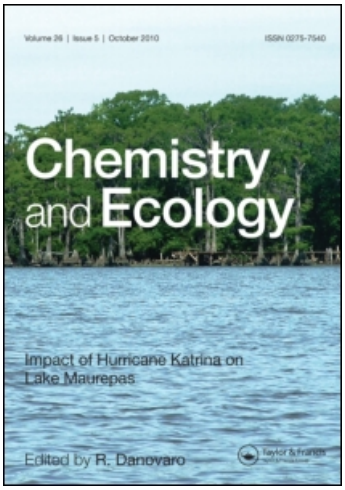

Chemistry and Ecology

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713455114

Nitrate reductase activity in soil under shelterbelt and an adjoining cultivated field

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Online publication date: 30 September 2010

To cite this Article Szajdak, Lech Wojciech and Gaca, Wioletta(2010) 'Nitrate reductase activity in soil under shelterbelt and an adjoining cultivated field', Chemistry and Ecology, 26: 4, 123 — 134
To link to this Article: DOI: 10.1080/02757540.2010.501028
URL: http://dx.doi.org/10.1080/02757540.2010.501028

Chemistry and Ecology
Vol. 26, Supplement, October 2010, 123–134

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Nitrate reductase activity in soil under shelterbelt and an adjoining cultivated field

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(*Received 15 September 2009; final version received 8 June 2010*)

Laboratory studies were conducted to evaluate the effect of moisture (field-moist and 15% moisture) and nitrogen concentrations (field content and after the addition of nitrogen in the form of 0.25 and 0.5% urea, respectively), pH and organic matter on the changes in nitrate reductase activity in soils under shelterbelt and an adjoining cultivated field. Shelterbelt consists mainly of *Robinia pseudoaccacia*. A first-order kinetics reaction model was fitted to the experimental changes in nitrate reductase activity over time data at different moisture and nitrogen content. Under shelterbelt, an increase in the soil moisture content from field-moist to 15% led to a 1.13-fold increase in the first-order reaction rate constant. However, under the adjoining cultivated field, an increase in the soil moisture content from field-moist to 15% increased the first-order reaction rate constant significantly more than in soil under shelterbelt. Changes in nitrate reductase activity for a 15% moisture content in soil under shelterbelt and in field-moist soil in the adjoining cultivated field were similar. Moreover, the addition of 0.25% nitrogen to the adjoining cultivated field with field-moist soil led to a 3.65-fold increase in the first-order reaction rate constant.

Keywords: shelterbelt; nitrate reductase activity; chemical properties; first-order reactions rate constant

1. Introduction

Shelterbelts (mid-field rows of tree afforestation) represent a basic and advantageous component in the agricultural landscape and play an important ecological role. They fulfil significant functions in the agricultural landscape, mainly by decreasing wind and soil erosion. In addition, they limit the spread of chemical compounds between ecosystems. Shelterbelt and stretches of meadow have been shown to help in collecting the water-borne movement of various chemical compounds from cultivated fields into the collecting water basin. Moreover, they improve the microclimate for agricultural production, regulate the water ratio in soils and help maintain biodiversity in agricultural fields [1–3].

Nitrate pollution, particularly that caused by the use of nitrogen fertilisers, is a large threat to rural areas. Many chemical, biochemical, physical and biological processes control the dispersion

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ISSN 0275-7540 print/ISSN 1029-0370 online

DOI: 10.1080/02757540.2010.501028
http://www.informaworld.com

of nitrates in soils, and these processes depend on the organic matter content, particularly humic substances [4]. At present, there is a lack of investigation into humic substances and their transformation in soils under shelterbelts.

Denitrification represents the reduction of nitrate and nitrite in gaseous products like molecular nitrogen (N_2) and nitrous oxide (N_2O), which are further evaporated from the soil [5]. This occurs under anaerobic conditions with the participation of denitrification bacteria (*Paracoccus denitrificans*, *Paracoccus halodenitrificans*, *Thiobacillus denitrificans*, *Bacillus licheniformis*, *Pseudomonas aeruginos* and *Pseudomonas denitrificans*) [6].

Several physico-chemical parameters such as temperature, moisture, oxygen and H^+ content, soil density, texture and structure, biochemical activity, plants and rainfall may impact either directly or indirectly on nitrification and denitrification in soil [7]. Moreover, high levels of moisture and organic matter, and neutral and basic pH favour denitrification [8]. In addition, a low oxygen content accelerates the reduction of nitrate to nitrite. This process is catalysed by nitrate reductase. NO_2^- is further reduced to N_2O by nitrite reductase [9,10]. Furthermore, the N_2O to N_2 pathway is catalysed by nitrous oxide reductase. Finally, this process leads to a loss of nitrogen from the soil, mainly in the form of N_2 and N_2O. Depletion of nitrogen from the soil represents another undesirable feature of denitrification. Thus, in most cases, it is desirable to minimise soil denitrification (although the resulting nitrate accumulation can cause other environmental problems likely connected with nitrate leaching) [5,9].

Soil enzymes form a quantitatively minute but very important part of the soil organic matter, because all biochemical action is dependent upon, or related to, enzymes. The frequently poor correlation between overall metabolic activity and the activity of a particular enzyme is probably due to the stabilisation of extracellular enzymes by their association with soil organic matter and clay surfaces [11]. It is well known that the role of enzymes in coupling reactions leading to polymerisation is limited to oxidation of the substrates.

Nitrate reductase is involved in the process of denirification. Nitrogen present in the structure of this enzyme, rather than molecular O_2, acts as a terminal electron acceptor by bacteria and this is irreversible once NO is formed [12]. In the international classification of enzymes [13], nitrate reductase has been assigned the identification number EC 1.7.99.4. The systematic name for nitrate reductase is reduced NADP:nitrate oxidoreductase. Flavoprotein (FAD) containing molybdenum creates a prosthetic group for this enzyme.

The potential for denitrification in soils shows a complex interaction among aeration, nitrate and carbon substrate availability and other intrinsic soil factors [14]. It is well known that the absence of O_2 or reduced O_2 availability is required for both the synthesis and activity of denitrification enzymes. However, quantification of O_2 availability and related rates of denitrification in soil is complicated by dynamic relationships between aeration potential (O_2 flux) and microbial oxygen use [15,16]. In addition, temperature, water content, matric potential and water holding capacity all serve as relative predictors of microbial activity in soil [17].

Although denitrification has been studied in a wide variety of agricultural soils, there is relatively little information available on denitrification in shelterbelt soils. Several investigators have studied denitrification in forest soils [18–20]; however, these studies have either measured the potential rate of denitrification or have been limited in temporal or spatial scale. Comprehensive investigations into denitrification are needed for a better characterisation of the role this process plays in shelterbelt nitrogen cycles and to assess the contribution of shelterbelt to regional [21,22] and global [23] nitrogen budget.

The object of this study was to estimate the influence of organic nitrogen and moisture levels on nitrate reductase activity in soil under shelterbelt and an adjoining cultivated field using a first-order kinetics reaction model.

2. Materials and methods

The investigations were carried out in Dezydery Chłapowski Agroecological Landscape Park in Turew (40 km south-west of Poznań, West Polish Lowland). Intensive agriculture is observed in this region, and the agricultural land is composed of 70% cultivated fields, 12% meadows and 14% shelterbelts. Characteristic features of this landscape are shelterbelts created in the nineteenth century by General Dezydery Chłapowski [2]. Shelterbelt and adjoining cultivated fields were introduced on Hapludalfs soils (according to FAO classification).

Soils were sampled from a *Robinia pseudoacacia* shelterbelt and an adjoining cultivated field. The shelterbelt consists mainly of *R. pseudacacia* and a small admixture of *Quercus robur* and *Larix deciduas*. It is 200 years old, 2 km long and 36 m wide. The humus horizon layer of this shelterbelt reaches a depth of 15 cm. All soil samples were taken at 10 sites in the adjoining cultivated field located 100 m from the shelterbelt and in the middle of the shelterbelt areas from 0 to 20 cm depth (humus horizon). Bulky root residues, stones and leaf litter were removed by hand. Samples were air dried and crushed to pass through a 1 mm mesh sieve. The 10 subsamples were mixed to prepare a 'mean sample'. Total nitrogen (Kjeldahl methods), N-NH_4^+, N-NO_3^-, dissolved organic carbon (DOC), total organic carbon (TOC 5050A with Solid Sample Module, SSM-5000A, Shimadzu, Japan), pH [potentiometric method in 1 N KCl (1:2.5 v/v)] and moisture content (infrared balance, Radwag, Poland) were determined in all samples.

A kinetics model was used for the changes in nitrate reductase in these soil samples. Field-moist soils passed through a sieve of 5 mm mesh size. Soil material from the shelterbelt and adjoining cultivated field was divided into two: (1) field-moist content; (2) moisture content adjusted to 15%. Parts (1) and (2) were then further divided into three parts, each of them weighing 10 kg. Parts (1a, 2a), native total nitrogen; (1b, 2b), addition of 0.25% nitrogen per 1 kg of soil in the form of urea; (1c, 2c) addition of 0.50% nitrogen per 1 kg of soil in the form of urea. The samples were put into closed vials and kept in a thermostat at 20 °C. The samples were collected at suitable time intervals and the nitrate reductase activity was determined. All kinetic experiments were run triplicate and the results were averaged.

Nitrate reductase was determined using the Kandeler method [24]. The calibration standards ranged from 0.0 to 1.0 μgN-NO_2^- $\cdot$ mL^{-1}. For colorimetric analysis, 5 mL of each standard, 3 mL of ammonium chloride buffer and 2 mL of colour reagent [sulphanilamide and N-(1-naphtyl)-ethylenediamine hydrochloride] were added and allowed to stand for 15 min at room temperature. Purple complex was determined at $\lambda_{max} = 520$ nm. The nitrate reductase activity in the soils was calculated from a previously prepared analytical curve according to the Beer–Walter light absorption law by means of the least squares Equation (1) (Table 1, Figure 1):

$$A = \varepsilon \cdot c \cdot l, \tag{1}$$

where A = absorbance, ε = molar absorption coefficient ($L \cdot mol^{-1} \cdot cm^{-1}$), c = concentration ($mol \cdot L^{-1}$) and l = thickness of the layer (1 cm). Similar calculations were used for ammonium and nitrate ions (see below).

Table 1. Molar absorption coefficients (ε), and correlation coefficients (r) according to the Bear–Walter law.

Compounds	ε ($L \cdot mol^{-1} \cdot cm^{-1}$)	r
Nitrite (for measurements of nitrate reductase activity)	21115 ± 1.00	0.999
N-NH_4^+	$1.47 \times 10^6 \pm 2.06 \times 10^4$	0.999
N-NO_3^-	$3.66 \times 10^3 \pm 2.20 \times 10^2$	0.996

Note: $A = \varepsilon \cdot c \cdot l$ for nitrite, N-NH_4^+ and N-NO_3^-.

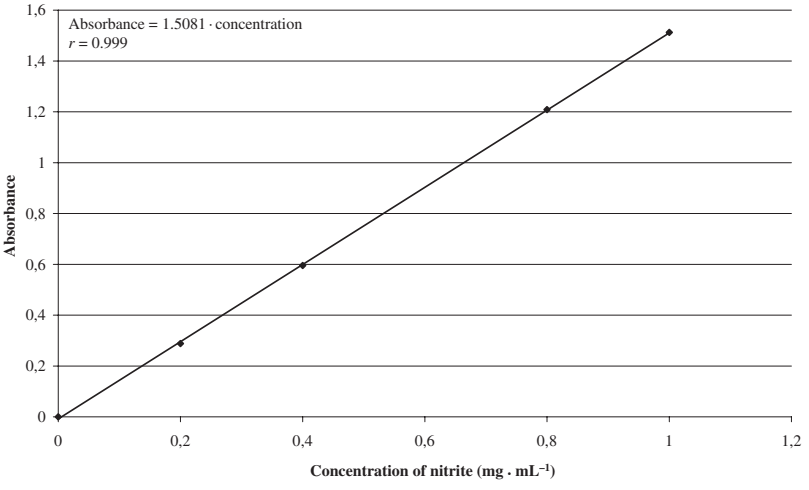

Figure 1. Analytical curve of the concentrations of nitrite ions.

Nitrate reductase activity was determined in field-moist soil samples. Briefly, 5 g soil was incubated for 24 h at 25 °C with 1 mL of 25 mM KNO_3 solution and 4 mL of 0.9 mM 2,4-dinitrophenol solution and 5 mL of deionised water. The control was incubated for 24 h at −20 °C. Nitrite reductase was inhibited by the addition of 2,4-dinitrophenol. Nitrate was released as a result of incubation extracted with 4 N potassium chloride. Colorimetric analysis was performed in a similar way to calibration standards with the difference that 5 mL of soil sample extract was used.

Ammonium ions were meausred using an ion chromatograph (Waters 1515) equipped with a 1515 Isocratic HPLC pump, conductivity detector (Waters 432), a rotary valve fitted with a 20 μL sample loop and a Hamilton column PRP-X200 (150 × 4.1 mm i.d.), protected with a guard column of the same material (25 × 2.3 mm i.d.). Detection was monitored at a sensitivity of 10 μS. The column was operated at 25 °C. The mobile phase consisted of 4 mM HNO_3 in water and methanol (70:30, v/v) at a flow-rate of 1 mL · min^{-1}. The calibration standards ranged from 0.5 to 9.0 mgN-NH_4^+ · L^{-1}.

Nitrate ions were measured using an ion chromatograph (HIC-6A Shimadzu, Japan) equipped with a LP-6A Isocratic HPLC pump, conductivity detector CDD-6A, a rotary valve fitted with a 20 μL sample loop and PRP-X100 (150 × 4.1 mm i.d.) column from Hamilton, protected with a guard column of the same material (25 × 2.3 mm i.d.). Detection was monitored at a sensitivity of 1 μS. The column was operated at 25 °C. The mobile phase consisted of 4 mM *p*-hydroxybenzoic acid with 2.5% methanol (pH 8.4) at a flow-rate of 1.5 mL · min^{-1}. The calibration standards ranged from 2.5 to 15.0 mgN-NO_3^- · L^{-1}.

Soil extracts were prepared by placing 10 g of air-dried soil, passed through a 1 mm mesh sieve, in a 250 mL beaker and adding 30 mL of deionised water. The samples were shaken vigorously for 30 min at room temperature. Next, the mixture was centrifuged and filtered by Whatman filter GF/C. Ammonium and nitrate as nitrogen were determined in these extracts.

To estimate the dissolved organic carbon (DOC), soil samples were heated in redistilled water at 100 °C for 2 h under a reflux condenser. Extracts were separated using the mean filter paper and analysed on TOC 5050A facilities (Shimadzu, Japan) [25].

Satisfactory precision based on replicate analyses were ±3.5% for nitrate reaductase activity, ±0.01 for pH measurements, ±3.5% for TOC, ±3.4% for DOC, ±4.3% for N_{total}, ±3% for N-NO_3^- and ±3% for N-NH_4^+.

Table 2. Chemical properties of soil under shelterbelt and an adjoining cultivated field.

Parameter	*Robinia pseudoacacia* shelterbelt	Adjoining cultivated field to *Robinia pseudoacacia* shelterbelt
pH	3.58	6.49
Field-moist content (%)	9.03	7.00
N-total ($g \cdot kg^{-1}$)	2.69 ± 0.03	0.78 ± 0.02
N-NH_4^+ ($mg \cdot kg^{-1}$)	35.34 ± 0.94	17.95 ± 0.97
N-NO_3^- ($mg \cdot kg^{-1}$)	18.86 ± 0.08	1.83 ± 0.21
TOC ($g \cdot kg^{-1}$)	32.04 ± 1.14	4.82 ± 0.13
DOC ($g \cdot kg^{-1}$)	3.03 ± 0.07	0.41 ± 0.10

Note: TOC, total organic carbon; DOC, dissolved organic carbon.

All experiments were replicated five times and the results averaged. All the chemicals used in this study were of analytical grade. A summary of the soil characteristics is presented in Table 2.

3. Results and discussion

Catalytic properties characterise chemical, biochemical, physical and biological processes in soil organic matter. Thus, these pathways and their mechanisms occurring in soil organic matter are significantly dependent on the properties of the environment. Significant differences in chemical properties between soil under shelterbelt and adjoining cultivated field were observed. Obviously, 200-year-old *R. pseudoaccacia* has very strong impact on the catalytic properties and chemical composition of the soil. The examined soil under shelterbelt with a pH of 3.58 (in 1 N KCl) belongs to very acidic soils (Table 2), whereas soil from the adjoining cultivated field was neutral (pH 6.49). Moreover, field-moist content of the soil under shelterbelt was equal to 9.03%, higher than for soil in the adjoining cultivated field, where it was 7.00%.

In comparison with the adjoining cultivated field, the 200-year-old shelterbelt marked an increase in the quantities of nitrogen forms. The total nitrogen content in the soil under shelterbelt was $2.69\,g \cdot kg^{-1}$; the value for soil from the adjoining cultivated field was 3.5 times lower. In addition, the concentrations of ammonium in soils under shelterbelt and the adjoining cultivated field were 35.34 and $17.95\,mg \cdot kg^{-1}$, respectively.

The principal regulatory mechanism of denitrification is a fluctuation in the partial pressure of molecular oxygen. Denitrification occurs when the O_2 is depleted from the vicinity of denitrifying microorganisms. Moreover, the concentration of nitrate or nitrite in the soil is another important regulator and the third main factor is the amount of available carbon [5,14,16]. The quantity of nitrates in the soil under shelterbelt was $18.86\,mg \cdot kg^{-1}$, 10.3 times higher than in the adjoining cultivated field. In addition, TOC for the soil under shelterbelt was also higher than in the adjoining cultivated field; in soil under shelterbelt the TOC value was $32.04\,g \cdot kg^{-1}$, whereas under the adjoining cultivated field the TOC value was $4.82\,g \cdot kg^{-1}$. Thus, accumulation of organic matter may proceed faster in shelterbelt soil than in the adjoining cultivated field.

Dissolved organic matter may contribute significantly to the cycling of soil nutrients. It can act as a substrate for microbial growth, but its production is also partly mediated by microbes. This fraction is responsible for microbiological activity [25]. In comparison with adjoining cultivated field, substantially higher DOC was available for microbiological and biochemical pathways in soils under shelterbelt. The DOC concentration in soil under shelterbelt was $3.03\,g \cdot kg^{-1}$, 7.4 times higher than in soil under the adjoining cultivated field.

Study of the relationship between point measurements of denitrification and chemical, biochemical and biological factors has been difficult. High spatial variability in denitrification rates, with

coefficients of variation exceeding 100%, has frequently been observed [21,26,27]. Significant relationships between denitrification and soil moisture [28–30] and soil nitrate [20,31] have been observed, but none of these variables has explained >50% of the variation in denitrification rates. Reasons for these poor results include difficulty in modelling interactions between the primary factors (oxygen, nitrate, available carbon) that regulate denitrification activity and an incomplete understanding of patterns of carbon availability in soil. Factors controlling denitrification in soils under shelterbelts are poorly characterised. Although irrigation, fertilisation and tillage have been emphasised as drivers in agricultural systems, other factors are important in shelterbelt. In the absence of irrigation, soil texture and drainage are key factors controlling aeration and have a direct effect on denitrification [21,30]. Soil nitrate appears to be more important in regulating denitrification in forests than in agricultural soils [18,20] and factors affecting carbon availability, such as freezing, thawing, wetting and drying, are also important regulators of denitrification in forest soils [21,32,33].

Kinetic studies modelling the changes in nitrate reductase activity in soils from shelterbelt and adjoining cultivated field at two moisture contents (field-moist and 15%) and three nitrogen concentrations (field concentration and after adding 0.25% and 0.5% of nitrogen in the form of urea) were performed.

The first-order kinetics model provided an excellent fit to the experimental nitrate reductase changes over time (Figure 2–4). Cumulative nitrate reductase activity as a function of time was characterised by exponential Equation (2) (Figure 3):

$$c_t = (c_\infty)(1 - e^{-kt}), \tag{2}$$

where c_t is the nitrate reductase activity, c_∞ is the maximum of the nitrate reductase activity, k is the first-order rate constant and t is the reaction time.

Its transformations lead to a linear relationship in agreement with the first-order kinetics reaction model (Figure 4) [34–37]:

$$\ln(c_\infty - c_t) = \ln(c_\infty) - kt. \tag{3}$$

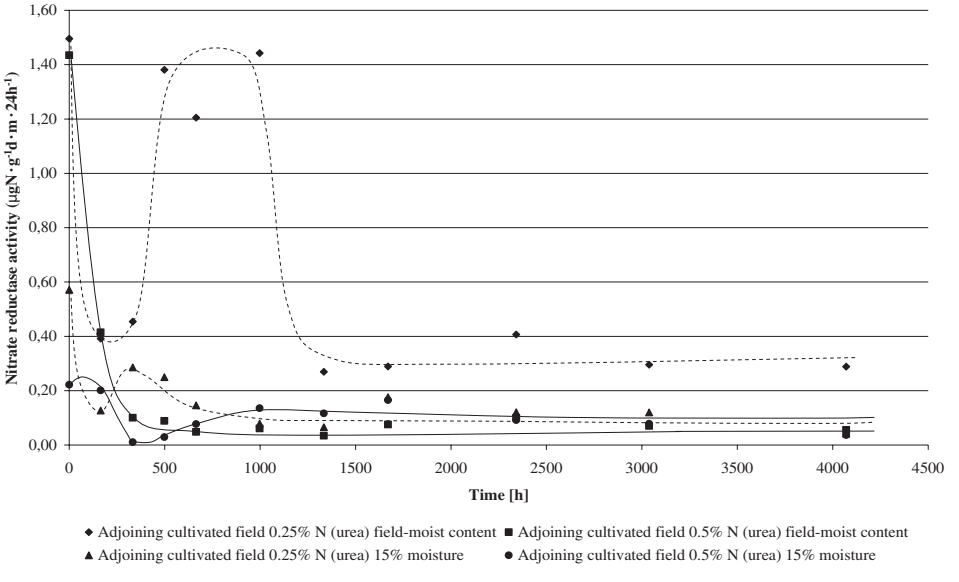

Figure 2. Changes in nitrate reductase activity in soil under adjoining cultivated field at two different moisture and nitrogen levels.

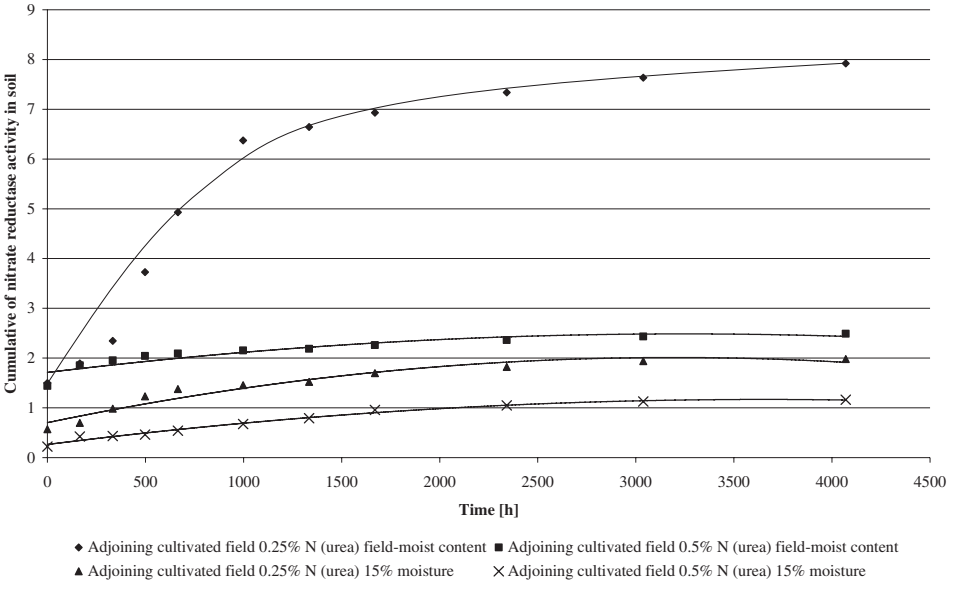

Figure 3. Cumulative nitrate reductase activity in soil under adjoining cultivated field at two different moisture and nitrogen levels.

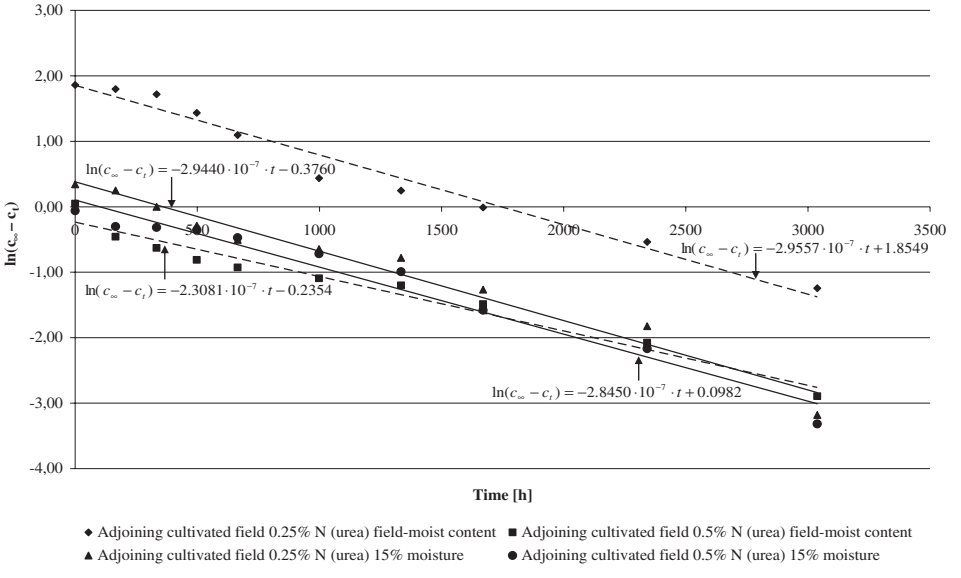

Figure 4. Semi-logarithmic functions of the changes in nitrate reductase activity in soil under adjoining cultivated field at two different moisture and nitrogen levels.

The first-order reaction rate constants were calculated as the slope of Equation (3) by means of the least squares formula (Equation 1).

Nitrate reductase activity measured over time showed a linear curve (Figure 4). The correlation coefficients varied from −0.982 to −0.998 (Table 3). The slopes of the Equation (3) describe the rate of change in nitrate reductase activity. The first-order reaction constants measured for the studied soils at two moisture contents and three nitrogen concentrations with the parameters of their statistical analysis are shown in Table 3. For all investigated samples, significant differences

Table 3. Pseudo first-order reaction rate constants ($k \times 10^{-7} \cdot s^{-1}$), half-lives ($t_{0.5}$, h), movements ($b$) and correlation coefficients (r) for the change in nitrate reductase activity in soils under shelterbelt and adjoining cultivated field with different moisture and nitrogen contents.

	Robinia pseudoacacia shelterbelt		Adjoining cultivated field to *Robinia pseudoacacia* shelterbelt	
	Field-moist content	Moisture content adjusted to 15%	Field-moist content	Moisture content adjusted to 15%
Native soil nitrogen	$k = 2.0942$ $t_{0.5} = 919.4$ $b = 1.2645$ $r = -0.994$	$k = 2.3591$ $t_{0.5} = 816.2$ $b = 0.3427$ $r = -0.998$	$k = 0.8099$ $t_{0.5} = 2377.2$ $b = 0.9885$ $r = -0.994$	$k = 2.4340$ $t_{0.5} = 791.1$ $b = 1.5114$ $r = -0.998$
Addition of 0.25% nitrogen (urea)	$k = 2.5139$ $t_{0.5} = 765.9$ $b = 4.0716$ $r = -0.985$	$k = 2.7156$ $t_{0.5} = 709.0$ $b = 3.1795$ $r = -0.998$	$k = 2.9557$ $t_{0.5} = 651.4$ $b = 1.8549$ $r = -0.994$	$k = 2.9440$ $t_{0.5} = 654.0$ $b = 0.3760$ $r = -0.994$
Addition of 0.5% nitrogen (urea)	$k = 2.7079$ $t_{0.5} = 711.0$ $b = 4.0233$ $r = -0.994$	$k = 1.5995$ $t_{0.5} = 1203.7$ $b = 3.9479$ $r = -0.982$	$k = 2.3081$ $t_{0.5} = 823.2$ $b = -0.2354$ $r = -0.994$	$k = 2.8450$ $t_{0.5} = 676.8$ $b = 0.0982$ $r = -0.984$

Note: Native soil nitrogen, concentrations of nitrogen under field conditions.

between the rate of change in nitrate reductase at the two moisture contents were observed. The first-order reaction rate constant ($2.0942 \times 10^{-7} \cdot s^{-1}$) for the change in nitrate reductase activity from soil under shelterbelt at field-moist content and a TOC content of $32.04\ g \cdot kg^{-1}$ was shown to be significantly higher than the first-order reaction rate constant ($0.8099 \times 10^{-7} \cdot s^{-1}$) calculated for soil under adjoining cultivated field with field-moist content and a TOC content of $4.82\ g \cdot kg^{-1}$ (Table 3).

The availability of organic carbon is one of the most important factors to affect denitrifying activity in soil, supplying a source of energy and a substrate for bacterial growth. In addition, organic carbon participates in an electron exchange [4,38–40]. Under aerobic conditions, denitrifying bacteria use a wide variety of organic compounds, but under denitrifying conditions, organic carbon sources may be restricted [41]. An electron supply from different organic carbon substrates may be one factor that determines their efficiency in denitrification, but the importance of this factor has rarely been isolated from its other potential effects. The capacity of soil organic carbon to release available e^- is revealed by the oxidation of soil organic matter [42].

It should be mentioned that in our experiment a significant relationship between nitrate reductase activity in field-moist soil and soil with a 15% moisture content under shelterbelt and adjoining cultivated field was observed. The increase in moisture content from field-moist soil to 15% in soil under shelterbelt led to an increase in the first-order reaction rate constant from $2.0942 \times 10^{-7} \cdot s^{-1}$ to $2.3591 \times 10^{-7} \cdot s^{-1}$ (1.13 times) and to a decrease in the half-life ($t_{0.5}$) of the reaction from 919.4 to 816.2 h.

However, an increase in the moisture content from field-moist to 15% in soil under adjoining cultivated field had a greater effect on the first-order reaction rate constant than was seen for soil under shelterbelt, with an increase from $0.8099 \times 10^{-7} \cdot s^{-1}$ to $2.4340 \times 10^{-7} \cdot s^{-1}$ (3 times); the half-life of the reaction decreased from 2377.2 h to 791.1 h. It seems that organic matter in soil under *R. pseudoaccacia* represents high stability in comparison with adjoining cultivated field.

Denitrification is favoured by high moisture content, high organic matter concentrations and basic conditions. The concentration of organic matter in soil under shelterbelt was 6.65 times higher than in soil under the adjoining cultivated field. The higher organic matter content in soil under shelterbelt in comparison with that under the adjoining cultivated field led to higher water

adsorption than mineral fractions of adjoining cultivated field. However, soil under shelterbelt showed acidic properties (pH 3.58) in contrast to soil under the adjoining cultivated field where pH was 6.58. These conditions markedly affect the redox potential in the investigated soils. Because of the acidic soil conditions under shelterbelt in comparison with the adjoining cultivated field, soil under shelterbelt may moderate changes in the nitrate reductase activity. Šimek et al. [8] and Firestone [14] suggested that in acidic soils populations of denitrifers are adapted to low pH, or more generally populations of denitrifers are adapted to prevailing soil pH (acidic, neutral and alkaline).

It should be mentioned that our findings are in agreement with other studies. Several crucial points were evident in the data by Binstock [43] with respect to the potential pattern of *in situ* denitrification of a forest soil. In contrast to the normal pattern found in agricultural soils of increasing denitrification with increased moisture content [44,45], forest soil incubated in the laboratory showed a lower production of N_2O-N at saturation than at field capacity. One possible explanation for this pattern would be retarded gas flow at moisture contents above field capacity. With a water holding capacity of 316%, based on oven-dry weight in this particular deciduous forest soil, compared with typical Iowa soil with a field capacity (30 kPa or 30 bar) of 23% moisture [46], this explanation seems plausible. Other possible explanations for a decrease in denitrification at saturation are increased non-dissimilatory nitrate reduction and alteration at higher moisture contents in the type and amount of available organic carbon, which is essential for denitrification. In forest soil, there appears to be a critical content of moisture beyond which denitrification slows, thus altering the timing of peat denitrification with respect to a typical agricultural soil [43].

Our results show that addition of 0.25 and 0.5% nitrogen to field-moist soil under shelterbelt significantly accelerated the rate of change in nitrate reductase activity. Estimated values for the first-order reaction rate constants increased from $2.0094 \times 10^{-7} \cdot s^{-1}$ to $2.5139 \times 10^{-7} \cdot s^{-1}$ and further to $2.7079 \times 10^{-7} \cdot s^{-1}$, however, the half-life of the reaction decreased from 919.4 to 765.9 h and further to 711.0 h (Table 3).

However, the direction of the change in nitrate reductase activity at 15% moisture in soil under shelterbelt and in field-moist soil from the adjoining cultivated field were similar. The addition of 0.25% nitrogen to soils under shelterbelt at 15% moisture resulted in an increase in the first-order reaction rate constants. The increase in the nitrogen content in soil under shelterbelt from the field amount to 0.25% was connected with an increase in the first-order reaction rate constant from $2.3591 \times 10^{-7} \cdot s^{-1}$ to $2.7156 \times 10^{-7} \cdot s^{-1}$ (1.15 times) and with a decrease in the half-life of the reaction from 816.2 to 709.0 h. Furthermore, addition of 0.5% nitrogen to this soil impacted on the decrease in the first-order reaction rate constant and increase in the half-life of the reaction from $1.5995 \times 10^{-7} \cdot s^{-1}$ to 1203.7 h.

Consequently, the addition of 0.25% nitrogen to the adjoining cultivated field with field-moist content led to a 3.65 times increase in the first-order reaction rate constant. However, further addition of 0.5% nitrogen to this soil revealed, similar to soil under shelterbelt with a 15% moisture content, a decrease in the first-order reaction rate constant and an increase in the half-life of the reaction from 651.4 to 823.2 h. Moreover, the addition 0.25 and 0.5% of nitrogen to soil from the adjoining cultivated field with 15% moisture resulted in a similar increase in the first-order reaction rate constants and half-lives.

Our findings are consistent with the results of Bremner and Blackmer [47,48]. They proposed a much higher N_2O yield (expressed as a proportion of urea-N or NH_4^+-N added) at high N addition rates ($400\ mgN \cdot kg^{-1}$ soil compared with $50–100\ mgN \cdot kg^{-1}$ soil). Bremner and Blackmer reported definitive evidence that N_2O is released on incubation of aerobic soils treated with NH_4^+. This was verified in subsequent studies [48–50]. They found that the ratio of N_2O-N released to NO_3^--N formed was $\sim 0.6 \times 10^{-3}$ and was fairly constant over 16 days. Ratios of N_2O to $(NO_2^- + NO_3^-)$-N produced ranged from 0.4 to 2×10^{-3}. Freney et al. [44] reported N_2O and

NO_3^- production data for one soil; the N_2O-N to NO_3^--N ratio was initially $\sim 0.12 \times 10^{-3}$, but was relatively constant after day 2 at $\sim 1 \times 10^{-3}$.

Rolston et al. [51] postulated that the application of fertiliser to agricultural soils is followed by an increase in the N_2O flux. They found that the N_2O flux increased when fertilisers were applied. The highest flux was found under the wettest soil conditions. Denmead et al. [52] suggested that the flux from grasslands increased after irrigation and rainfall. Conrad and Seiter [53] and Christensen [54] showed that the increase in N_2O-flux after fertilisation is dependent on the type of fertiliser. Mosier et al. [55] pointed out a close relationship between N_2O-flux and soil moisture for a shortgrass prairie. They also postulated that an application of urea increased the flux.

4. Conclusions

Our study on nitrate reductase activity in soil under 200-year-old shelterbelt (*R. pseudoaccacia*) and an adjoining cultivated field showed excellent fit of the modelling kinetic studies to the experimental nitrate reductase changes over time at two moisture contents (field-moist and 15%) and three nitrogen concentrations (field concentration and after addition of 0.25 and 0.5% nitrogen in the form of urea).

The increase in the moisture content from field-moist to 15% in soil under shelterbelt and in adjoining cultivated field led to significantly higher values for the first-order reaction rate constants in soil under an adjoining cultivated field compared with soil under shelterbelt. Changes in nitrate reductase activity at 15% moisture in the soil under shelterbelt and at field-moist content in an adjoining cultivated field were similar.

The addition of 0.25% nitrogen to the adjoining cultivated field with field-moist soil led to a 3.65 times increase in the first-order reaction rate constant. However, further addition of 0.5% nitrogen to this soil revealed, similar to the soil under shelterbelt with a 15% moisture content, a decrease in the first-order reaction rate constant.

Acknowledgements

This study was supported by the grants N N305 121934 and N N305 320436 founded by the Polish Ministry of Education.

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