominant O_2 consumption because the use of NO_3^- was higher than that of O_2 until it disappeared completely from the soil solution. Bonin and Michotey, (2006) measured the denitrification rates and found complete NO_3^- use during the first hour of incubation.

N_2O was the third electron acceptor source that was used from the headspace, although O_2 and NO_3^- were still available.

The EAu ratio for O_2 and N_2O showed that O_2 was preferred over N_2O as an electron acceptor at the beginning of the period of net N_2O use, except for one of the sandy soils where the use of both acceptors was comparable (No. 39). During the following days of incubation in three silty soils (Nos 922, 951 and 984) and one loamy soil (No. 351) N_2O was utilized more efficiently than O_2.

There are several possible explanations for this phenomenon. One is that there may be an additional branch in the transfer of electron acceptors which is transiently used under hypoxic environmental conditions. Some groups of denitrifiers are capable of using both O_2 and NO_3^- or nitrite as the electron acceptor simultaneously (Carter *et al.*, 1995). Therefore, denitrification by those organisms may occur under aerobic conditions if a rate-limiting step in the transfer of electrons from its substrate to O_2 is present. The provision of a second electron acceptor, in this case NO_3^-, enables the utilization of an additional branch in the electron transport chain (Robertson and Kuenen, 1991). These findings indicate that the provision of a second electron acceptor, in this case N_2O, allows for the utilization of an additional branch in the electron transport chain, as is the case with NO_3^-. Therefore, the presence of O_2 in the headspace changed the aeration status of the studied system when compared to anaerobic conditions and, as a consequence, the sequence of electron acceptors used. Johnson *et al.* (2012) suggested that coupling between C and N in agricultural streams involves potentially complex interactions and possibly indirect biogeochemical pathways.

A second explanation may be N_2O being consumed more rapidly than O_2, resulting from the depletion of the O_2 concentration to a certain level in the headspace and a decreased rate of supply to the soil suspension. The greater rate of net use of N_2O compared to that of O_2 may be due to greater N_2O solubility in water and a higher concentration and accessibility, especially in the later part of the incubation when the O_2 concentration was very low. However, when the N_2O content in the headspace decreased to a certain level, O_2 was again utilized to a higher degree than N_2O (soils No. 947).

The uniqueness of soil No. 351 should be emphasized. On the seventh day of incubation, net N_2O use was observed, at the same time the utilization of O_2 and NO_3^- as an electron acceptor clearly declined. Over the next 7 days, intense net N_2O use was observed. On day 21 of the incubation, when the N_2O concentration dropped to 0.01 mmol N_2O-N kg^{-1}, O_2 and NO_3^- utilization increased sharply, thereby indicating that this soil preferably utilized N_2O as opposed to the remaining two electron acceptors. The results contradict the study of Chapuis-Lardy *et al.* (2007), which stated that denitrifiers use N_2O as an electron acceptor and reduce it to N_2 when soil nitrate concentrations are very low.

The use of electrons to change the state of oxidation, *i.e.* from more oxidized to less oxidized, may have been the result of the microbial community structure and activity of the investigated soil, the genetic configuration of N_2O reductase, or the history of the soil prior to sampling (*i.e.* the oxygenation status of the soil, availability of NO_3^-, humidity, crop production and rotation, *etc.*). The history of the soil prior to sampling and the prolonged drought before flooding may have caused differences in the species composition of the denitrifying community and affected the preferences in the use of electron acceptors. The respiratory reduction chain of denitrification depends on the environmental conditions or genetic configuration (Dodla *et al.*, 2008; Philippot *et al.*, 2011; Pauleta *et al.*, 2013; Hatano, 2019). Johnson *et al.* (2012) suggested that coupling between C and N in agricultural streams involves potentially complex interactions with the microbial community structure, and may also involve indirect biogeochemical pathways. Bouwman *et al.* (2002) reported that limited information was available with regard to the bulk reduction of N_2O to N_2 by N_2O-reductase, which was regulated by various soil and environmental factors. N_2O reductase has been isolated in different forms depending on the oxidation state and molecular forms of its Cu centres (Pauleta *et al.*, 2013). Furthermore, soil microorganisms maintain their microbiological activity (capacity for memory) for many years from the time of sampling agricultural soils (Włodarczyk, 2000).

Thirdly, in some soils under consideration, an additional result of preferential O_2 use versus NO_3^- was observed at the time at which N_2O began to be used from the headspace, resulting from the fact that N_2O uses the same biochemical pathway as NO_3^-, and this led to the following reaction: ($N_2O \rightarrow N_2$). Moreover, in this case a synergistic effect occurred resulting from the simultaneous use of O_2 and N_2O. The lower concentration of O_2 creates improved conditions for the production of N_2O reductase, which is particularly sensitive to the presence of O_2 (Włodarczyk *et al.*, 2011).

The initiation of net N_2O utilization was observed simultaneously with NO_3^- consumption when the NO_3^--N concentration (mmol kg^{-1} of soil) in the soil solution was much higher than that of N_2O-N. The average concentration of NO_3^--N at the start of net N_2O use was 5.9, 2.3 and 3.6 mmol kg^{-1}, and N_2O-N was 0.5, 1.8 and 1.1 mmol kg^{-1} in the case of sandy, silty and loamy soils, respectively.

A one-way analysis of variance (LSD test) showed the significance of the effect of soil texture on the $NO_3^-:N_2O$ ratio which was calculated from the day of the initiation of net N_2O use from the headspace. There were differences between the sandy soils (coarse-grained soils) and silty and loamy soils (fine-grained soils). The high ratio of $NO_3^-:N_2O$ in sandy soils (12.2) compare to silty (3.6) and loamy (1.7) soils should be noted. In sandy soils, which had better aeration, N_2O reductase was probably less adapted to hypoxic conditions in the soil under natural conditions as compared to the fine-grained soils. Bouwman *et al.* (2002) reported that N_2O reductase has been isolated in different forms depending on the oxidation state and molecular forms of its Cu centres.

In general, the statistical analysis clearly shows a correlation between the EAu value and particle-size distribution (sand, silt and clay) of soils, this is particularly the case with silty soils ($p < 0.001$). Liptzin *et al.* (2011) investigated humid tropical forest soils and found a correlation between the content of O_2 and N_2O. Harrison-Kirk *et al.* (2013) found that soil texture was one of the important factors affecting nitrogen (N) mineralization under constant soil moisture conditions.

Based on the ratio of NO_3^- and N_2O utilization, soils may be divided into two groups: (i) those for which the beginning of net N_2O use occurs at low levels of NO_3^- or after their complete consumption and (ii) those which show a preference for the use of N_2O (the less oxidized compound) as an electron acceptor with a simultaneously high content of NO_3^- (the more highly oxidized compound) in the soil solution.

The first group included two silty soils. The net N_2O use in the two soils started when the NO_3^--N concentration was below one and where N_2O use was observed after the complete consumption of NO_3^-.

When the ratio of NO_3^- and N_2O use for the second group were considered, it was found that the net N_2O use in these soils was initiated when the NO_3^--N concentration was above 1.0 and ranged from 2.5 to 6.65 mmol kg^{-1} while the concentration of N_2O ranged from 0.41 to 2.4 mmol kg^{-1}. These findings indicate that the appearance of N_2O as an electron acceptor even at relatively low content, may limit NO_3^- use in some soils. The sequence of electron acceptors used in the soils described above is consistent with the sequence described in several other studies. Specifically, Cho and Sadkinan, (1978) reported that the effect of NO_3^- upon the reduction of N_2O to N_2 was due to the competition between NO_3^- and N_2O as electron acceptors. Glatzel and Stahr, (2001) found that a soil medium with a low content of NO_3^- acted as an N_2O sink because NO_3^- is preferred over N_2O as an electron acceptor. Wagner-Riddle *et al.* (1997) and Ryden (1983) also connected the events of N_2O uptake to low soil NO_3^- concentration. Vor *et al.* (2003) stated that the final step of denitrification (the reduction of N_2O to N_2) would not occur so long as more efficient electron acceptors (*e.g.* NO_3^-) were still available. Finally, Włodarczyk *et al.* (2004) investigated the stability of NO_3^- in agricultural soils under hypoxic conditions and found that N_2O could be used as an electron acceptor in the presence of low and moderate NO_3^- concentrations, while the presence of NO_3^- at concentrations greater than 100 mg NO_3^--N kg^{-1} inhibited the consumption of N_2O by the loess soils. When the headspace-N_2O dropped slightly to a value less than 0.5 mmol N kg^{-1} and distinctly dropped in soil No. 351 (0.01 mmol N kg^{-1}) and the NO_3^--N concentration was still comparably high (an average value was 4.6 mmol N kg^{-1}), NO_3^- utilization increased sharply. As stated above, NO_3^- inhibited the reduction of N_2O to N_2, but in this case, the appropriate conditions for the investigation of this statement may not apply. In general, under hypoxic conditions, an alternative sequence of electron acceptor preference was noted. Specifically, net N_2O utilization was not limited by the relatively high or very high NO_3^- content in the soil solution. This was because, in the case of these soils, N_2O was a more preferable electron acceptor than NO_3^-, especially at the beginning of the N_2O disappearance from the headspace. The exceptions to this were two silty soils No. 951 and 984, which showed a decrease in NO_3^- concentration to 0.0 and 0.15 mmol N-NO_3^- kg^{-1} in the soil solution, thereby indicating that the low level of NO_3^- may have limited its availability and affected the preference for N_2O use. In some soils, the low O_2 concentration in the headspace probably had a greater effect on the net use of N_2O as opposed to NO_3^-. Specifically, these conditions probably resulted in the activation of N_2O reductase, which is extremely sensitive to O_2, rather than high concentrations of NO_3^-. In the other soils, the preferential net use of N_2O or NO_3^- might have been due to the microbial activity of the investigated soil, the genetic configuration of N_2O reductase, and the history of the soil prior to sampling, in particular the oxygenation status of the soil before sampling.

One very important finding of this study was that, during NO_3^- use, N_2O was a preferable electron acceptor compared to NO_3^- when O_2 was present in the headspace for most of the investigated agricultural soils. Accordingly, even when there is a relatively high concentration of NO_3^- in the soil suspension a higher net N_2O use compared to NO_3^- use may be expected. These findings indicate that, in the natural agricultural soil environment, under conditions that are satisfactory for denitrification, intensive net N_2O utilization can occur during its migration from the deeper parts of the profile to the atmosphere when there are high concentrations of NO_3^- present. Zaman and Nguyen, (2010) found that, unlike N_2O, the emission of N_2 to the atmosphere does not pose any environmental problems and it can be recycled through symbiotic and non-symbiotic N_2 fixation.

CONCLUSIONS

1. Considering the sequence of electron acceptor use, the microorganisms showed some preferences in the use of electron acceptors.

2. The results showed that the use of products during NO_3^- reduction which occurred under hypoxic conditions did not follow the pattern of the sequence which occurs during denitrification: $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$, (under completely anoxic conditions).

3. It is likely that the presence of O_2 in the headspace changed the oxygenation status of the studied system and, as a consequence, the sequence of electron acceptors used, especially in the case of NO_3^- and N_2O use.

4. The calculated percentage of EAu confirms that the particle-size distribution had a significant impact on preferences in the use of electrons in the process of nitrate respiration.

5. The electron acceptors, which had a higher degree of oxidation (O_2, NO_3^-) are used more effectively in soils with coarser particle sizes, but the increase in fine fraction (clay) caused a significant increase in the use of electron acceptors, with a lower degree of oxidation (N_2O).

6. Taking into account N_2O emissions, fine-grained soils are less of a threat to the environment.

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