UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
ACADEMIC STUDY IN BIOTECHNOLOGY

Anton GOVEDNIK

DYNAMICS OF DENITRIFYING SOIL MICROBIAL COMMUNITY AND GREENHOUSE GAS EMISSIONS UNDER CONVENTIONAL AND ORGANIC SYSTEM OF WINTER WHEAT PRODUCTION IN DEPENDENCE OF PRE-CROP AND FERTILIZATION

M. SC. THESIS

Master Study Programmes

Ljubljana, 2016

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DINAMIKA DENITRIFICIRAJOČE TALNE MIKROBNE ZDRUŽBE IN EMISIJE TOPLOGREDNIH PLINOV V KONVENCIONALNEM IN EKOLOŠKEM SISTEMU PRIDELAVE OZIMNE PŠENICE V ODVISNOSTI OD PREDPOSEVKA IN GNOJENJA

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Commitee for evaluation and defence of M. Sc. Thesis (komisija za oceno in zagovor):

Chair (predsednica):	Prof. Dr. Branka Javornik University of Ljubljana, Biotechnical Faculty, Department of Agronomy
Member (članica):	Assist. Prof. Dr. Marjetka Suhadolc University of Ljubljana, Biotechnical Faculty, Department of Agronomy
Member (član):	Assist. Prof. Dr. Andreas Gattinger Research Institute of Organic Farming (FiBL), Department of Soil Sciences
Member (članica):	Prof. Dr. Ines Mandić Mulec University of Ljubljana, Department of Food Science and Technology

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AB Nitrous oxide (N_2O) is a long-lived greenhouse gas which is also a potent ozone depleting substance. Main anthropogenic contributor to N_2O emissions is the agricultural sector with its land usage and fertilization practices. In order to develop effective mitigation measures, it is important to understand the processes which contribute to N_2O production. This study was conducted to examine impact of different fertilizer forms (mineral and organic) and pre-crop history (rapeseed and soybean) under conventional (CONFYM) and organic (BIOORG) farming system on microbial community composition and N_2O production. Abundance of denitrifier's functional genes and gene transcripts (*nirS*, *nirK*, *nosZ*, *nosZ II*) as well as N_2O emissions were monitored for 19 days after fertiliser application in spring 2015 at the long-term DOK trial (Therwil, Switzerland). There were no differences observed between the two farming systems, while pre-crop had an effect on N_2O emissions. Higher emissions were detected in soybean which was reflected by increased soil water content. At the same time increases of denitrifier community (especially *nir*, *nosZ*) were observed on the transcript, but not on the gene level. While results presented in this thesis may play a role in understanding the pre-crop effect on emissions, further studies are needed to explain the mechanisms completely.

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AI Didušikov oksid (N_2O) je v atmosferi dolgoživ toplogredni plin, ki uničuje ozon. Glavni antropogeni vir le-tega je kmetijski sektor z obdelovanjem tal in gnojenjem. Če želimo zmanjšati emisije N_2O, moramo poznati mehanizme in procese pri katerih nastaja. To študijo smo zastavili z namenom razlikovanja vpliva različnih gnojil (mineralnega in organskega), zgodovine predposevkov (oljna ogrščica in soja) v konvencionalnem (CONFYM) in ekološkem (BIOORG) kmetijskem sistemu na mikrobno združbo in emisije N_2O. Spremljali smo spremembe številčnosti genov in transkriptov genov (*nirK*, *nirS*, *nosZ*, *nosZ II*) denitrificirajoče mikrobne združbe ter emisije N_2O 19 dni po spomladanskem gnojenju na dolgoletnem preiskusu DOK v Therwilu v Švici leta 2015. Med kmetijskima sistemoma nismo opazili razlik v preučevanih parametrih, medtem ko je bil vpliv predposevka statistično značilen. Večje emisije smo izmerili pri soji, ki jih lahko povežemo s povečanjem vsebnosti vode v tleh. Prav tako se je povečala transkripcijska aktivnost denitrifikatorjev (številčnost transkriptov *nir* in *nosZ*), medtem ko njihov potencial (številčnost genov) ni bil odvisen od vsebnosti vode v tleh. Rezultati predstavljeni v tej študiji so pokazali vpliv predposevka na emisije N_2O, vendar so za poglobljeno razumevanje mehanizmov potrebne nadaljne študije.

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1 INTRODUCTION

Soils contribute to the fluxes of greenhouse gases (CO_2, N_2O and CH_4) by acting as sources or sinks (Conrad, 1996). Nitrous oxide (N_2O) is one of the most potent ozone depletion substances; it has 298 times stronger greenhouse warming potential than CO_2 and has a lifetime of 114 years (WMO, 2014). N_2O from cultivated agricultural soils covers the biggest share of all agricultural greenhouse gas (GHG) emissions, excluding CO_2 resulting from land use change (Smith et al., 2008). Developing strategies to reduce N_2O emissions is a key challenge for the agricultural sector.

The fact that N_2O production is closely linked to the fertilizer application has important implications for management of N_2O emissions (Smith et al., 2008). As the extent of N_2O emissions from soils largely depends on microbial activity, and especially denitrification, mitigation of N_2O emissions will not only be achieved by reducing fertilizer use but will also require measures to alter microbial activities associated with N_2O production.

Although the impact of single parameters, such as nitrate (NO_3^-) and dissolved organic carbon (DOC) concentrations, soil water content, temperature, and pH, on N_2O emissions are quite well studied (Wallenstein et al., 2006), there is still a knowledge gap concerning the impact of complex farming system (Butterbach-Bahl et al., 2013). In order to close this knowledge gap, *in situ* experiments on long term trials comparing farming systems are necessary to untangle the complexity of processes leading to N_2O emissions.

One example of such experiment is long term DOK trial, established in 1978 in Therwil, near Basel (Switzerland). Four farming systems (CONMIN, CONFYM, BIOORG and BIODYN) together with negative control (NOFERT) are compared, differing in fertilization strategy and concept of plant protection (Mäder et al., 2002; Mayer et al., 2015). Our study was conducted on fields managed according to the two farming systems: CONFYM and BIOORG, differing in the application rate and type of fertilization. Conventional CONFYM system was fertilized with higher rate of mineral fertilizer, while organic BIOORG system was fertilized with lower rate and organic fertilizer (slurry). All the test fields were sown by winter wheat and were one season prior to our study sown by two different crops (pre-crops): soybean and rapeseed. The experimental set up for this study covered four different treatments: soybean BIOORG and CONFYM and rapeseed BIOORG and CONFYM. It allowed to evaluate effects of two pre-crops (rapeseed and soybean) under two distinct farming systems (conventional – CONFYM and organic – BIOORG) on N_2O emissions and abundance of denitrifying microbial community functional genes and transcripts (*nirK*, *nirS*, *nosZ*, *nosZ II*) in addition to the abundance of the whole bacterial community (16S). Based on the state of the art the following hypotheses were postulated:

(i) Farming system will have an effect on N_2O emissions. Significantly higher emissions are expected in CONFYM treatment.

(ii) Pre-crop will have an effect on N_2O emissions. Significantly higher emissions are expected in soybean as its residues have lower C: N ratio.

(iii) Since denitrification is heterotrophic process the organic carbon added in BIOORG treatment will increase N_2O emissions.

(iv) Water content in the soil is expected to have positive impact on denitrification and that should be observed by N_2O emissions and on the transcriptional activity level of denitrifiers.

(v) N_2O reducers will have the biggest impact in BIOORG system because of the organic carbon addition and relatively low contents of quickly available nitrogen.

(vi) Transcript ratio of *nir* / *nos* will be positively correlated with N_2O emissions.

2 LITERATURE REVIEW

Nitrous oxide (N_2O) is a greenhouse gas that contributes ~ 6% to global long lived greenhouse emissions. Atmospheric N_2O has an atmospheric lifetime of 114 years and its emissions have increased for 21% since pre-industrial times (WMO, 2014). It is the third most important anthropogenic greenhouse gas with a global warming potential ~ 300 times higher than carbon dioxide (Forster et al., 2007) and single most potent substances depleting ozone layer (Ravishankara et al., 2009). Prior to industrialization the atmospheric N_2O was generally balanced by production from soils and oceans and chemical losses in the stratosphere (WMO, 2014). With industrialization new anthropogenic sources of N_2O emissions emerged mostly from agriculture. This is a consequence of synthetic nitrogen fertilizers application which act as a source of N_2O emissions directly from the field and indirectly from ammonia or nitrate when emitted/leached to the atmosphere or aquatic systems. Other mankind's sources are fossil fuel combustion, biomass burning and some other minor processes. Currently anthropogenic sources represent ~ 40% of total emissions (WMO, 2014). Main pathway of nitrous oxide production is contributed to microbial transformations in soil, water and sediments (Syakila and Kroeze, 2011).

2.1 N_2O PRODUCTION PATHWAYS

Main source of agricultural N_2O emissions are microbial transformations in soil called nitrification, denitrification and nitrifier denitrification (Kool et al., 2011), which are all part of the nitrogen cycle. Nitrogen is introduced into the soil with fixation by nitrogen fixing procaryotes (bacteria and archaea). In this reaction elementary nitrogen (N_2) is reduced to ammonium (NH_4^+) under anoxic conditions (Canfield et al., 2010). Ammonium can then be oxidized in aerobic conditions into nitrit (NO_2^-) and further on to nitrate (NO_3^-) in the process called nitrification. NO_3^- is the most oxidised form of nitrogen with redox state of +5 and can be reduced in anaerobic conditions to N_2 through NO and N_2O intermediates in the process of denitrification or to NH_4^+ through dissimilatory NO_3^- reduction to ammonium (DNRA). Annamox which is reduction of NO_2^- to N_2 using NH_4^+ as an electron donor was shown to occur in different environments but has minor role in the soil ecosystems (Figure 1).

In the natural systems different reductions of nitrogen oxides occur but they are not all denitrification processes. Clear criteria were established by Mahne and Tiedje (1995) for distinguishing denitrifiers from NO_3^--respiring and NH_4^+-producing bacteria:

1. N_2O and/or N_2 must be the major end product of NO_3^- or NO_2^- reduction.

2. This reduction must be coupled to an increase in biomass which greater than if NO_3^- or NO_2^- served just as an electron sink.

Referring to those non-denitrifying pathways, which can still be a source of N_2O or N_2, the following can be distinguished: (i) dissimilative NO_3^- reduction to NH_4^+, where NO_3^- serves as an electron sink but can also provide energy, (ii) (chemical) oxidation of NH_2OH to N_2O, (iii) Annamox (Crowe et al., 2012), (iv) NO reduction to N_2O, to reduce stress induced by this oxide (Philippot, 2005).

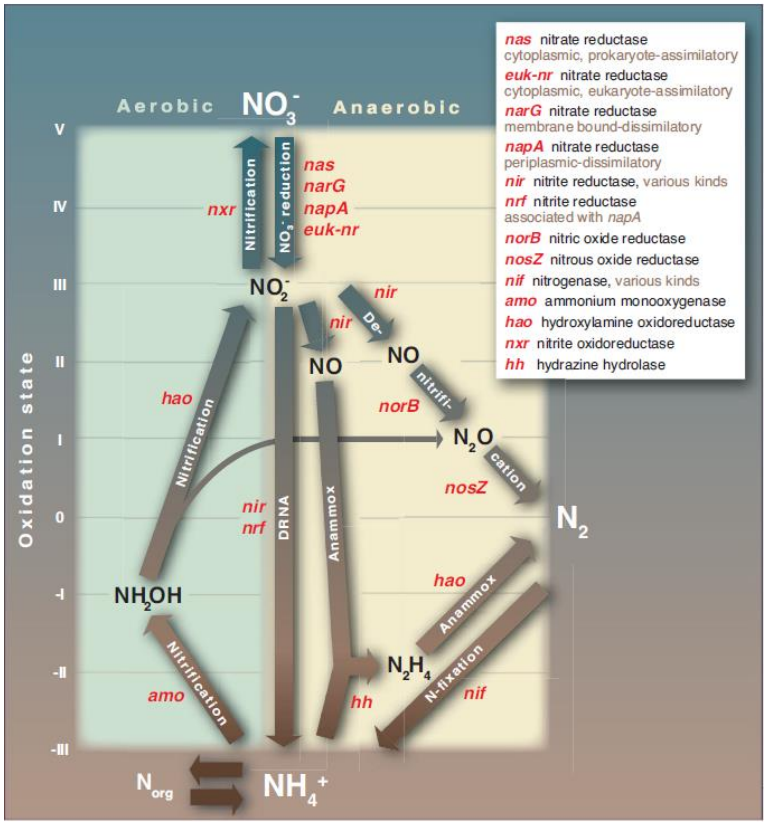

Figure 1: Nitrogen cycle (Canfield et al., 2010).
Slika 1: Dušikov cikel (Canfield in sod., 2010).

2.1.1 Denitrification

Denitrification is the form of heterotrophic microbial respiration where soluble nitrogen oxides are used as electron acceptors when oxygen itself is not available.

First step of denitrification (Figure 2) is reduction of NO_3^- to NO_2^- (244 kJ) which is catalysed by enzyme NO_3^- reductase either membrane bound (Nar) coded by *narG* or periplasmic (Nap) coded by *napA* gene. Both can be observed in the same strain (Roussel-Delif et al., 2005). The key step in denitrification pathway is reduction of NO_2^- to gaseous NO by NO_2^- reductase (Nir) which exists in two evolutionary distinct forms but which are functionally equivalent and mostly exclusively observed in single strains (Graf et al., 2014): copper containing Nir encoded by *nirK* gene and cytochrome cd_1 containing Nir encoded by *nirS*. In the next step of denitrification NO is reduced to N_2O which is

performed by two enzymes which receive electrons from two different donors: from cytochrome *c* (cNor) and quinol pool (qNor) which are encoded by *norB* and *qnorB* gene respectively. Final denitrification step is forming of N_2 and is catalysed by NosZ (*nosZ* and *nosZ II* genes) which is homodimeric multicopper enzyme in the periplasm of Gram-negative bacteria (Philippot et al., 2007). It is known that N_2O by itself is not triggering expression of denitrifying genes. Rather low O_2 and high NO concentration had been observed to influence *nir* and *nos* expression. O_2 competes as electron acceptor and therefore reduces need for denitrification. NO is on the other hand a toxic product that should not be accumulated and has to be reduced further. However exact mechanisms are not universal and different strains have different regulations of denitrification (Spiro, 2012).

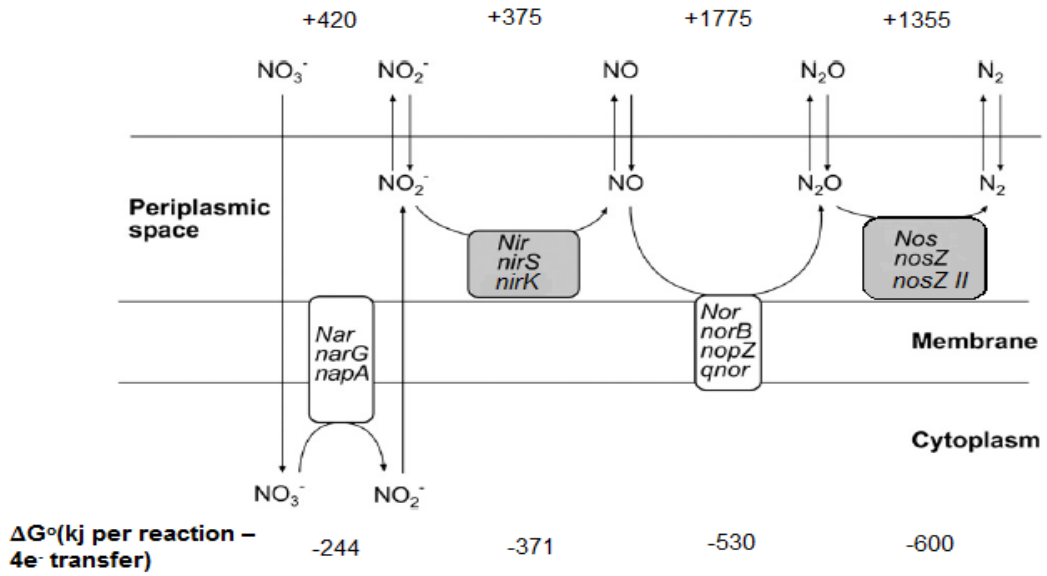

Figure 2: Overview of denitrification pathway genes (*narG, napA, nirS, nirK, nosZ, nosZ II*) enzymes with their cell location (Nar, Nir, Nor, Nos) responsible for each reaction accompanied by redox potentials (above) and energy yield (below) per each reaction (modified after: Saggar et al., 2013).
Slika 2: Pregled denitrifikacijske poti z geni (*narG, napA, nirS, nirK, nosZ, nosZ II*) in celično lociranimi encimi (Nar, Nir, Nor, Nos) potrebnimi za le-te ter redoks potenciali (zgoraj) in energetskimi donosi (spodaj) na posamezno reakcijo (prirejeno po: Saggar in sod., 2013).

2.1.2 Microbial community responsible for denitrification

The ability to denitrify is widely distributed among taxonomic and phylogenetic groups of microorganisms (Jones et al., 2008). Since the development of molecular methods for detecting microbial community responsible for denitrification scientists are trying to link N_2O emissions with denitrification community structure which is usually determined by its hallmark genes *nirS, nirK nosZ* and *nosZ II*. Since molecular methods are not very robust different findings were made from conclusions ranging from no connection between community structure and function (Dandie et al. 2008; Boyle et al. 2006; Rich and Myrold

2004) to results where community structure was related to its function (Kandeler et al., 2009; Németh et al., 2014; Philippot et al., 2011). The first group of conclusions originate mostly from field experiments, where determining factors, such as rain and temperature, are hard to control and therefore eliminate spatial and temporal heterogeneity. The second group of conclusions mostly represent mesocosm incubation experiments where environment is more controlled, but for this reason realistic field conditions are not fully mimicked (Németh et al., 2014). Denitrifier community generally consist of one third of only *nirS* and *nirK* bearing organisms, which could be assessed by detecting *nirS* and *nirK* genes, and two thirds of complete denitrifiers, which contain either *nosZ* or *nosZ II* additionally to one of the *nir* genes (Jones et al., 2013; Philippot et al., 2011; Sanford et al., 2012). Interestingly *nosZ* and *nosZ II* could be detected in organisms possessing neither of the previous denitrification genes in the pathway. Portion of those is more than half among the ones possessing *nosZ II* and around 17% among *nosZ*, which means that the greater the ratio of *nosZ II*:*nosZ* is, the likelier a specific community will act as a N_2O sink (Jones et al., 2014; Sanford et al., 2012). Genes *nirS* and *nirK* represent two distinct phylogenetic clades of *nir* genes, which are exclusively present in individual strain of bacteria and also ecological niches (Jones et al. 2008; Jones and Hallin 2010). Similar niche partitioning as of *nir* bearing community was detected among *nosZ* and *nosZ II* clades (Jones et al., 2014), which was at the same time reflected by phylogenetic analysis of bacteria constituting both clades (Sanford et al., 2012).

Different communities and even phylogenetically closely related microorganisms are showing different functions that are not consistent, which means they are hard to predict or model (Braker et al., 2012). However, especially phylogenetic diversity of denitrification genes (*nosZ II*) bearing bacteria and *nirS*:*nirK* ratio was shown to reduce N_2O formation in soil. This could be due to the fact that *nosZ* is less abundant among *nirK* compared to *nirS* denitrifiers (Jones et al., 2014), which means that with increasing *nirS*:*nirK* ratio also the relative abundance of *nosZ* in community is increasing. The same authors also observed that soils with lower *nosZ* diversity are dominated by *nirK* type denitrifiers, which again supports previous thesis.

Finally, increased diversity means not only broad range of metabolisms, but also diverse regulation network, which makes it harder to identify relationships between community structure and function (Sanford et al., 2012; Braker and Conrad, 2011). In an experiment where *nir* abundance and consequently *nosZ* portion were manipulated, an increased denitrification potential was observed and N_2O emissions followed this pattern to some extent. Increase in denitrification potential was not completely reflected by N_2O emissions, which means that indigenous community somehow adjusted to increased N_2O flux with higher Nos activity (Philippot et al., 2011). This is another proof that there are other factors besides community structure which are influencing denitrification starting with soil type (Philippot et al., 2011). A meta study performed by (Rocca et al., 2014) showed correlation between abundance of denitrifier genes *nirK*, *nirS*, *nosZ* and the process of N_2O formation.

However, no correlation was observed between the process and activity of the same genes on RNA level, which could be due to the small data set available for the study or methodological constraints during RNA analysis. In the same study agricultural soil was the only habitat with the single highest statistical significance ($p < 0.001$) where those differences could be found.

2.2 FACTORS INFLUENCING N_2O EMISSIONS

Denitrification, being one the main causes for N_2O emissions in arable land, is influenced by several proximal and distal environmental factors (Figure 3). The first two proximal factors (soil nitrate and carbon content) are mostly influenced by fertilization and land management practices, while the second two (temperature and soil oxygen saturation) mostly depend on environment and climate.

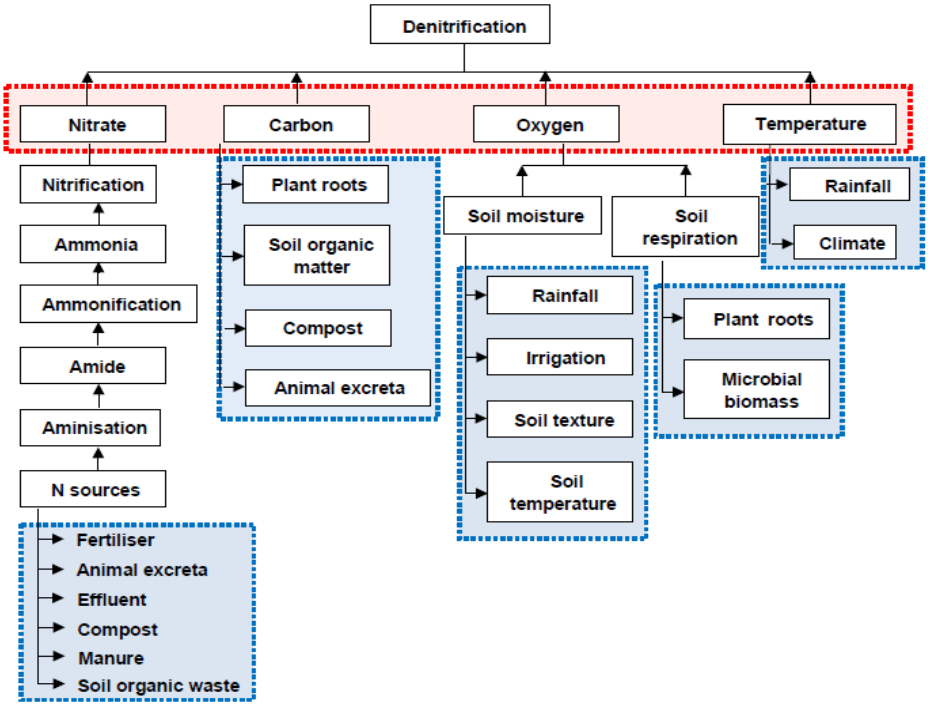

Figure 3: Factors influencing denitrification in agricultural soils, proximal (red box) and distal (blue boxes) (Saggar et al., 2013).
Slika 3: Proksimalni (rdeči) in distalni (modri) faktorji, ki vplivajo na denitrifikacijo v kmetijskih tleh (Saggar in sod., 2013).

2.2.1 Agricultural systems

Organic and conventional farming systems usually differ by their land management, fertilization and crop protection strategies. On one hand, organic farming is usually associated with lower energy inputs, reduced pesticide usage, higher biodiversity and enhanced fertility. However, due to the lower inputs also the crop yields are lower (Mäder

et al., 2002). On the other hand, conventional farming systems are associated with the opposite: higher energy inputs, especially in the form of applying mineral fertilizers and crop protection agents (pesticides). The major factor that influences N_2O emission with regard to different farming systems is N fertilization (see 2.2.2). In organic farming mineral N fertilizers are not allowed (Bio Suisse, 2016), which is the reason why only organic fertilizers are applied, while in conventional farming the combination of both is usually applied (IP-Suisse, 2016).

2.2.2 Fertilization

Fertilization is agricultural practice that contributes the biggest share to anthropogenic N_2O emissions. Since the development of the Haber Bosch process, that fixes molecular dinitrogen (N_2) from the air as NH_3 fast and easy application of mineral N was possible to ensure plant nutrition. As nitrogen is the most limiting macronutrient for plant growth and development if applied reasonably, it increases crop yield and quality. However, considering that it is mostly applied in the form of synthetic fertilizer, consisting of easily leachable forms (NO_3^-, NH_4^+, urea), fertilization event (application rate, time of application, vegetation dynamics) should be carefully planned. All these facts were proved to have an impact on N_2O emissions (Shcherbak et al., 2014). When applied excessively, the efficiency of plants to use N is decreased and more N_2O is emitted which is an economical loss and an environmental problem at the same time (Liu et al., 2015). As the biggest contributor to N_2O emissions it should be carefully studied in order to mitigate N_2O emissions.

Study by Smith et al. (2012) showed that there is little difference in N_2O emission rate depending on the form of nitrogen (ammonium nitrate, calcium ammonium nitrate, urea, urea ammonium, sulphate and urea ammonium nitrate) applied. Even further in the study of Louro et al. (2015) where organic and mineral fertilizations were compared the results showed that both fertilizer forms caused the same amount of N_2O emissions when the application rate (200 kg N ha^{-1}) was the same for both treatments. Therefore lower N_2O emissions usually assumed in organic farming systems (which mostly use organic fertilization) are therefore on account of lower N inputs in those systems (Skinner et al., 2014). Since fertilizer formulation doesn't really have an impact on N_2O emissions other factors such as timing, rate and depth of application (below or on the soil surface) which have greater impact should be manipulated in order to decrease emissions. For example in the study of Velthof et al. (2003) it was shown that sub-surface (5 cm soil depth) application of fertilizer leads to higher N_2O emissions for both mineral and organic fertilizer in comparison to the soil surface application. Timing and rate of fertilizer application usually depends on vegetation dynamics of each individual crop and site conditions (soil mineral N content). In the case of winter wheat, the total amount of N that will be applied is usually split in two or more applications (Smith et al., 2012; Gu et al.,

2013; Mayer et al. 2015). First of the three applications, which was also subject of our investigation, is usually applied at the beginning of winter wheat tillering which is normally in the middle of March. A study by Smith et al. (2012) showed that from the three fertilizer applications the first one produced the lowest peak in winter wheat. That was probably also due to the low dose (40 kg N ha^{-1}) that was applied in comparisson to the second and third fertilizations when 70-90 kg N ha^{-1} was applied. If all of the nitrogen would be applied in one setting this could lead to an exponential increase of emissions. In the example of meta-analysis by Shcherbak et al. (2014) it was shown that N_2O emissions start to increase exponentially after the point when N inputs are exceeding crop needs. Timing of separate fertilizer applications should therefore be carefully studied firstly in order not to exceed or underestimate crop needs and secondly to reduce nitrogen leaching and N_2O emissions (after a big rain event and sequential water logging).

2.2.3 Crop residue management

Another agricultural practice for increasing macronutrient content in the soil is crop residue incorporation. Macronutrients are released into the soil via mineralization which is stimulated in optimal conditions (water content, temperature). This can consequently lead to N_2O emissions (Laville et al., 2011). Crop residues differ in C: N ratio depending on their plant source. However if the N mineralization or immobilization will take place depends on the value of C:N ratio. Breakeven point was experimentally determined at 41 (Vigil and Kissel, 1991). Higher C:N ratio stimulates N immobilization, while lower stimulates N mineralization. Review by Shan and Yan (2013) showed no increase in N_2O emissions when only crop residues were incorporated. However when additional fertilizer was applied significantly higher emissions were measured compared to controls. The authors concluded that this could be due to the supply of easily available C that enhances denitrifyer activity and thereby N_2O emissions. Nitrogen for denitrification was provided from both mineral fertilizer N and crop residue N. At the same time mineralization increases local O_2 consumption which makes conditions suitable for denitrification (Velthof et al., 2002). This goes in hand with the findings from the study of Huang et al. (2004) where low C: N ratio was positively correlated with increased N_2O emissions. Pre-crop residues with high C: N ratio were accordingly shown to even reduce N_2O emissions compared to controls due to immobilization of N (Baggs et al., 2000). Therefore it can be concluded that N_2O emissions from soil can be influenced by C:N ratio of crop residues especially in the period immediately following incorporation when mineralization occurs.

2.2.4 Soil chemical factors

Denitrification is generally promoted in anaerobic environments when carbon as a source of electrons, NO_3^- as an initial substrate and microbes capable of catalysing this pathway are readily available (Philippot et al., 2007).

Mineral nitrogen in the form of NO_3^- as initial substrate was shown to have a positive impact on denitrification. Sources of NO_3^- in soil are mostly fertilizer applications and mineralization with coupled nitrification, while sinks are mostly denitrification, plant uptake, microbial immobilization and leaching.

Concentration of NO_3^- is one of the factors that positively influence the N_2O:N_2 ratio (Zaman et al., 2007). This means that higher NO_3^- concentrations favour incomplete denitrification as Nos activity is suppressed due to the fact that denitrifiers obtain more energy with the first three steps in denitrification pathway (Figure 2) than with one additional step (Saggar et al., 2013). N_2O is the first nontoxic product which has to be produced during denitrification but N_2 is produced optionally. When a lot of substrate (NO_3^-) is available the microbes use it as fast as possible and the pathway runs till the first nontoxic product. However when NO_3^- concentration decreases conversion of N_2O to N_2 is favoured again (Swerts et al., 1996).

Given that denitrification is a heterotrophic process it is obvious that it will be heavily dependent on available organic carbon in the soil as an electron donor. Soil organic matter (SOM) provides microbes with labile organic carbon and inorganic N via mineralisation of organic substrates. Inorganic N in the form of NH_4^+ serves as substrate for nitrification where final product NO_3^- serves as a substrate further on for denitrification. Even though SOM provides substrates for nitrification and denitrification it is still not clear if this has a positive or negative impact for N_2O emissions (Gu et al., 2013; Snyder et al., 2009). In fact when soil was supplied with large quantity of labile carbon or urea fertilizer was applied with an organic carbon source comparably low N_2O emissions were observed. Reason for that could be that some of the added N was immobilized or because more N_2O was reduced to N_2 (Baggs et al., 2000; Senbayram et al., 2009). However it was shown that dissolved organic carbon (DOC) rather than the amount of total organic carbon has a positive effect on denitrification. This could be explained by higher biodegradability of low molecular weight organic acids which may account for substantial portion of DOC (Castaldelli et al., 2013; Saari et al., 2009). In addition to that higher DOC levels are in general related to lower N_2O:N_2 ratio (Vallejo et al., 2006). The same was observed in the study of Senbayram et al. (2012) where at low NO_3^- concentration and high available labile carbon concentrations lower N_2O:(N_2O+N_2) denitrification ratio was detected. On the other hand in the meta-analysis by Schcherbak et al. (2014) higher emission rates were associated with soil having bigger carbon pools (> 1.5%). From all this it can be concluded that soil carbon content obviously influences denitrification and N_2O emissions. However the role of action depends on the type of carbon involved and soil nitrate concentration.

Electrons provided by organic carbon oxidation are in denitrification received by nitrogen oxides (NO_3^-, NO_2^-, NO and N_2O). Oxygen (O_2) is competing with them as an alternative electron acceptor. In this case O_2 is preferred as a terminal electron acceptor because higher energy yields are obtained. This is also the reason why denitrification is inhibited at

O_2 concentrations higher than 5% (Hochstein et al., 1984). Nature of inhibition is through Nos enzyme inhibition which catalyses the last step of denitrification (Figure 2) which positively affects N_2O:N_2 ratio (Spiro, 2012). This means that denitrification can run in suboptimal conditions but it's incomplete (N_2O instead of N_2 is the final product). O_2 concentration in the soil is decreasing with increasing soil water content due to reduced diffusivity of gasses. Therefore soil water content acts as an indirect factor influencing denitrification (Saggar et al., 2013). The optimum for denitrification was estimated at around 80% water filled pore space (WFPS) (Kool et al., 2011).

Denitrification occurs in a wide temperature range between sub-zero to 75 ^oC with the most optimal at around 30 ^oC. Beside denitrification kinetics temperature influences gas diffusivity, mineralization and substrates availability which altogether influence denitrification (Saggar et al., 2013). The temperature, together with pH, is one among the more stable soil proximal factors influencing denitrification (Figure 3). Denitrification can occur in a wide range of pH but it was shown that N_2O emissions were increased in soils with pH lower than 7 (Shcherbak et al., 2014). However this doesn't mean that also complete denitrification (N_2 as final product) is stimulated in soils with lower pH. Low pH was found to increase N_2O:N_2 ratio due to: (i) post-transcriptional Nos sensitivity (Bergaust et al., 2010), (ii) increased abundance of denitrifying fungi compared to bacteria which lack N_2O reduction enzymatic pathway (Bååth and Anderson, 2003; Bergaust et al., 2010) and (iii) abiotic transformations of NO_2^- to NO_3^- (Clough et al., 2001) which then represent substrate for denitrification.

2.2.5 Pre-crop legacy

One of the research questions of our study was if there is pre-crop effect that influences microbial community and sequentially N_2O emissions. Not many studies were done in this field but the ones published (Gulden et al., 2015; Hossain et al., 2015; Philippot et al., 2013; Majchrzak et al., 2010) show conflicting results. In the study of Gulden et al. (2015) it was shown that pre-crop history has an effect on microbial community and also on potential denitrification (DEA). It was suggested that this was caused by increased number of weeds due to reduced herbicide use. This goes well with the conclusions of Philippot et al. (2013) where it was reported that rizosphere effect has presumably a big effect on microbial community. On the other hand results of the study by Hossain et al. 2015 show that planting Brassicacea plants (family to which also rapeseed belongs) have no influence on N cycling genes of microbial community. Since pre-crops from two different families (Brassicacea and Fabaceae) are tested in our experiment more distinct results are expected.

3 MATERIALS AND METHODS

3.1 DOK EXPERIMENTAL SITE

DOK trial was set up in 1978 in Therwil in the vicinity of Basel, Switzerland to compare different farming systems: organic, biodynamic, and conventional. The organic farming system (BIOORG) is managed according to the guidelines of Bio Suisse (Bio Suisse, 2016), while biodynamic farm system (BIODYN) is managed according to Demeter Suisse guidelines (Demeter-Schweiz, 2016). Two conventional systems (CONFYM and CONMIN) included are managed according to the Swiss integrated management standard since 1985 (IP-Suisse, 2016). The two differ in the form of fertilizers: CONFYM is fertilized with farm yard manure or mineral fertiliser, while CONMIN is fertilized solely with mineral fertilizer. BIOORG, BIODYN, CONFYM are fertilized at the two different fertilization levels corresponding to 0.7 and 1.4 livestock units ha^{-1} (50 and 100% of recommended fertilization rate) and marked 1 and 2, respectively. Also a negative control as unfertilized treatment (NOFERT) is included. Finally, all the treatments are replicated 4 times and each is additionally divided into 3 different stages of the same crop rotation, which gives in total 96 plots of 100 m^2 (5mx20m). The soil type is Haplic Luvisol on deep deposits of alluvial loess and contains 12% sand, 72% silt and 16% clay (Mäder et al., 2002; Mayer et al., 2015).

3.2 EXPERIMENTAL SETUP

Our research question was whether a farming system and pre-crop have an effect on N_2O emissions and abundance of functional microbial community responsible for it. According to that we chose the most suitable plots in the DOK trial: BIOORG and CONFYM plots, all fertilized with higher fertilizer application rate (100% – 1.4 livestock units ha^{-1}) and sown by winter wheat. The two different pre-crops (soybean and rapeseed) were sown in the same plots previous year as seen on Figure 4 (left). The main focus of our investigation was the first of the three fertilizations events planned for the winter wheat (Table 1), which is usually performed at the beginning of tillering (around middle of March). Soil and greenhouse gas (GHG) emissions were sampled for 19 days following a fertilization event (Table 2).

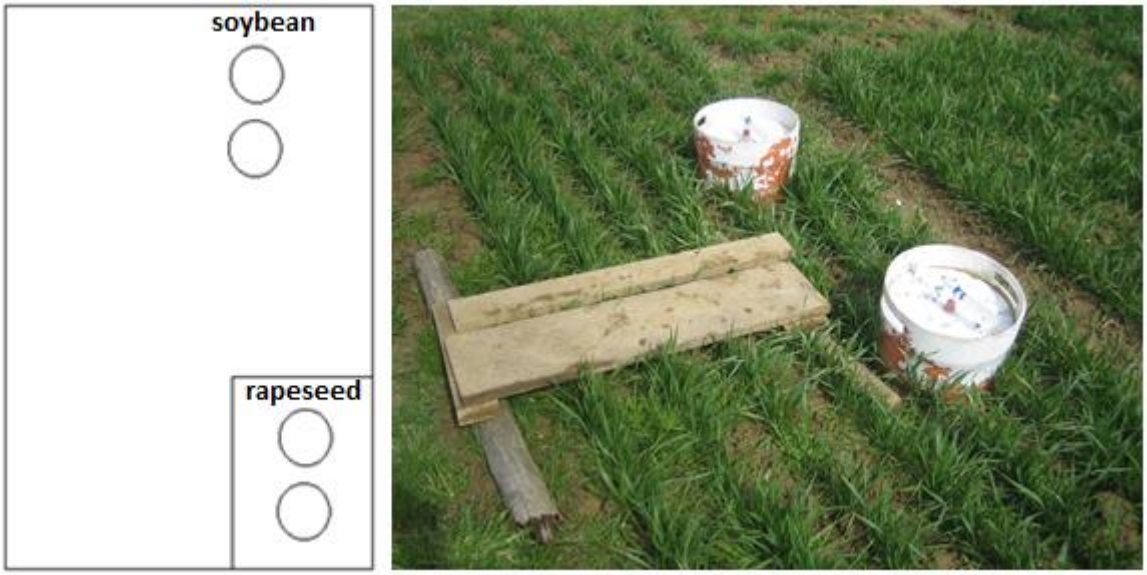

Figure 4: GHG (greenhouse gas) sampling points set up (left) for one parcel and closed chambers in the field (right). Both of the two subplots (left) – soybean and rapeseed were sown by winter wheat (right) in the time of our investigation.
Slika 4: Točke za vzorčenje toplogrednih plinov (levo) in zaprte statične komore namenjene vzorčenju plinov (desno). Obe manjši parceli (levo) – posejani s sojo in oljno ogrščico sta bili v času naše raziskave posejani z ozimno pšenico (desno).

Table 1: Fertilization setup
Preglednica 1: Gnojilni načrt.

Treatment	**Fertilizer type**	**Chemical composition [%]**			**Application rate**
		NH_4^+	NO_3^-	org N	
BIOORG2	Slurry, organic	56	0	44	36 kg N/ha
CONFYM2	Amonium nitrate, mineral	50	50	0	60 kg N/ha

Table 2: Sampling setup where GHG stands for GHG sample, G for geochemical and M for molecular sample acquisition. All the possible samples for GHG and G were analyzed whereas only selected (x) were analyzed for gene abundance and transcription activity with molecular analyses.
Preglednica 2: Načrt vzorčenja, kjer GHG, G in M pomenijo vzorčenja za analizo plinov, fizikalno-kemijskih in molekularnih parametrov. Od vseh zajetih vzorcev so bili za molekularne raziskave izbrani le štirje označeni z (x).

Date	**Days after fertilization**	**Event**	**Samples selected for molecular analyses**
18.03.2015	-1	GHG, G, M	
19.03.2015	0	*fertilization*	
20.03.2015	1	GHG, G, M	
22.03.2015	3	GHG, G, M	
23.03.2015	4	GHG	
24.03.2015	5	GHG, G, M	x
25.03.2015	6	GHG	
26.03.2015	7	GHG, G, M	x
27.03.2015	8	GHG	
29.03.2015	10	GHG, G, M	x
31.03.2015	12	GHG, G, M	
03.04.2015	15	GHG, G, M	x
07.04.2015	19	GHG, G, M	

3.2.1 Gas sampling

Gas sampling was done with static chamber method according to Hutchinson and Mosier (1981). This method allows determination of gas fluxes in or out of the soil based on depletion/accumulation in the chamber headspace respectively.

The chambers are vented to balance inner and outer pressure, because this has an impact on the measurements especially in the well-drained soil with high air permeability (Conen and Smith 1998).

Chambers are made from two parts (Figure 4). A collar with 30 cm diameter and 25 cm height was inserted in the soil down to 15 cm. This part of the chamber stayed in the soil for the whole sampling period while the headspace part with the sampling needle was only mounted air-tightly on the ring at the sampling event. There were two sampling points with chambers per plot (Figure 4). To calculate the volume of the chamber the height of the soil as compared to the ring protrusion was measured at five random points and from that the volume of the whole chamber was calculated with adding the volume of the headspace with internal height of 11 cm.

At each sampling event the chambers were closed for ca. 60-70 minutes in which four gas samplings were done in evenly spaced intervals of about 15-20 minutes. Samples were taken with a 20 ml syringe and injected into an evacuated 12 ml vial with first taken directly after the chamber closing. Sampling time for each sample was precisely recorded in the field protocol. This data was later used to calculate the flux of the nitrous oxide (see 3.3.6).

Soil and air temperatures were measured every time at the beginning and end of the sampling event. To exclude daily variations of temperature and gas fluxes samples were taken always at the same time between 9 and 11 a.m. (CET).

3.2.2 Soil sampling

Soil was sampled in 0-12 cm depth namely 8 and 10 cores (around 100-140 g) at randomly chosen points in soya and rapeseed plot respectively. It was immediately homogenized and split in two parts: first part (~10 g) was frozen in liquid nitrogen for RNA/DNA extractions and the rest was stored in the cooling box for geochemical analyses.

3.3 GEOCHEMICAL ANALYSIS METHODS

3.3.1 pH

pH was determined using pH electrode (Xylem, WTW, inoLab pH 7110, Weilheim, Germany). First 5 g of overnight dried soil (on 105 ^{o}C) was mixed with 50 ml of 0.01 M $CaCl_2$ in 100 ml plastic bottle. Prior to measurement electrode was calibrated with two standard solutions with pH of 4 and 9. pH measurement was carried out immediately after 24 hours of shaking (Kuhner shaker, Birsfelden, Switzerland) in the settling solution and repeated 24 hours after first measurement according to ISO 10390 standard.

3.3.2 Bulk density

Bulk density ρ_b [g cm^{-3}] determination was done in order to calculate water filed pore space (WFPS) (eq. 1). Undisturbed soil samples were taken 2 weeks prior to experiment with core cutters where cylinders with the volume V [cm^3] of 100 cm^3 were used to capture the soil. For average 3-4 soil cores were taken in the 10-15 cm depth. After drying for 24 hours at 105 ^{o}C dried soil weight m_{dry} [g] was collected.

$$\rho_b = \frac{m_{dry}}{V} \quad \text{... (1)}$$

3.3.3 Water filled pore space

Water filled pore space (WFPS) was determined from soil bulk density ρ_b [g cm^{-3}] and water gravimetric content data using equations from (Yanai et al., 2007) (eq. 2 and 3). Water gravimetric content θ_w [g g^{-1}] was determined by subtracting the mass of dry soil samples from freshly weighted samples. For water density ρ_{H2O} value 1 g cm^{-3} was used. For porosity n [-] calculation particle density ρ_p of 2.65 g cm^{-3} was taken.

$$WFPS = \frac{\theta_w}{\rho_{H_2O}} \cdot \frac{\rho_b}{n} \quad \text{... (2)}$$

$$n = 1 - \left(\frac{\rho_b}{\rho_p} \right) \quad \text{... (3)}$$

3.3.4 Mineral N forms

For nitrate (NO_3^-), nitrite (NO_2^-) and ammonium (NH_4^+) determination 20 g of soil (m [g]) was weighted in 250 ml plastic bottles mixed with 80 ml (V [ml]) 0.02 M $CaCl_2$ and shaked for 1 hour on a shaker (Edmund Bühler, Hechingen, Germany). The solution was then filtered through folded filter (Macherey-Nagel, MN 619 EH ¼ d=185 mm, Düren, Germany) and analyzed photometrically for concentration c [mg-N l^{-1}] (Skalar, The San ++ Continuos Flow Analyzer, Breda, Netherlands). Because soil was not dried prior to extraction the water volume from soil V_s [ml] and dry matter fraction dm [-] was accounted for when calculating final mass concentration γ [mg-N kg dry $soil^{-1}$] (eq. 4).

$$\gamma = \frac{c \cdot (V + V_s)}{m \cdot dm} \quad \text{... (4)}$$

3.3.5 Dissolved organic C

Dissolved organic carbon (DOC) was measured on TOC/ TN_b analyzer (Analytik Jena AG, Multi N/C 2100 S, Jena, Germany). Samples from N_{min} extractions (exact procedure is described in chapter 3.3.4) were used. Eq. 4 was used to calculate final DOC concentration which was then converted to mg kg dry $soil^{-1}$.

3.3.6 Greenhouse gas (GHG) determinations

N_2O emissions were measured using gas chromatograph with electron capture detector (ECD) (Agilent Technologies, 7890A, Santa Clara, CA, USA) and auto sampler (GERSTEL GmbH & Co.KG, MultiPurpose Sampler MPS2 XL, Mülheim an der Ruhr, Germany). Gas samples (5 ml) were taken out of 12.5 ml vials.

Standard curve was constructed with three different standard gases (standard gas 1: 0.295 ppm N_2O, 1 ppm CH_4, 296 ppm CO_2; standard gas 2: 1 ppm N_2O, 5 ppm CH_4, 600 ppm CO_2; standard gas 3: 2.94 ppm N_2O, 2 ppm CH_4, 2960 ppm CO_2) measured before and after the sample set measurement.

Areas under the peak of the chromatographs were determined using Agilent ChemStation Revision C.01.04 software which was assigned to a molar amount n [ppm] of N_2O using the standard curve. This value was then corrected to n_c [ppm] (eq. 5) for an error because of the remaining pressure p_r [mbar] before the sampling in the vial and overpressure p_v [mbar] after sampling. Average overpressure (1630 mbar) in the vial was determined by measuring pressure in 20 separate vials.

$$n_c = n \cdot \frac{p_v - p_r}{p_v} \quad \text{... (5)}$$

Data obtained with Agilent software were extracted with RStudio software (version 0.98.1103) running with R (version 3.0.2 – 2013-09-25) using HMR package (version 0.3.1.). HMR package was used to ascribe the best fitting function model for regression (Hutchinson-Mosier nonlinear function, robust regression function and linear regression function) over sampling points within the chamber to calculate gas fluxes for each chamber (Figure 5).

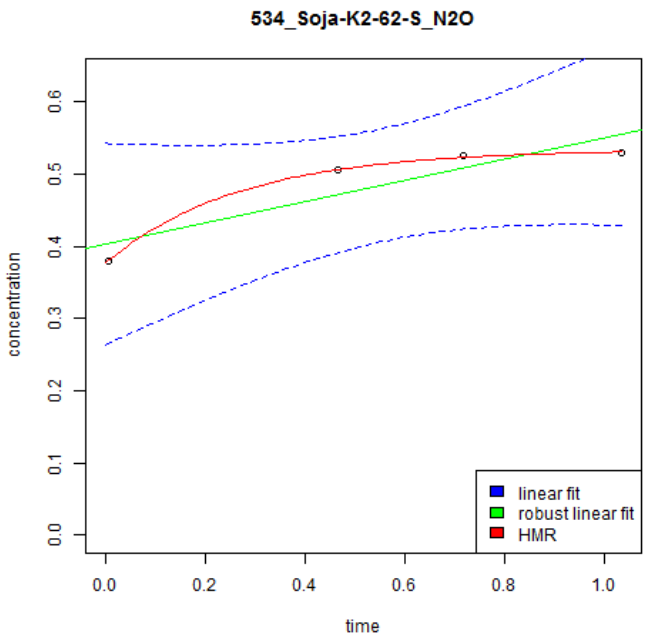

Figure 5: Comparison of different possible functions for N_2O flux calculation. At presented chamber Hutchinson-Mosier nonlinear (HMR) function was selected as the best fit.
Slika 5: Primerjava različnih prilegajočih funkcij fluksu N2O. Pri prikazani komori je bila izbrana kot najbolj ustrezna nelinearna Hutchinson-Mosierjeva funkcija.

3.4 MOLECULAR BIOLOGICAL ANALYSIS METHODS

3.4.1 Chemicals

Substance:	Specification:
Kapa Sybr Green	Kapa SYBR FAST qPCR Master Mix, Kapa Biosystems, Wilmington, MA, USA
$CaCl_2 \cdot 2H_2O$	Carl Roth Gmbh + Co. KG, Karlsruhe, Germany
Tris buffer 10 mM, pH 8	Carl Roth Gmbh + Co. KG, Karlsruhe, Germany
T4 gp32	MoBio, Carlsbad, CA, USA
T7 polymerase	Thermo Fisher Scientific, Waltham, MA, USA

3.4.2 DNA and RNA extraction

DNA and RNA co-extraction was performed using RNA PowerSoil Total RNA Isolation Kit (MoBio, RNA PowerSoil® Total RNA Isolation Kit cat.: 12866-25, Carlsbad, CA, USA) according to manufacturer's instructions but with some modifications: all centrifugation steps prior to elution were performed at 4 ^{o}C, incubation on ice after SR3 addition, isopropanol precipitation was prolonged to 20 minutes. After RNA elution DNA elution was performed from the same column with DNA elution buffer followed by isopropanol precipitation step. After centrifugation the pellet was re-suspended in Tris 10 mM (pH 8) buffer.

A plasmid with an inserted fragment of a cassava mosaic virus gene (GenBank AJ427910) served as internal DNA standard ($6.32 \cdot 10^9$ copies) and was added at the beginning to the extraction mix to quantify DNA extraction efficiency (Thonar et al., 2012). The same was done for RNA extraction efficiency. RNA standard was produced from DNA standard by T7 transcription of a linearized plasmid using T7 polymerase (Thermo Fisher Scientific, T7 RNA Polymerase; Waltham, MA, USA) according to manufacturer's instructions and added to the extraction mix ($2.35 \cdot 10^{11}$).

Both DNA and RNA concentrations of the samples were measured after extraction with Qubit (Invitrogen, Qubit 1.0, Carlsbad, CA, USA) using Qubit dsDNA HS (Invitrogen, Qubit dsDNA HS Assay Kit) and Qubit RNA HS (Invitrogen, Qubit RNA HS Assay Kit; Carlsbad, CA, USA) assay respectively. Finally samples were stored at – 80 ^{o}C (RNA) and – 20 ^{o}C (DNA).

3.4.3 Complementary DNA synthesis

Co-extracted mRNA was reverse transcribed into complementary DNA (cDNA) with QuantiTect Reverse Transcription Kit (Quiagen, QuantiTect Reverse Transcription Kit, Venlo, Netherlands) according to manufacturer's instructions. Prior to reverse transcription inhibitors were removed with inhibitor removal kit (Zymo Research, OneStep PCR Inhibitor Removal Kit, Irvine, CA, USA) according to manufacturer's instructions. Genomic DNA removal step was tested with 16S qPCR of 1:100 diluted samples while keeping all the samples at 1 °C. After successful confirmation of DNA removal reverse transcription was done according to kit's manual. cDNA samples were stored at -20 °C.

3.4.4 Quantitative polymerase chain reaction (qPCR)

To demonstrate the effect of fertilization and pre-crop on denitrification genes (*nirK, nirS, nosZ, nosZ II*) quantification with qPCR was performed with Rotor-Gene Q (Quiagen, Rotor-Gene Q 5plex HRM, Venlo, Netherlands). Standards for each gene were used from organisms as shown in Table 3.Primers and protocols can be viewed in Table 4 and 5 respectively.

Table 3: Standard sources and vectors for each gene.
Preglednica 3: Izvor standardov in vektorji za posamezen gen.

Gene	Organism	GeneID	Vector
nirK	*Ensifer meliloti 1021*	1235717	pCR4
nirS	*Ralstonia eutropha H16*	4456658	pCR4
nosZ	*Ensifer meliloti 1021*	1235679	pCR4
nosZ clade II	*Gemmatimonas aurantiaca*	not annotated yet	pEX-A
16S	*Pseudomonas sp. RR62*	not annotated yet	Pjet1.2
APA9	*African cassava mosaic virus*	not annotated yet accession number: AJ427910.1	Pjet1.2

All used standards (*nirK, nirS, nosZ, nosZ II*) were produced by insertion of vector for ampicilin resistance into *E. coli* DH5 α for multiplication grown in LB medium with ampicillin which impedes all other growth for 24 hours at 37 °C on a shaker (Infors HT, Ecotron, Bottmingen, Switzerland) at 180 rpm.

Plasmid DNA was isolated using a kit (peqLab, peqGOLD Plasmid Miniprep Kit II, Erlangen, Germany) according to manufacturer's instructions with all additional washing steps. After isolation the plasmids were linearized with restriction enzymes: pCR4 with NotI (New England BioLabs, Ipswich, MA, USA) and pEX-A with HindIII HF (New England BioLabs, Ipswich, MA, USA) according to manufacturer's instructions.

Table 4: Primers used for quantification of functional genes.
Preglednica 4: Začetni oligonukleotidi uporabljeni za kvantifikacijo funkcionalnih genov.

Gene	Primer	Sequence	Target size [bp]	Reference
nirK	nirK876C	ATYGGCGGVCAYGGCGA	162	(Henry et al., 2004)
	nirK1040	GCCTCGATCAGRTTRTGGTT		(Harter et al., 2014)
nirS	nirScd3af	AACGYSAAGGARACSGG	413	(Throbäck et al., 2004)
	R3cd	GASTTCGGRTGSGTCTTGA		
nosZ	nosZ2F	CGCRACGGCAASAAGGTSMSSGT	267	(Henry et al., 2006)
	nosZ2R	CAKRTGCAKSGCRTGGCAGAA		
nosZ II	nosZ-II-F	CTIGGICCIYTKCAYAC	690-720	(Jones et al., 2013)
	nosZ-II-R	GCIGARCARAAITCBGTR		
16S	341F	CCTACGGGAGGCAGCAG	466	(Muyzer et al., 1993)
	797R	GGACTACCAGGGTATCTAATCCTGTT		(Nadkarni et al., 2002)
APA9	APA9-F	CGAACCTGGACTGTTATGATG	80	(Thonar et al., 2012)
	APA9-R	AATAAACAATCCCCTGTATTTCAC		

Table 5: Composition of the master mix and protocol for qPCR for the genes analysed with qPCR.
Preglednica 5: Sestava master mixov in protokoli za posamezne gene.

Gene	Substance	Volume [µL] Σ=10 µL	Protocol	Reference
nirK	Kapa Sybr Green	5	95°C – 3 min	Modified after (Babic et al., 2008)
	nirK876C (2 µM)	1	6x	
	nirK1040 (2 µM)	1	95°C – 15s	
	Tris (10mM)	2	62-57°C – 20s	
	sample	1	80°C – 10s	
			35x	
			95°C – 15s	
			57°C – 20s	
			80°C – 10s	
			melt 55-95°C	

Continued

Continuation of Table 5:

nirS	Kapa Sybr Green	5	95°C – 3 min	
	nirScd3af (5 µM)	1	40x	
	R3cd (5 µM)	1	95°C – 15s	Modified after (Towe et al., 2010)
	Tris (10mM)	2	58°C – 30s	
	sample	1	80°C – 10s	
			melt 55-95°C	
nosZ	Kapa Sybr Green	5	95°C – 3 min	
	nosZ2F (5 µM)	1	6x	
	nosZ2R (5 µM)	1	95°C – 15s	
	Tris (10mM)	2	65-60°C – 20s	
	sample	1	80°C – 10s	Modified after (Babic et al., 2008)
			35x	
			95°C – 15s	
			60°C – 15s	
			80°C – 10s	
			melt 55-95°C	
nosZ II	Kapa Sybr Green	5	95°C – 10 min	
	nosZ-II-F (10 µM)	1	40x	
	nosZ-II-R (10 µM)	1	95°C – 15s	
	DMSO	0.5	54°C – 30s	(Jones et al., 2013)
	Tris (10mM)	1.5	72°C – 30s	
	sample	1	80°C – 10s	
			melt 54-95°C	
16S	Kapa Sybr Green	5	95°C – 3 min	
	341F (2 µM)	1	35x	
	797R (2 µM)	1	95°C – 15s	Modified after (Nadkarni et al., 2002)
	Tris (10mM)	2	61.5°C – 20s	
	sample	1	80°C – 10s	
			melt 55-95°C	
APA9	Kapa Sybr Green	5	95°C – 3 min	
	APA9-F (2 µM)	1	35x	
	APA9-R (2 µM)	1	95°C – 10s	(Thonar et al., 2012)
	Tris (10mM)	2	50°C – 20s	
	sample	1	melt 55-95°C	

3.4.5 Copy and transcript number calculation

Initial plasmid DNA concentration in the standard solution was measured fluorometrically with Qubit (Invitrogen, Qubit 1.0, Carlsbad, CA, USA) using Qubit ds DNA BR assay (Invitrogen, Qubit dsDNA BR Assay Kit, Carlsbad, CA, USA).

Number of copies for each plasmid per µl N [μl^{-1}] were calculated using initial plasmid concentration γ_p [ng/µl], Avogadro's number N_a ($6.022 \cdot 10^{23}$) [mol^{-1}] and plasmid molecular mass M [g mol^{-1}] (eq. 6). Molecular mass of each plasmid with inserted standard fragment was calculated by multiplying the number of individual nucleotide within the plasmid with its respective molecular mass (A: 313.2 g/mol, T: 304.2 g/mol, C: 289.2 g/mol, G: 329.2 g/mol) and then summing it up.

$$N = \frac{\gamma_p \cdot N_a}{M_p \cdot 10^9} \quad \text{... (6)}$$

Standard curve was obtained from the standard 1:10 serial dilution steps. For each qPCR seven duplicated dilutions of individual standard were used to cover the range of possible results (between 10^8 to 10^2 copies per reaction).

To determine concentration of the samples where inhibitors don't have an impact anymore, sample dilution series (1:1, 1:10, 1:100, 1:1000) were performed and tested with qPCR for *nosZ* gene. Finally 1:1000 and 1:10 dilutions were used for DNA and cDNA samples respectively.

Raw data from the qPCR cycler were analysed using LinReg algorithm which estimates the baseline by reconstructing the log linear phase downward from the early plateau phase of the PCR reaction. Efficiency is determined for every reaction separately with high reproducibility (Ruijter et al., 2009).

Afterwards linear regression was performed between log N and threshold cycle ct [-] with slope m [-] and y-intercept n [-] which was then used for calculation of copy numbers of individual samples N_s [-] by their ct_s (eq. 7):

$$N_s = 10^{m \cdot ct_s + n} \quad \text{... (7)}$$

Corrections of gene copy numbers and transcripts were performed based on the recovery rates of DNA and RNA internal standard respectively. Because similar DNA yields were observed (~ 200 ng μl^{-1}) but selective losses of DNA internal standard were detected, mean DNA recovery rates per each treatment were taken as a correction coefficient.

Corrected gene copy numbers and transcripts N_s [-] were normalized as N_{ds} [copies g^{-1} dry soil] using dry mass of the extracted soil m_{dry} [g] (eq. 8).

$$N_{ds} = \frac{N_s}{m_{dry}} \quad \text{... (8)}$$

3.4.6 Quality assessment

To inspect the quality of the qPCR assay standard series and amplification processes were analysed (Table 6). A negative template control (NTC) was performed with Tris 10mM buffer as a template. For analysis of the samples it was assured that amplified NTC *ct* was at least 5 cycles higher than sample *ct* and unspecific as examined on the melt curve. Otherwise amplification of the samples was repeated. After each amplification melt curve analyses were performed as well as 1% agarose gel analyses for selected samples (Figure 6) in order to verify size of the amplification product.

Table 6: Efficiencies of qPCR and quality of the standard curve as analyzed with Rotor Gene Q Series Software (Quiagen, Rotor Gene Q Series Software, Venlo, Netherlands). Each sample was replicated twice in the same run. If the difference between ct_s of the duplicates exceeded 0.5 cycle then that sample was repeated.
Preglednica 6: Učinkovitost in kvaliteta standardne krivulje analizirana z Rotor Gene Q Series Software (Quiagen, Rotor Gene Q Series Software, Venlo, Netherlands). Vsak vzorec je bil v dveh ponovitvah znotraj ene verižne reakcije. Če je bila razlika v ct_s med posameznima ponovitvama istega vzorca več kot 0.5 cikla potem je bil le-ta ponovljen.

	DNA	
Gene	**efficiency[%]**	**R^2**
nirK	86, 85, 89	0.998, 0.999, 0.999
nirS	87, 87, 89	0.999, 0.999, 0.999
nosZ	81, 79, 81	0.999, 0.999, 0.999
nosZ II	81, 79, 79	0.999, 0.999, 0.998
16S rRNA	88, 87, 87	0.998, 0.999, 0.999
APA9	78, 76, 78	0.999, 0.999, 0.999
	cDNA	
Gene	**efficiency[%]**	**R^2**
nirK	84, 82, 83, 85	0.999, 0.999, 0.999, 0.999
nirS	89, 88, 86	0.998, 0.999, 0.998
nosZ	100, 93, 89	0.999, 0.998, 0.998
APA9	88, 92, 89	0.999, 0.999, 0.999

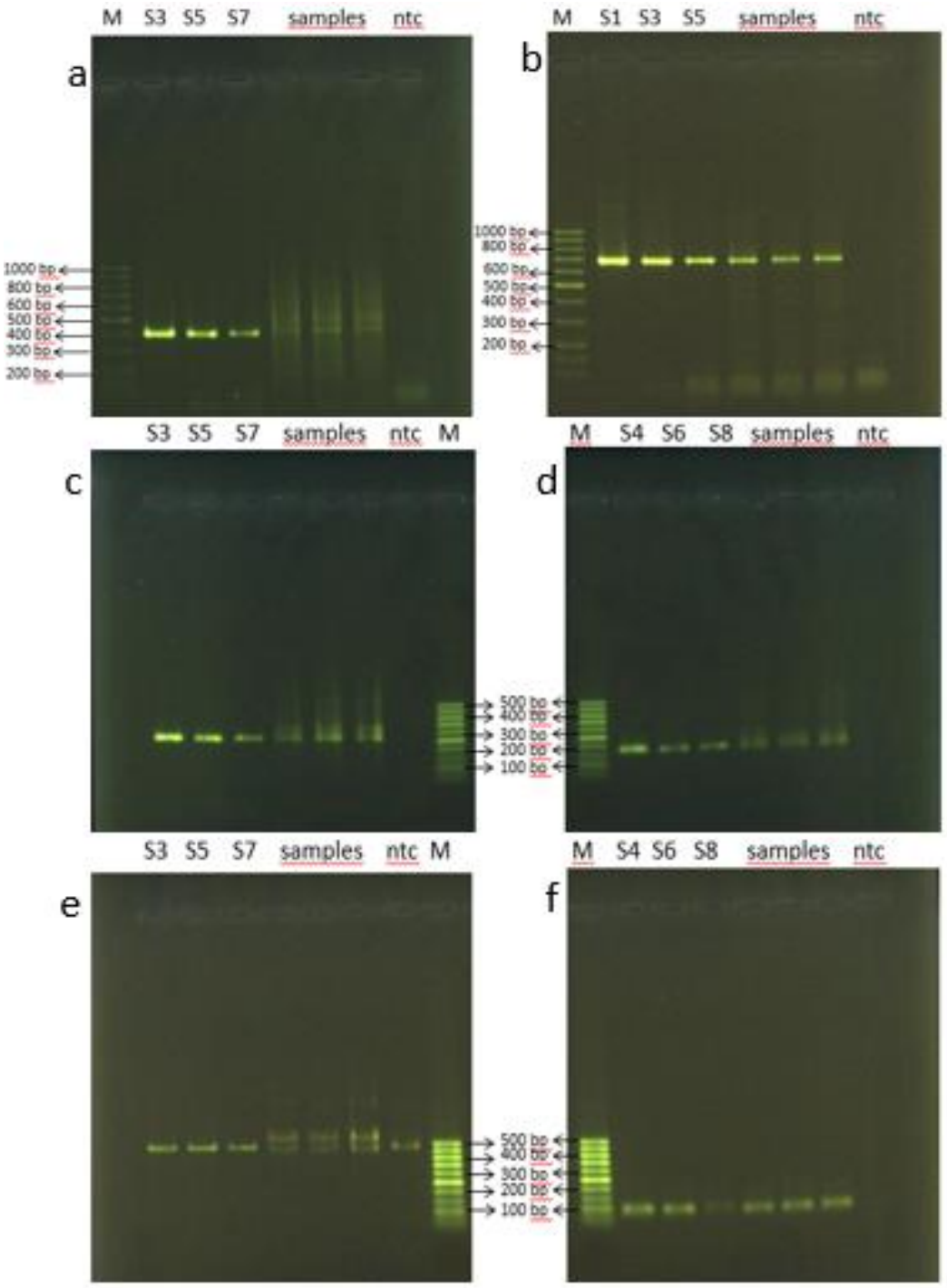

Figure 6: Photos of qPCR amplification products on 1% agarose gel. For each gel three standards from dilution series were used (S) with number marking step in the serial dilution, three individual samples (samples) and no template control (ntc). Length was determined comparing with the ladder (M). Fragments presented are coming from *nirS* (a), *nosZ II* (b), *nosZ* (c), *nirK* (d), *16S* (e) and *APA9* (f) genes.
Slika 6: Fotografije s qPCR pomnoženih produktov na 1 % agaroznem gelu. Za vsak gel so bili uporabljeni trije standardi iz oštevilčenega koraka redčitvene vrste (S), trije posamezni vzorci (samples) in negativna kontrola (ntc). Dolžina posameznih fragmentov je bila določena z velikostno lestvico fragmentov (M). Fragmenti predstavljeni prihajajo od *nirS* (a), *nosZ II* (b), *nosZ* (c), *nirK* (d), *16S* (e) and *APA9* (f) genov.

3.5 STATISTICAL ANALYSIS

For identifying effects of different farming systems and pre-crops on soil properties (DOC, mineral N forms, water content), microbial community composition (bacterial 16S and denitrifiers gene and transcripts abundances) and N_2O emissions, statistical analysis over the whole data set using SPSS statistical software (IBM, SPSS Statistics 22, Armonk, NY) was performed. Each time point and each parameter was checked for statistical differences by performing an univariate analysis of variance (ANOVA). In order to identify which of the four different treatments differed significantly Duncan's Post Hoc test was performed. This resulted in a, b and c labels which mark statistically significant differences ($p < 0.05$).

4 RESULTS

Our study examined the effect of two different farming systems in combination with two different pre-crops (soybean and rapeseed). Farming systems differed in the amount and type of fertilizer: conventional system (CONFYM) was fertilized with ammonium nitrate in the rate 60 kg N ha^{-1} while organic system (BIOORG) was fertilized with slurry in the rate of 36 kg N ha^{-1}. N_2O emissions and denitrifier gene and gene transcript abundances were followed for 19 days after the first fertilization event in spring 2015 at the DOK long-term trial in winter wheat fields.

4.1 SOIL WATER CONTENT

Soil water content played an important role in our experiment. Two distinct phases (before and after day 5) were observed in soil water content dynamics, expressed as water filled pore space (WFPS) (Figure 7). The reason for substantial WFPS increase on day 6 and 7 was the rain event on day 5. No significant differences were found between the two pre-crops, however when comparing the farming systems, BIOORG had significantly higher WFPS at the beginning of the experiment: days 1 ($p < 0.001$), 3 ($p = 0.011$) and 5 ($p = 0.004$) due to the liquid addition from slurry. In the second phase (after day 5) WFPS was fluctuating around 80%, which is optimal for denitrification (Kool et al., 2011). The highest soil water content (83% WFPS) during our study was detected on day 15 in rapeseed BIOORG, which mirrors slightly higher WFPS in BIOORG compared to CONFYM, although statistically insignificant.

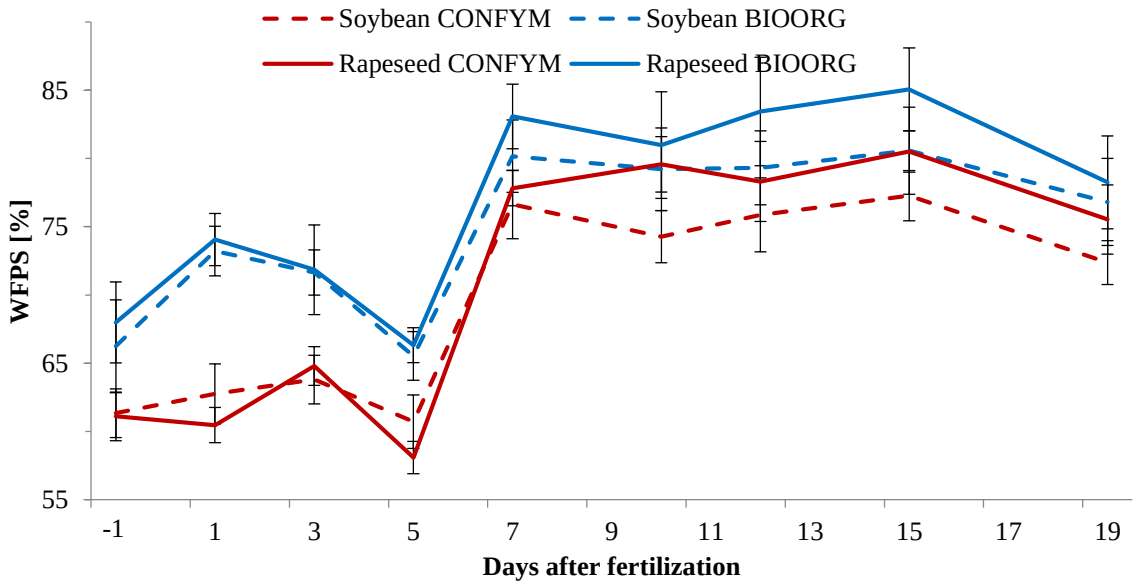

Figure 7: Soil water content dynamics, expressed as water filled pore space (WFPS) during the experiment. Means and standard errors of four plot replicates are shown.
Slika 7: Dinamika vsebnosti vode v tleh, izražene kot % por napolnjenih z vodo (WFPS)) med poskusom. Predstavljena so povprečja in standardne napake štirih ponovitev.

4.2 N_2O EMISSIONS

N_2O emissions detected in our study (Figure 9) were quite low (peak between 20 and 50 µg N_2O-N m^{-2} h^{-1}), however they are in accordance with other studies investigating emissions under winter wheat (Gu et al., 2013; Smith et al., 2012). A clear pre-crop effect can be observed as only in soybean (BIOORG and CONFYM) emissions increased above the background level (~ 5 µg N_2O-N m^{-2} h^{-1}). Emission peak started at day 7 in plots under both farming systems, which was clearly coinciding soil water content increase at the same time (Figure 7). Significantly higher emissions in soybean were detected at days 8, 10 and 12 after fertilization (all three $p < 0.001$). Farming system effect was not so pronounced but detected anyway. On day 10 and 15 the emissions in CONFYM were significantly higher compared to BIOORG ($p = 0.037$ and $p = 0.001$ respectively).

Cumulative N_2O emissions (Figure 8) reflected daily measurements, meaning that significant pre-crop effect was again detected. Soybean clearly showed significantly higher cumulative emission values ($p < 0.001$). Differences observed between the two farming systems were insignificant.

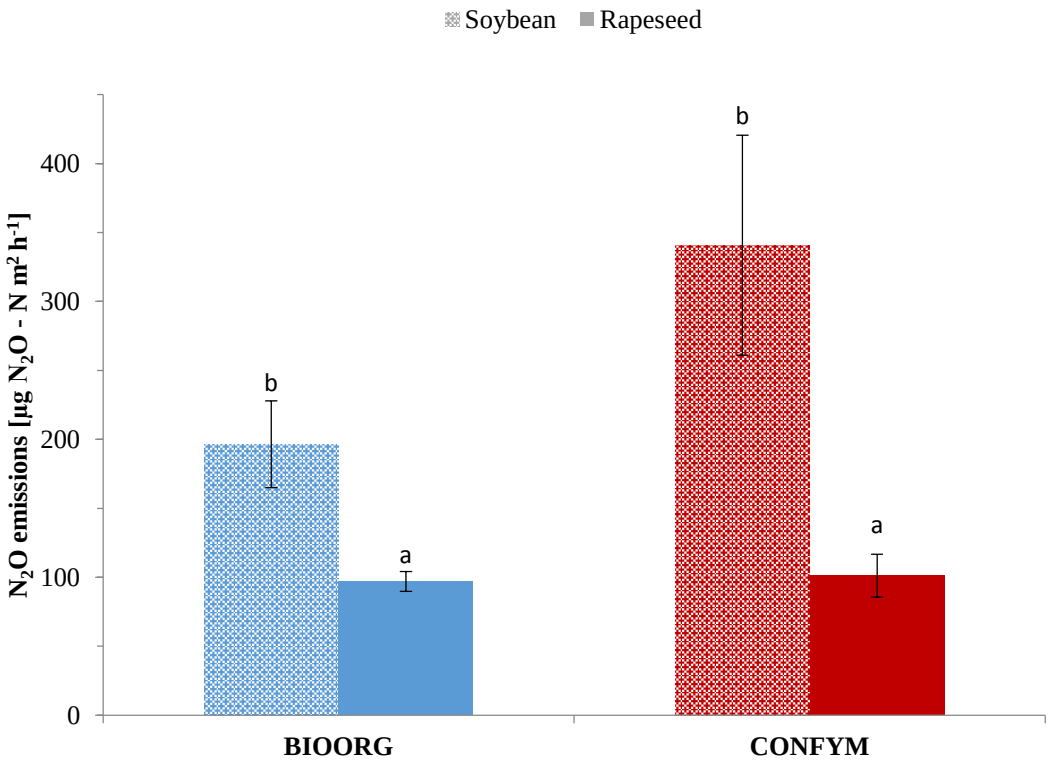

Figure 8: Cumulative N_2O emissions. They were calculated from 12 emission measurements in 21 days. Means and standard errors of four plot replicates are shown. Different letters indicate significant differences between the treatments according to Duncan's test. For statistical analysis data was log transformed.
Slika 8: Kumulativne emisije N_2O. Izračunali smo jih iz 12 merjenj emisij v 21ih dneh. Predstavljena so povprečja in standardne napake štirih ponovitev. Različne črke predstavljajo statistično značilne razlike med obravnavanji z Duncanovim testom. Za namene statistične analize so bili podatki logaritemsko transformirani.

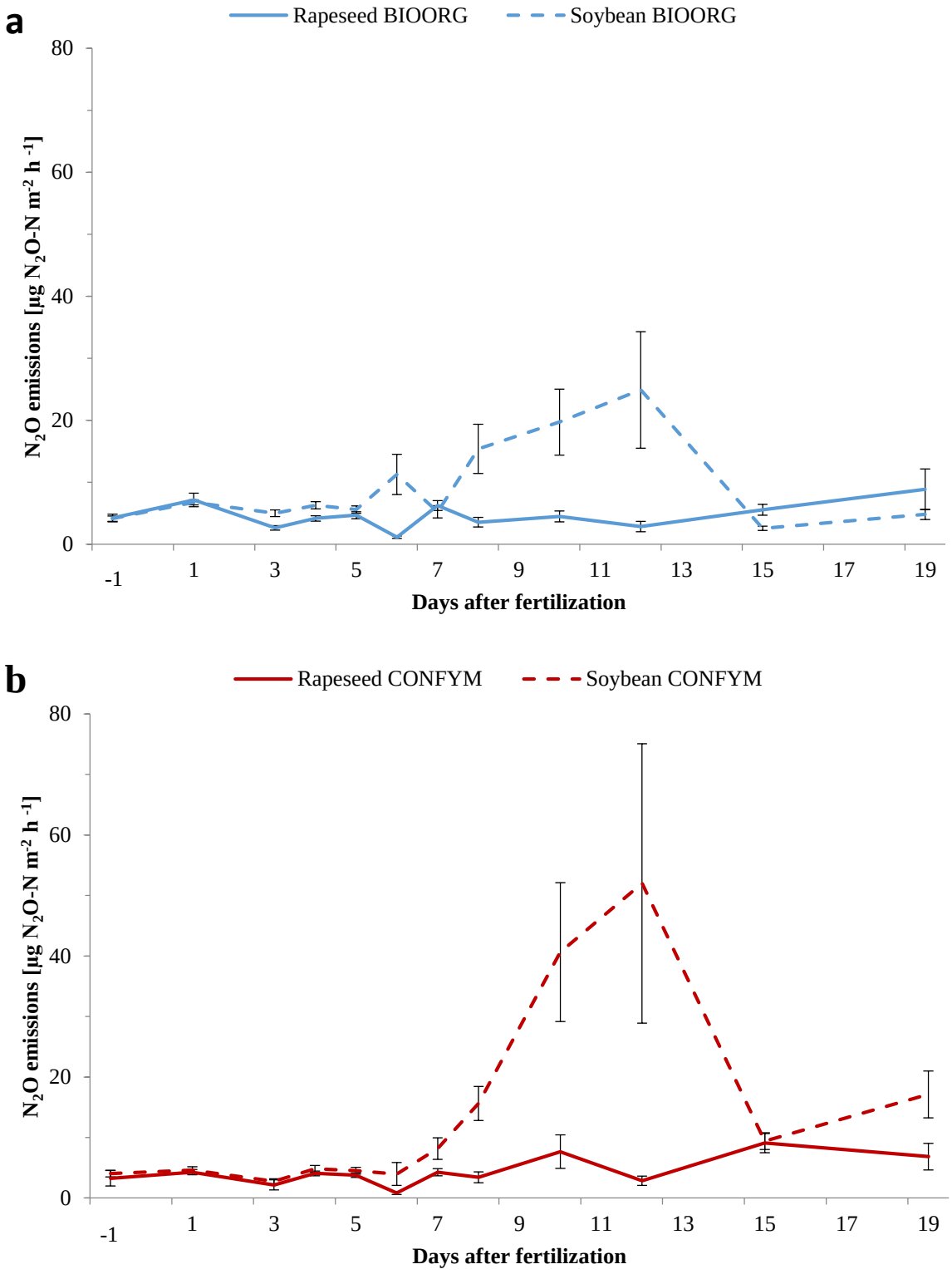

Figure 9: N_2O emissions for BIOORG (a) and CONFYM (b) system. Means and standard errors of four plot replicates are shown. For statistical analysis data was log transformed.
Slika 9: N_2O emisije za ekološki (BIOORG) (a) in konvencionalni (CONFYM) (b) sistem. Predstavljena so povprečja in standardne napake štirih ponovitev. Za namene statistične analize so bili podatki logaritemsko transformirani.

4.3 SOIL MINERAL N FORMS

Mineral nitrogen forms (NO_3^-, NH_4^+) showed higher values in conventional (CONFYM) farming system (Figure 10), which is due to the higher fertilizer application rate in this system (Table 1). However, significant differences were mostly observed for nitrate (NO_3^-), while for ammonium (NH_4^+) they were not statistically significant. NO_3^- concentrations were higher in CONFYM compared to BIOORG through the whole course of the experiment with the exception of the first data point (1 day before fertilization). On the contrary to nitrate, NH_4^+ dynamics did not differ much among systems. In fact only once (at day 15) significantly higher ammonium concentrations were detected in CONFYM compared to BIOORG system ($p = 0.003$). Not surprisingly, as mineral fertilizer applied in CONFYM system contained both mineral N form, whereas slurry in BIOORG treatment contained only NH_4^+ (Table 1). Thus, in BIOORG system no initial NO_3^- concentration was observed, but its concentration then increased with time. Simultaneous decrease of NH_4^+ concentration and appropriate soil water content conditions with WFPS at 70% (Figure 7) indicated nitrification in the first phase of our experiment. In CONFYM system after fertilisation high increase of both NO_3^- and NH_4^+ was observed, which were then gradually decreasing towards the end of experiment. High error bars in CONFYM system indicate nonhomogeneous distribution of both forms in the fertilized plots at the beginning of the experiment. Despite CONFYM system received higher dose of NH_4^+ than BIOORG, the differences in the field NH_4^+ concentrations between the farming systems were not significant.

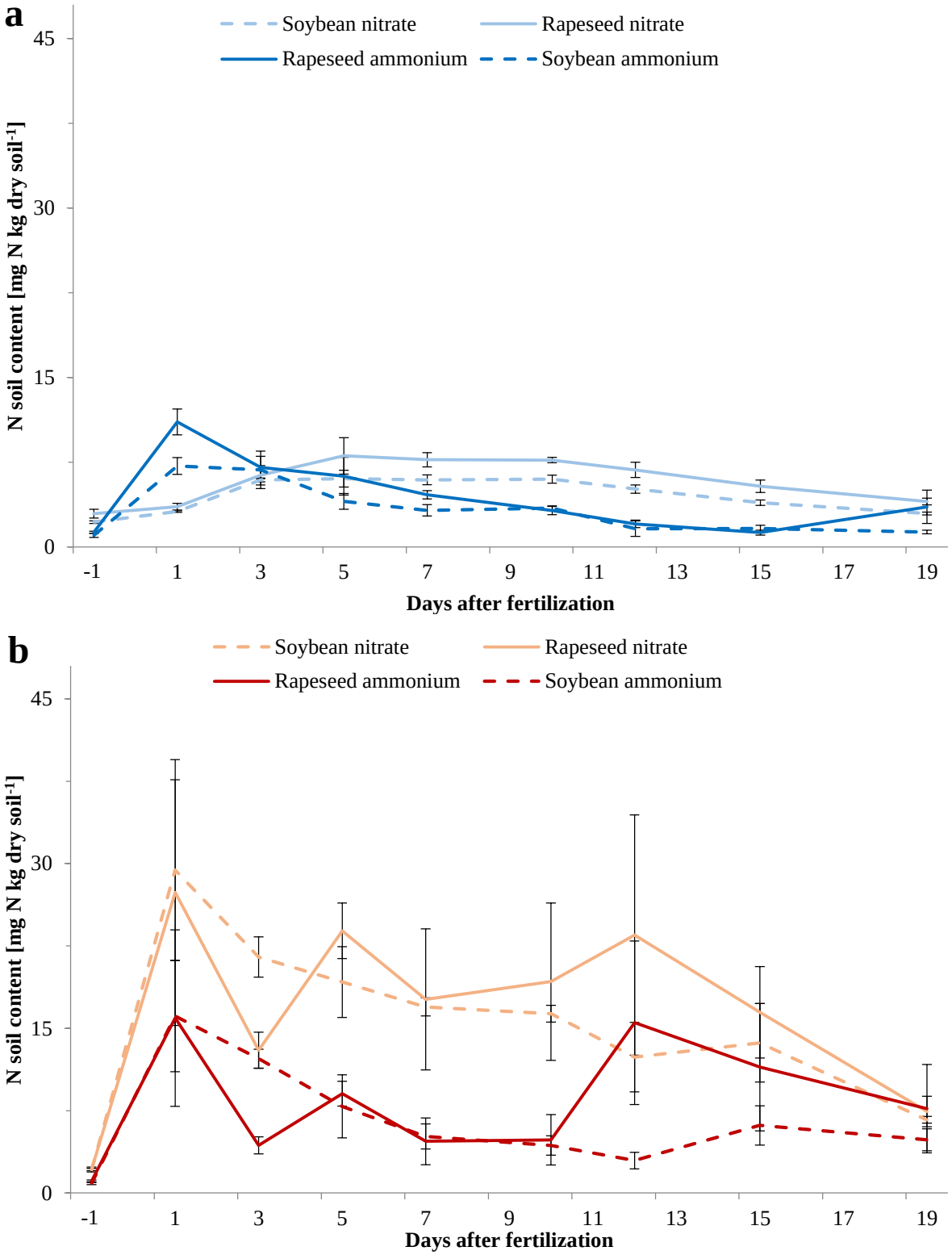

Figure 10: NO_3^- and NH_4^+ dynamics in BIOORG (a) and CONFYM (b) system. Means and standard errors of four plot replicates are shown. For statistical analysis data was log transformed.
Slika 10: Dinamika NO_3^- in NH_4^+ koncentracij v BIOORG (a) in CONFYM (b) sistemu. Predstavljena so povprečja in standardne napake štirih ponovitev. Za namene statistične analize so bili podatki logaritemsko transformirani.

4.4 SOIL ORGANIC CARBON

Organic matter was added with fertilization only in BIOORG system through slurry. Thus, an initial increase to around 68 mg C kg dry soil^{-1} can be observed only in organic system (Figure 11). Significantly higher DOC contents in BIOORG than in CONFYM were found at days 1 ($p < 0.001$) and 3 ($p = 0.017$)). Afterwards, DOC decreases back to background level (~ 50 mg kg dry soil^{-1}) probably because it's used up by microbes. In CONFYM system it stayed on the background level throughout the whole experiment. No significant differences between pre-crops (soybean and rapeseed) were detected.

Soil organic carbon (SOC) is part of soil organic matter (SOM) and it represents organic carbon stocks in soil. It influences soil structure and soil water properties. SOC data for our study (Table 7) were collected from internal FiBL database since the latest analysis was done in 2012. There is no need to do it more often since they don't change greatly in time. However no significant differences were detected between the two farming systems.

Source of organic matter in the soil are also the crop residues (Table 7). As expected were soybean residues significantly lower in C:N ratio than rapeseed at the time of incorporation ($p < 0.001$). Interestingly rapeseed residues had significantly higher C:N ratio in BIOORG treatment compared to CONFYM ($p = 0.002$).

pH didn't show any significant differences between the two farming systems or pre-crops.

Table 7: pH and crop residue C:N ratio values measured at the beginning of the experiment and before sowing winter wheat in autumn 2014 respectively. SOC was measured in 2012. Different letters indicate significant differences between the treatments according to Duncan's test (D). Means and standard errors of four plot replicates are shown.
Preglednica 7: Podatki za C:N razmerje vključenih rastlinskih ostankov, pH in SOC. Le-ti so bili določenih pred setvijo ozimne pšenice (C:N razmerje), na začetku našega eksperimenta (pH) in leta 2012 (SOC). Različne črke predstavljajo statistično značilne razlike med obravnavanji z Duncanovim testom (D). Predstavljena so povprečja in standardne napake štirih ponovitev.

System	Pre-crop	pH ($CaCl_2$)			Crop residue C:N ratio			SOC (g kg^{-1})		
		Mean	.se	D	Mean	.se	D	Mean	.se	D
BIOORG	Rapeseed	5.63	0,07		126.01	3.03	c	11.80	0.38	
	Soybean	5.74	0,06		69.63	2.25	a			
CONFYM	Rapeseed	5.75	0,05		94.81	3.02	b	11.33	0.33	
	Soybean	5.61	0,09		61.85	1.56	a			

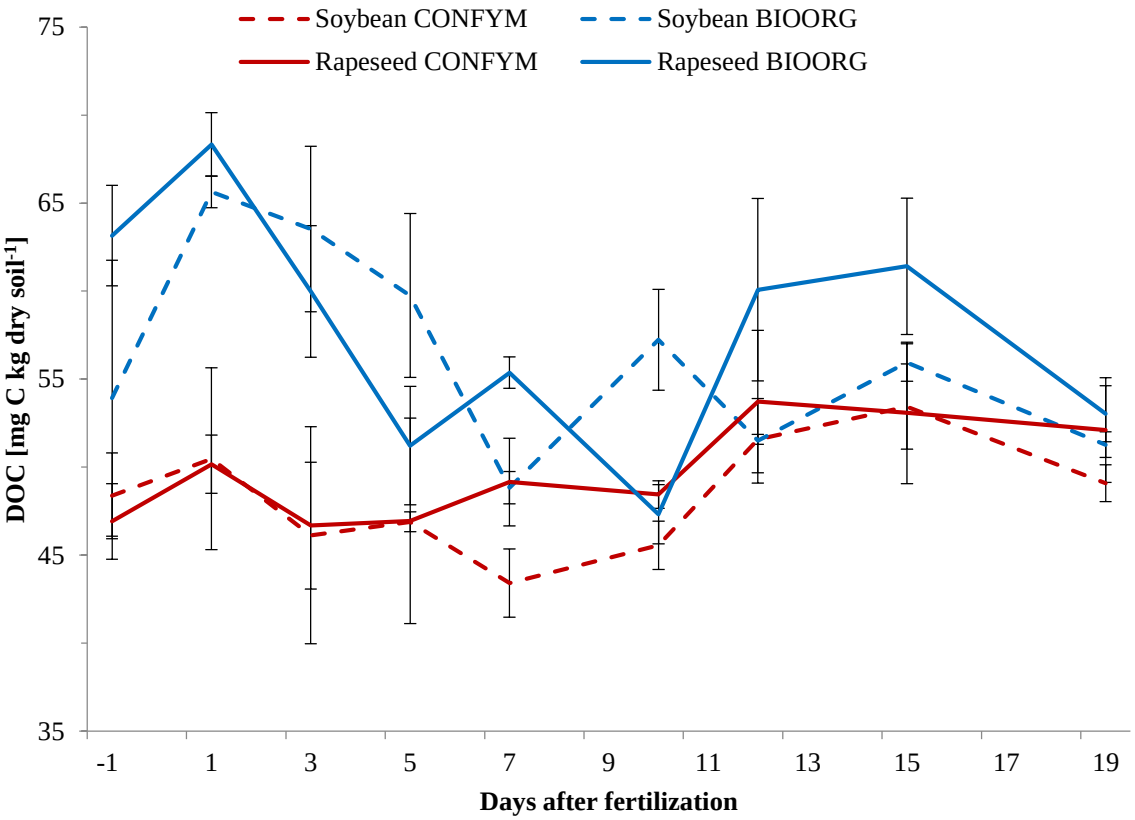

Figure 11: DOC dynamics during the experiment. Means and standard errors of four plot replicates are shown. For statistical analysis data was log transformed.
Slika 11: Dinamika topnega organskega ogljika (dissolved organic carbon – DOC) med poskusom. Predstavljena so povprečja in standardne napake štirih ponovitev. Za namene statistične analize so bili podatki logaritemsko transformirani.

4.5 MOLECULAR ANALYSES OF MICROBIAL COMMUNITY

For molecular biological analysis only four time points out of nine soil samplings were considered. First examined time point was 5 days after fertilization, which was before the rain event at day 6 when WFPS increased to around 80% in day 7 (Figure 7). Three other time points were chosen referring to N_2O emissions coinciding with the beginning, middle and end of the emission peak at 7, 10 and 15 days after the fertilization respectively. Taking middle instead of highest emissions time point was justified by assumption that higher transcription rate for denitrifying genes was expected in the point where emissions were still increasing.

4.5.1 Abundance of bacterial community

Abundance of bacterial 16S rRNA genes was similar between the treatments (Figure 12). Greater dynamics can be detected in BIOORG than in CONFYM system, where a more steady increase can be observed. The lowest bacterial abundance was found in rapeseed

under BIOORG system, being significantly lower than in all other treatments at day 7 ($p < 0.001$).

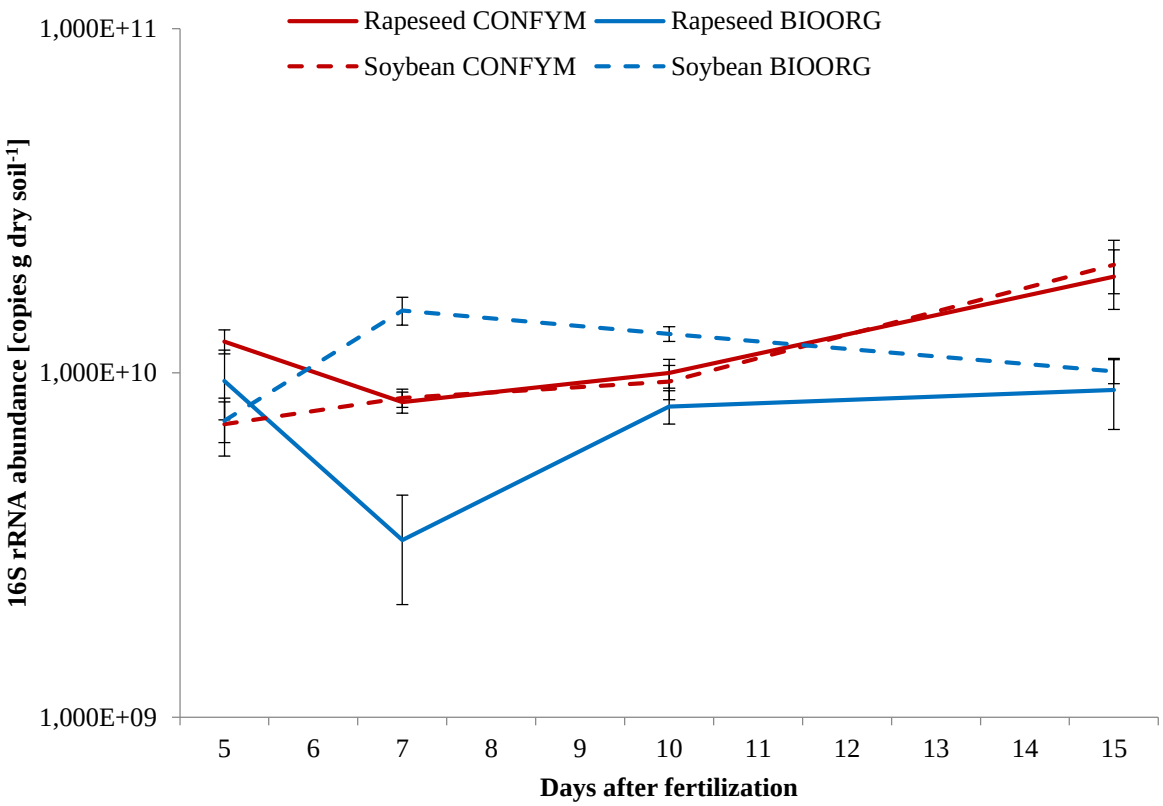

Figure 12: Bacterial 16S rRNA gene copy numbers during experiment. Means and standard errors of four plot replicates are shown.
Slika 12: Število kopij bakterijskega 16S rRNA gena med poskusom. Predstavljena so povprečja in standardne napake štirih ponovitev.

4.5.2 Denitrifier gene abundance

Both *nirK* and *nirS* gene and transcript copy numbers were summed up together (Figure 13) in *nir* as enzymes from both genes serve the same function (Philippot, 2002). *nirK* was roughly 5 times less abundant than *nirS* which is in accordance with *nirK*:*nirS* ratio measured in wetlands (Ligi et al., 2013).

Under both farming systems (Figure 13) higher *nir* gene abundances were observed compared to *nosZ* (Annex G), which is in accordance with the literature (Kandeler et al., 2006; Ligi et al., 2013). In BIOORG *nir* abundances were in general higher in soybean compared to rapeseed, but in CONFYM the reverse trend was seen.

By all functional genes roughly similar dynamics are observed (Figure 13): On days when significant differences were detected rapeseed CONFYM usually shows the highest abundances and rapeseed BIOORG the lowest. Both systems with soybean were

somewhere in between. This was reflected by 16S rRNA (Figure 12) which could indicate that differences on the level of the whole community abundance could influence single functional genes abundances.

The abundance of *nir* gene was at only one time point (15 days after fertilization) influenced by pre-crop (Figure 13) when significantly higher values were detected in soybean as compared to rapeseed ($p = 0.032$). On day 7 significantly higher *nir* copy numbers were detected in CONFYM system as compared to BIOORG. Significant differences on the level of separate treatments were detected on day 10 when rapeseed CONFYM was significantly higher than soybean CONFYM ($p = 0.003$). The latter is however in discordance with N_2O emissions (Figure 9) which were significantly higher in soybean CONFYM.

By *nosZ* abundance farming system (CONFYM > BIOORG) and pre-crop (rapeseed > soybean) effects were observed. Significantly higher values of *nosZ* were detected in rapeseed CONFYM compared to the other three treatments on days 5 ($p = 0.002$) and 10 ($p < 0.001$) which influenced the system effect – higher copy numbers of *nosZ* in CONFYM than BIOORG – observed on both days ($p = 0.001$ and $p = 0.001$) and pre-crop effect on day 5 (rapeseed > soybean, $p = 0.038$). Abundance of *nosZ* repeated the trend of *nir* when rapeseed BIOORG showed significantly the lowest values on day 7 ($p = 0.008$).

In both BIOORG and CONFYM systems around 1.5 order of magnitude higher abundances of *nosZ II* compared to *nosZ* were detected which was already previously reported (Jones et al., 2014). Farming system influenced *nosZ II* abundance on days 7 ($p = 0.001$) and 10 ($p = 0.002$) when also statistically significant differences on the level of single treatments were detected. On day 7 rapeseed CONFYM treatment had the highest and rapeseed BIOORG the lowest *nosZ II* abundances ($p = 0.001$). Soybean BIOORG and CONFYM were in between but insignificantly different from one another. On day 10 rapeseed CONFYM was again the highest ($p < 0.001$) from the other three treatments which also influenced the system effect (CONFYM > BIOORG).

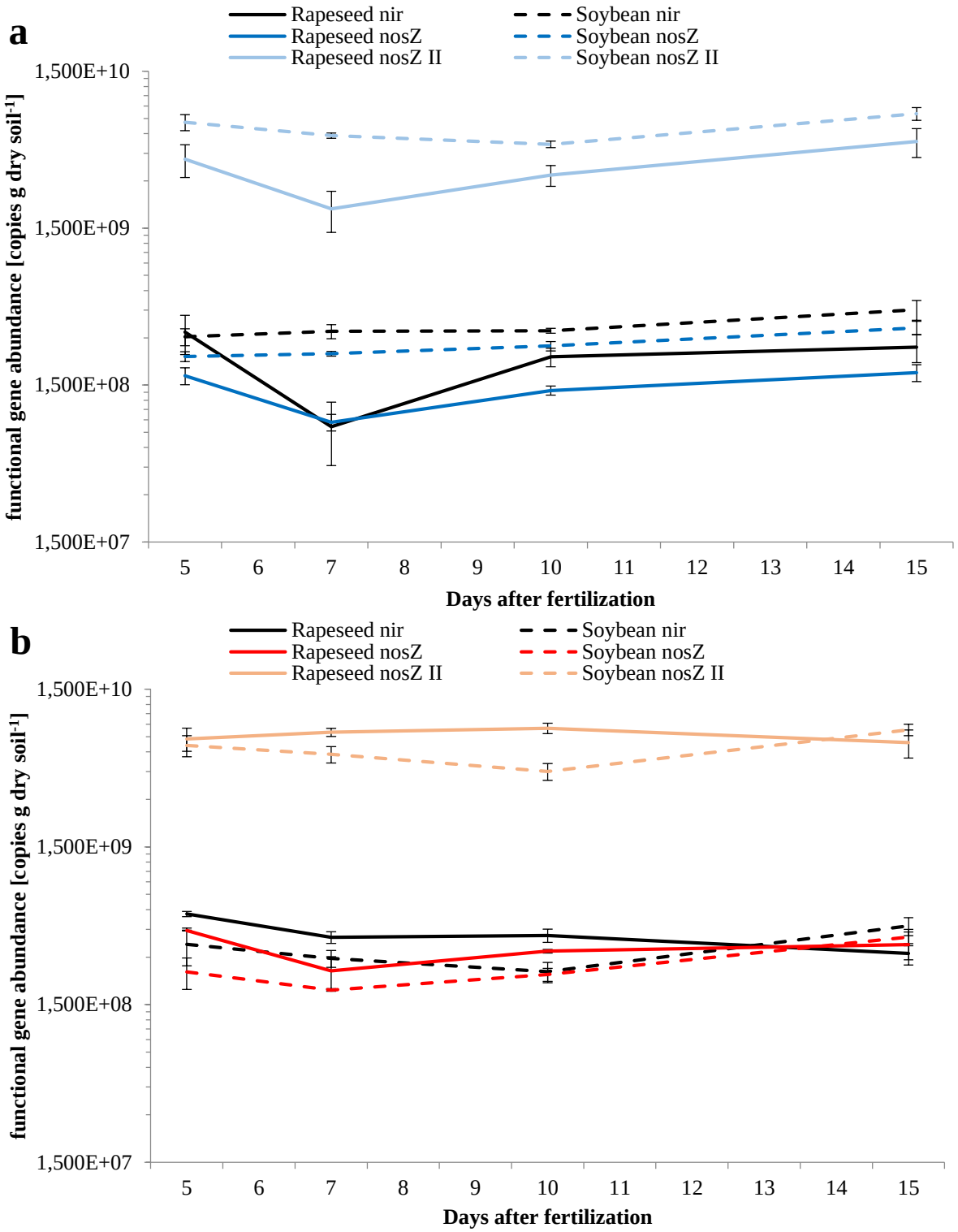

Figure 13: Functional gene copy numbers for BIOORG (a) and CONFYM (b) systems. *nirK* and *nirS* copies are summed up in *nir* group which is presented on the graph. *nosZ* and *nosZ II* are presented separately because there is more than one order of magnitude difference between them. Means and standard errors of four plot replicates are shown.

Slika 13: Kopije funkcionalnih genov v BIOORG (a) in CONFYM (b) sistemu. *nirK* in *nirS* sta združena skupaj v *nir* skupino, ki je predstavljena na grafu. *nosZ* in *nosZ II* sta na grafu predstavljena ločeno, ker je med njima več kot en velikostni razred razlike. Prikazana so povprečja in standardne napake štirih ponovitev.

4.5.3 Denitrifier gene transcript abundance

Functional gene transcription showed greater dynamics than their abundance (Figure 14). Increase in the size of around one and a half order of magnitude was detected among 3 of 4 treatments for both *nir* and *nosZ* genes. Stable levels of *nir* and *nosZ* transcripts were observed in rapeseed BIOORG treatment. Other three treatments (rapeseed CONFYM, soybean BIOORG and CONFYM) are following the trend of increase after the initial time point which could be related to coinciding WFPS increase (Figure 7). Soil water content increase established more anoxic environment due to waterlogging and probably also because of the mobilized nutrients and sequentially increased respiration. The increase of *nir* and *nosZ* transcripts in BIOORG and CONFYM soybean treatment is in concordance with increased N_2O emissions at the same time points. However the increase of both functional gene transcript numbers in rapeseed CONFYM treatment did not result in observable N_2O emissions.

In CONFYM system (Figure 14) *nir* transcript abundances were higher than *nosZ* in the first time point for both pre-crops rapeseed and soybean. Following an increase coinciding with WFPS increase both gene transcripts stabilized on the similar level for both pre-crops. This could explain non-detectable N_2O emissions in rapeseed because it could mean that all N_2O produced (*nir*) is reduced (*nosZ*). However this logic couldn't explain high N_2O emissions in soybean pre-crop.

nir transcripts detected showed significant farming system and pre-crop effect. Abundance of *nir* transcripts were higher in CONFYM compared to BIOORG at days 5 ($p = 0.027$), 10 ($p = 0.009$) and 15 ($p = 0.029$). Significant pre-crop effect was observed on day 7 ($p = 0.050$) when higher transcript values were detected in soybean compared to rapeseed. At the same time point on the level of treatment soybean BIOORG showed the highest value of *nir* transcript abundance ($p = 0.037$). At day 10 rapeseed BIOORG demonstrated the lowest transcript rate compared to other three treatments ($p = .016$).

On *nosZ* transcript level no significant pre-crop, system or treatment effect was detected at all four time points.

No transcripts of *nosZ II* were detected. The reason for that could be the length of the qPCR amplified fragment (~ 690-720 bp) because mRNA of this length couldn't be conserved. Other reason could be that *nosZ II* was not being transcribed in the first place. This is more probable explanation as 628 bp fragments from *amoA* could be detected with qPCR.

4.5.4 Dependence between molecular biology markers and emissions

To examine whether there is a dependence between gene and gene transcript data and N_2O emissions that can be quantified, correlation analysis between *nir*:*nos* ratio and the product of incomplete denitrification N_2O was performed. Ratio *nir*:*nos* was taken as it represents ratio between genes responsible for producing N_2O (*nirK*, *nirS*) and reducing N_2O (*nosZ*, *nosZ II*).

All correlations between *nir*:*nos* ratio and N_2O emissions were insignificant on both gene and transcript level (Table 8). In general, higher correlations were observed for cDNA (transcript) compared to DNA (gene) level ratios. For correlation on DNA level similar numbers of observations were collected for all the treatments. Soybean in BIOORG treatment showed the highest correlation at the lowest *p* value at the same time. Similar trend was observed on the cDNA level, where soybean BIOORG showed the highest correlation at the lowest *p* value. Second highest correlation was observed in rapeseed CONFYM treatment with the same low *p* value. However, number of observations differed markedly on the cDNA level which could have an influence on Spearman's coefficient. For soybean BIOORG only 8 observations were collected while for the other 3 treatments between 11 and 14 observations were collected.

Table 8: Correlations between nir:nos ratio and N_2O emission for different treatments. On DNA level values for *nirK* and *nirS* were summed up in *nir* group while *nosZ* and *nosZ II* were summed up in *nos* group. On cDNA level *nosZ II* were not added to *nos* as no respective transcripts were detected.
Preglednica 8: Korelacija med *nir*:*nos* razmerjem in N_2O emisijami za različne tretmaje. Na DNA nivoju so bile vrednosti za *nirK* in *nirS* seštete v *nir* skupino medtem ko so bile *nosZ* in *nosZ II* vrednosti seštete v nos skupino. Na nivoju genskih transkriptov je bil postopek ponovljen s to razliko, da je *nos* skupina obsegala samo vrednosti za *nosZ*, ker *nosZ II* transkripti niso bili detektirani.

Treatment	**DNA**			**cDNA**		
	Spearman's coefficient	**p**	**Number of observations**	**Spearman's coefficient**	**p**	**Number of observations**
Rapeseed CONFYM	0.17	0.51	16	0.46	0.10	14
Soybean CONFYM	0.08	0.76	15	0.26	0.44	11
Rapeseed BIOORG	0.08	0.79	15	0.02	0.95	13
Soybean BIOORG	0.41	0.13	15	0.62	0.10	8

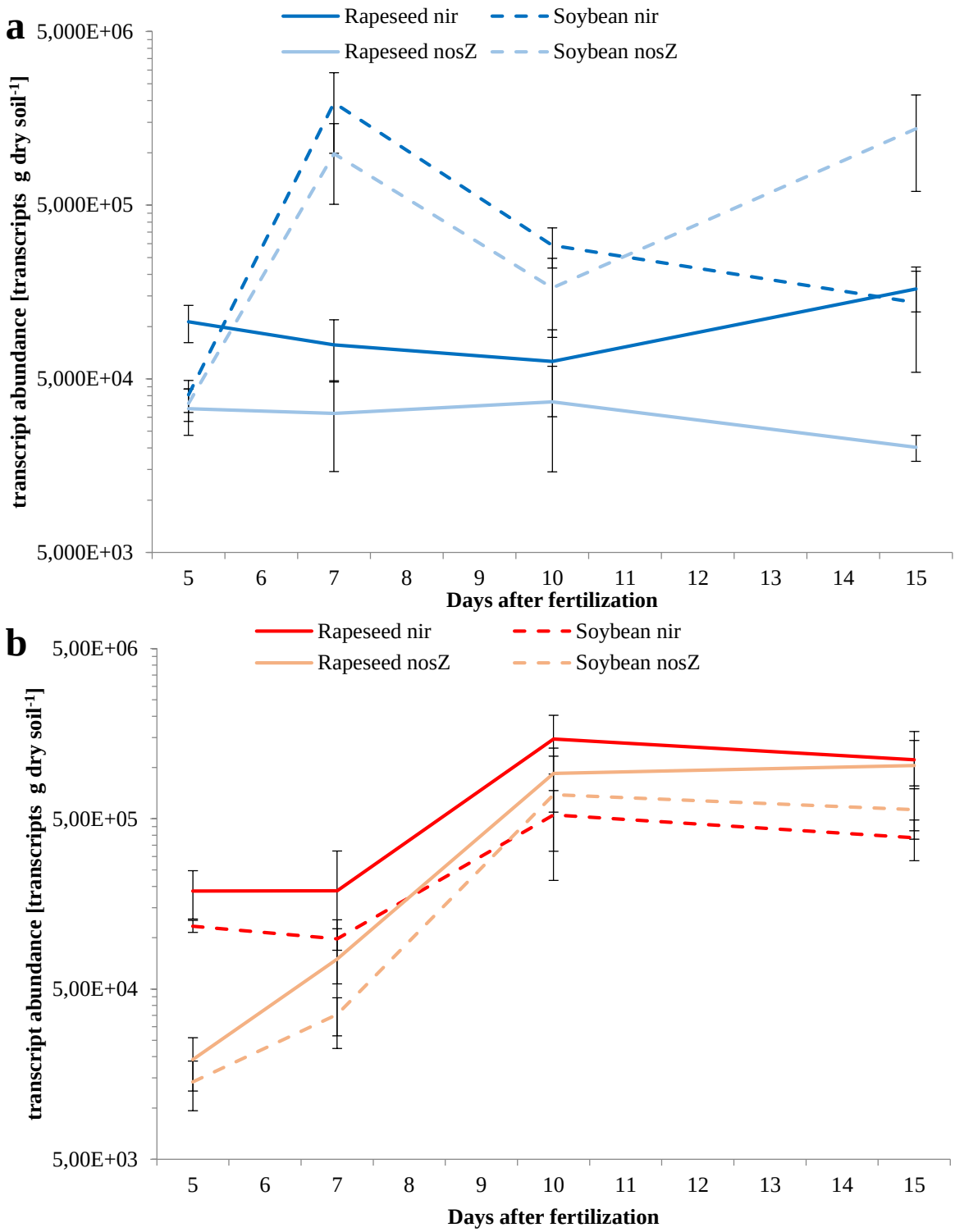

Figure 14: Transcript numbers for BIOORG (a) and CONFYM (b) system. *nirK* and *nirS* transcripts are summed up in *nir* group which is presented on the graph. From the *nos* gene group only transcripts for *nosZ* were detected. Means and standard errors of four or less (depending on if the transcripts were detected) plot replicates are shown.

Slika 14: Vrednosti za transkripte funkcionalnih genov v BIOORG (a) in CONFYM (b) sistemu. Vrednosti za *nirK* in *nirS* so združene v *nir* skupino, ki je predstavljena na grafu. Od *nos* skupine so bili detektirani samo *nosZ* transkripti. Prikazana so povprečja in standardne napake štirih ali manj ponovitev (odvisno, če so bili transkripti detektirani).

5 DISCUSSION

The goal of this study was to identify the impact of different farming systems and pre-crops on N_2O emissions produced by microbial denitrification.

5.1 FARMING SYSTEM EFFECT ON N_2O EMISSIONS

Our results showed small but significant differences in daily N_2O emissions between the two farming systems (Figure 9), however this was not reflected by the cumulative (19 days) emissions (Figure 8). Our results are in accordance with Smith et al. (2012) and Louro et al. (2015), who showed that not the form (either different mineral forms or organic) but N application rate dictates the cumulative amount of N_2O emissions. Emissions detected in our study were on one hand quite low (20-50 $\mu g\ m^2\ h^{-1}$) compared to emissions from fields with other agricultural crops (corn) (~ 250 $\mu g\ m^2\ h^{-1}$) (Snider et al., 2015), while on the other hand they were in accordance with reported emissions from winter wheat fields after first fertilization. This is especially true if we take into account lower application rate (150 kg N ha^{-1}) in our experiment as compared to other studies (170 – 220 kg N ha^{-1}) (Smith et al., 2012; Gu et al., 2013).

Soil water content (i), temperature (ii) and mineral nitrogen (iii) availability were identified as the main factors influencing N_2O emissions in the field (Saggar et al., 2013; Butterbach-Bahl et al., 2013; Smith et al., 2012). Optimal soil water content (i) for denitrification was estimated at 80% WFPS (Kool et al., 2011). In our study N_2O emissions started increasing 7 days after fertilization, shortly after the rain event, at which soil water content reached around 80% +/- 5% WFPS and stayed there till the end of experiment (Figure 7). Soil temperature (ii) didn't play a significant role in our experiment as it was always in the range between 7 and 11 °C (Annex I) due to our rather short term sampling schedule (21 days in March 2015). Contents of mineral nitrogen (iii) especially those of NO_3^- were significantly different among the farming systems (Figure 10), which could be explained by the fertilizer application rate and type: CONFYM received mineral fertilizer in the form of NH_4NO_3 (ammonium nitrate) and BIOORG organic fertilizer in the form of slurry constituting of organic N and NH_4^+. This was not completely reflected by N_2O emissions since only once (on day 10 after fertilization) were values in CONFYM significantly higher than in BIOORG. Based on these results we can conclude that we detected a small but existing farming system effect on daily but not cumulative N_2O emissions.

5.2 PRE-CROP EFFECT ON N_2O EMISSIONS

Significant pre-crop effect on daily N_2O emissions was observed (at 3 out of 5 time points) during the emission peak. Higher N_2O emissions were found in soybean than in rapeseed (Figure 9). Consequently also a significant pre-crop effect on cumulative N_2O emissions was observed (Figure 8). Interestingly, this does not have a basis in mineral nitrogen

species availability, as there were no significant differences among NO_3^- and NH_4^+ dynamics between soybean and rapeseed. One reason for different emission patterns could be different C:N ratio of the crop residues (Table 7) incorporated before sowing of the winter wheat. Soybean residues had lower C:N ratio which could mean releasing the nitrogen and stimulating N_2O emissions, whereas rapeseed residues would immobilize nitrogen due to the higher C:N ratio and therefore decrease emissions (Huang et al., 2004; Baggs et al., 2000). This goes well with the observation of Vigil and Kissel (1991) where the breakeven point between nitrogen net immobilization and release was determined at C:N ratio of 41. However, C:N ratios of both crop residues examined in our study were well above the breakeven point, ratios were high with 61 – 70 and 94 – 126 for soybean and rapeseed, respectively (Table 7). We expected lower C:N ratio for soybean residues. Although a clear pre-crop effect was observed in our study, it could be hardly explained by incorporation of pre-crop residues with different C:N ratios, especially as emissions were measured 7 months after incorporation and no differences in NO_3^- and NH_4^+ dynamics in soil between pre-crops were found. It can be concluded that C:N ratio of crop residues didn't play a role in N_2O emissions in our experiment. More probable cause for differences in emissions could be the rhizosphere effect which influences microbial community and its transcription activity (Philippot et al., 2013). For example in the study of Gulden et al. (2015) it was observed that different pre-crop history in combination with weed management history influenced differences in potential denitrification (DEA), but the exact cause was not unveiled. Therefore it can be concluded that significant pre-crop effect on N_2O emissions was observed but the reason for it has yet to be identified in future investigations.

5.3 DISSOLVED ORGANIC CARBON EFFECT ON N_2O EMISSIONS

DOC concentration in the soil was shown to stimulate denitrification and N_2O emissions (Saari et al., 2009). Sources of DOC in the soil could be: (i) residues with higher C:N ratios which release DOC or (ii) fertilization with organic fertilizers. No organic carbon in the form of DOC was released from crop residues at the time of our measurement. This was detected as no difference in soil DOC content between the two pre-crops was found (Figure 11). The only detected DOC increase in BIOORG system was attributed to slurry fertilization (second source). Despite higher DOC contents measured in BIOORG system in the first 10 days (Figure 11), no corresponding increase of N_2O emissions was detected (Figure 9). This could be explained by the findings of Huang et al. (2004) and Senbayram et al. (2012) that higher DOC levels in combination with low NO_3^- content decrease N_2O:N_2 ratio and therefore detectable N_2O emission. It can be concluded that DOC measured in our experiment didn't influence N_2O emissions.

5.4 SOIL WATER CONTENT EFFECT ON N_2O EMISSIONS AND MICROBIAL COMMUNITY

Soil water content measured as water filled pore space (WFPS) played a major role in our field experiment (Figure 7). It was expected that N_2O emissions (Figure 9) will coincide with the WFPS increase as then denitrification will become more dominant due to the optimal conditions. Initial WFPS of around 60% predominantly stimulated nitrification of ammonium in BIOORG system, where gradual decrease in NH_4^+ concentration and concurrent increase in NO_3^- concentration was observed (Figure 10). In the second part of the study, after the rain event, WFPS increased to around 80% which was optimal for denitrification (Kool et al., 2011) and resulted in N_2O peak. This confirmed our first hypothesis of positive connection between increased soil water content and N_2O emissions. Further it was expected that increased WFPS will increase denitrifier community abundance and transcription of functional genes. When examining functional gene abundances (Figure 13) at the time of WFPS increase and simultaneous N_2O peak, it can be observed that they are more or less stable. Only in rapeseed BIOORG treatment, temporary decline for all functional genes was observed following the WFPS increase. 16S rDNA gene abundance showed similarly stable pattern with slight increase in CONFYM system (Figure 12). On the transcript level, WFPS increase was followed by a transcript increase for all functional genes for almost all treatments except rapeseed BIOORG (Figure 14). While this increase happened immediately in soybean BIOORG, it was more gradual in CONFYM system. Speculative reason for this could be a faster nutrient release in the BIOORG, whereas mineralization had to occur first in CONFYM system. In the same system there was an observed delay in *nir* transcription. Faster increase in *nosZ* transcription could be explained by reduced O_2 presence (increased soil water content), which is one of the inhibitors for its transcription (Spiro, 2012). Generally an increase in the abundance of functional gene transcripts coinciding with WFPS increase supports our hypothesis of increased denitrifier transcription activity after increased soil water content.

5.5 EFFECTS ON N_2O REDUCERS

A major but unresolved question in microbial ecology is whether microbial community structure and function are interlinked. A study by Philippot et al. (2011) showed that by increasing the portion of denitrifiers incapable of N_2O reduction (bacteria bearing *nirS* or *nirK* but lacking *nosZ* and *nosZ II*), the potential for denitrification is increasing. However, when looking into microbial community structure and function connection, it was observed that (*nirK*+*nirS*):*nosZ* ratio was not correlated to the denitrification product ratio N_2O:(N_2O+N_2). Potential denitrification was higher than increase in N_2O emissions, which suggests that indigenous community acted as a sink for a part of produced N_2O. Our hypotheses was that since lower N_2O:(N_2O+N_2) ratio is expected in limiting NO_3^- conditions and labile carbon addition (Senbayram et al., 2012), higher impact of N_2O

reducers containing either *nosZ* or *nosZ II* should be observed on gene and/or gene transcript abundances in BIOORG system. Our results showed the opposite (Figure 13): at two of four time points higher *nosZ* gene abundances were detected in the CONFYM system compared to the BIOORG. Similar was observed for *nosZ II* gene abundances. On the transcript level of *nosZ* (*nosZ II* was not transcribed), no significant differences were detected among different systems and pre-crops. Differences observed on the DNA level (*nir*, *nosZ*) between soybean and rapeseed could be assigned to different impacts of growing cultures on indigenous microbial community. For *Brassicacea* family, to which rapeseed belongs, it is known that it releases secondary metabolites glucosinolates, which cause changes in microbial community, especially in the family of *Rhizobiaceae* from the class *Alphaproteobacteria* (Bressan et al., 2009), which also constitutes of denitrifiers (Jones et al., 2008). However, when the effect on soil bacterial community particularly involved in N cycling was tested, no differences were found in gene abundances of ammonia oxidizing and N_2 fixing bacteria (Hossain et al., 2015). Although some indications of pre-crop effect on N_2O reducers were observed (Annex G), no clear conclusion could be made.

5.6 LINKING N_2O EMISSIONS WITH GENE AND GENE TRANSCRIPT ABUNDANCES

For any enzyme catalysed reaction occurring, information on DNA and sequentially RNA level for corresponding gene is needed. This is known as a sequence hypothesis and is the central dogma of molecular biology. As this is true in its most fundamental meaning, different variations of this process could be observed in natural systems. Rocca et al. (2014) observed that functional denitrifiying gene (*nirK*, *nirS*, *nosZ*) abundances show weak correlations with the processes (enzymatic reactions) they are involved in. However, correlation was not significant for gene transcript abundances. The first reason for that could be that RNA is harder to detect due to its short half-life and the second reason could be the small amount of RNA studies, as RNA analysis are more challenging. Further on, when examining different habitats, agricultural soil was shown to be one of the two habitats where this significant correlations were detected. In our study ratio of N_2O producing (*nir*) and reducing (*nos*) gene and gene transcripts and not solely abundance was analysed for correlation with N_2O emissions. Correlations between N_2O emissions and *nir*:*nos* ratio showed higher values for the gene transcript in comparisson to the gene abundances ratio (Preglednica 8). However, they were all insignificant. Reason for that could be too small dataset. Perhaphs with bigger dataset for both DNA and cDNA level more significant connections could be established. Since all the measurements were performed in the field, spatial and temporal heterogeneity, together with gas diffusivity, played a big role. Altogether this probably accounts for the most part of the standard error for the molecular-biological as well as geochemical data. As soil is a very diverse matrix all possible interactions between posttranscriptional effects, enzyme kinetics, diffusion rate

of gaseous products take place in different niches, which are occupied by different bacterial communities. With adding the bias from the methods this makes it even more difficult to see the clear picture. However with obtaining new data sets and performing new experiments more meaningful conclusions could be made in the future.

6 CONCLUSIONS AND OUTLOOK

Although differences in soil chemical data (DOC, NO_3^-) were observed between the two farming systems (BIOORG and CONFYM), no corresponding differences in cumulative N_2O emissions were found. Expected higher N_2O emissions in CONFYM treatment were only observed once during the emission peak by the daily measurements. In BIOORG treatment higher *nosZ* abundances were expected but not detected either. Based on our results and previous studies we may conclude that the fertilization rate (kg N ha^{-1}) is the single most important factor influencing N_2O emissions.

Many studies have been conducted underlining the effect of crop residues on N_2O emissions. However most of those studies were made under short term timescale. Our study was first to our knowledge which inspects the legacy of the pre-crop on N_2O emissions several months after their incorporation. Even though differences in N_2O emission on the level of pre-crop were observed, they could not be explained by the measured soil data (DOC, mineral N forms, WFPS). For the purpose of explaining pre-crop effect, biogeochemical soil properties should be investigated to a greater extent, especially on the level of signalling molecules and exudates, and thus trying to determine the interactions between plants and bacteria that might influence N_2O emissions. Microbial community responsible for producing N_2O and N_2 need to be further investigated, perhaps by measuring enzymatic activities.

This is also the first study to our knowledge which tried to correlate *nir*:*nos* activity ratio with N_2O emitted. Although no significant correlations were detected, a tendency for correlation with decreasing *p* value was observed on cDNA level. For more concrete conclusion more extensive data set would be needed.

Soil water content was a major factor influencing microbial processes in the soil. However, spatial and temporal heterogeneity combined with gas diffusivity were probably the factors which influenced results of this study the most. For experiments conducted in the field this is normal and will continue to influence the results. For next experiments it might be needed to consider segregating smaller micro plots in the field where the influence of those factors could be reduced.

7 SUMMARY (POVZETEK)

7.1 SUMMARY

Soils contribute to the fluxes of greenhouse gases. Among those N_2O is the most represented from agricultural sources. In order to mitigate N_2O emissions it is important to unravel reasons and mechanisms for its formation.

In our study we compared the effect of two different farming systems differing in amount and type of fertilization: conventional system with mineral fertilizer at the rate of 60 kg N ha^{-1} and organic system with slurry at the rate of 36 kg N ha^{-1}; combined with two different pre-crops (soybean and rapeseed) on N_2O emissions and denitrifying genes.

To detect those differences a 19 days experiment was set up in the DOK trial in Therwil, Switzerland where those requirements were met (long-term paralel comparison of conventional, organic and biodynamic farming systems).

Gas samples collected were quantified with GC and later analyzed with R software. Soil samples were analyzed for different geochemical parameters namely NO_3^-, NH_4^+ and DOC (dissolved organic carbon) content, pH and WFPS (water filled pore space). Abundance of bacterial community bearing denitrification genes and transcripts of *nirS*, *nirK*, *nosZ*, *nosZ II* and the whole community (16S) were determined by qPCR. Important parameter for denitrification was soil water content, where the optimum at 80%+/- 4% WFPS (water filled pore space) was reached in the second phase of the experiment. Thus it was assumed that incomplete denitrification will act as a main source of N_2O emissions.

While significantly higher daily N_2O emissions were detected in soybean compared to rapeseed, only once during the peak (day 10) were the emissions significantly higher in the CONFYM system compared to BIOORG system. Higher daily emissions in soybean were reflected by higher cumulative emissions for this pre-crop in both farming systems. Cumulative N_2O emissions didn't show any differences on the level of a farming system. DOC, NH_4^+ content didn’t show any or very small differences among different farming systems and pre-crops. Both NO_3^- and WFPS results were in accordance with our assumptions. Higher levels of NO_3^- in CONFYM system were due to the higher fertilizer application rate in this system, while higher WFPS at the beginning of the experiment in BIOORG system can be attributed to the liquid addition by slurry application.

The abundance of microbial community (*nirK*, *nirS*, *nosZ*, *nosZ II*) was influenced by farming system and pre-crop. Higher *nir* and *nosZ* gene abundances were detected in rapeseed CONFYM treatment and lower in rapeseed BIOORG. Soybean in both systems was somewhere in between (order of magnitude between 10^7 and 10^8). The whole microbial community (16S) reflected those results. Transcriptional activity of the community increased with WFPS. The increase (1-1,5 order of magnitude) was

significantly higher in CONFYM system (at 3 out of 4 time points). On the level of pre-crop no clear trend for gene transcripts was observed. Gene and gene transcript abundances could not be correlated with measured N_2O emissions even though tendency in this direction was observed.

Since our study was done as a field experiment spatial and temporal heterogeneity played a huge role for our measurements. Therefore we propose use of semi controlled experimental environment in the future which should make it easier for repeatable measurements and more conclusive results.

7.2 POVZETEK

UVOD

Tla so lahko tako vir kot tudi ponor toplogrednih plinov (CO_2, N_2O and CH_4) (Conrad, 1996). Če ne upoštevamo sproščanja CO_2 zaradi sprememb v rabi tal, je med emisijami kmetijskega izvora najpomembnejši didušikov oksid (N_2O) (Reay in sod., 2012). Le-ta nastaja v procesu denitrifikacije v tleh, zato je pomembno, da razumemo dinamiko denitrificirajoče talne mikrobne združbe v odvisnosti od različnih sistemov kmetijske pridelave.

N_2O je dolgoživeč toplogredni plin (razpolovna doba znaša 114 let), ki prispeva 6 % k celokupnim emisijam toplogrednih plinov in katerega atmosferska koncentracija se je zvišala za 21 % od pred-industrijskih časov (WMO, 2014). Poleg tega je tudi najbolj pogost »uničevalec« ozona (Ravishankara in sod., 2009). Nastajanje N_2O je tesno povezano z gnojenjem, zato je ta ukrep, skupaj z razumevanjem dinamike mikrobnih združb, bistven za upravljanje emisij N_2O iz tal (Smith in sod., 2008). Četudi je vpliv posameznih parametrov na emisije N_2O že dobro preučen (Wallenstein in sod., 2006), je še vedno veliko neraziskanega na nivoju kompleksnejših sistemov (Butterbach-Bahl in sod., 2013). Za celovito poznavanje procesa denitrifikacije v tleh so nujni dolgoletni poskusi različnih kmetijskih sistemov, v katerih spremljamo tako učinke različnih kmetijskih ukrepov (gnojenje, obdelava tal, kolobar, predposevki,...), kot tudi primerjamo konvencionalno in ekološko kmetijstvo. Eden izmed takšnih dolgoletnih testnih sistemov je poskusno polje DOK v Therwilu v Švici, kjer že od leta 1978 vzporedno primerjajo različne konvencionalne in ekološke kmetijske sisteme (Mäder in sod., 2002).

Glavni vir emisij N_2O so mikrobne transformacije v tleh (Syakila in Kroeze, 2011) (Slika 1). Od teh je najbolj zastopana denitrifikacija, obstajajo pa še nitrifikacija, kjer pride do kemične oksidacije vmesnega intermediata hidroksilamina (NH_2OH) in denitrifikacija nitrifikatorjev. Zadnja dva procesa sta predvsem pogosta pri manjši vsebnosti vode v tleh (od 50 do 70 % por napolnjenih z vodo (WFPS)) (Kool in sod., 2011). Denitrifikacijo sta Mahne in Tiedje (1995) opredelila kot proces:

- ki vodi do končnih produktov N_2 ali N_2O,
- pri katerem pride do večjega prirasta bakterijske biomase kot če bi NO_3^- ali NO_2^- služila samo kot donorja elektronov.

Denitrifikacija je heterotrofen proces, ki uporablja dušikove okside kot donorje elektronov, kadar ni na voljo kisika (O_2) (Slika 2). Prvi korak denitrifikacije je redukcija NO_3^- do NO_2^- s pomočjo encima NO_3^- reduktaze, ki je kodirana z *narG* in *napA*. Naslednji korak obsega redukcijo z NO_2^- redukatazo iz NO_2^- v plinasti NO. Le-ta je kodirana z *nirS* in *nirK* genoma. NO je toksičen plin, ki ga bakterije encimsko reducirajo v N_2O s pomočjo encima NO reduktaze, v genomu zapisane z *norB* in *qnorB*. Zadnji korak denitrifikacije je redukcija N_2O do N_2 z N_2O reduktazo v genomu bakterij kodirano z *nosZ* in *nosZ II* genoma (Philippot 2007).

Mikrobna združba sposobna denitrifikacije je opredeljena z geni, ki nosijo zapise za ključne encime denitrifikacije. Najbolj pogosto kvantificirani in ovrednoteni so geni: *nirK*, *nirS*, *nosZ* in *nosZ II*. Tretjina denitrifikatorjev nosi genski zapis samo za *nirK* in *nirS*, kar pomeni, da so sposobni samo nepopolne denitrifikacije (do N_2O). Ostali dve tretjini nosita dodatno še gene za zadnji korak denitrifikacije, torej za *nosZ* ali *nosZ II* (Philippot in sod., 2011). Od postavitve molekularnih orodij za detekcijo funkcionalnih genov se poskuša povezati strukturo mikrobne združbe z njenim delovanjem. To povezavo je lažje vzpostaviti v bolj kontroliranih poskusih. Na primer, Philippot s sod. (2011) je z manipuliranjem deleža *nir* gena dokazal vpliv sestave združbe na emisije N_2O. Zanimivo je, da emisije niso toliko narasle, kot je narasel delež *nir* gena v populaciji, kar pomeni, da je avtohtona združba delovala tudi kot porabnik nastalega N_2O. Iz tega sledi, da imajo na denitrifikacijo, poleg strukture mikrobne združbe, vpliv še drugi dejavniki. Glavni fizikalno-kemijski dejavniki, ki vplivajo na denitrifikacijo, so koncentracija NO_3^-, topni organski ogljik v tleh, O_2 kot fukcija vsebnosti vode v tleh, ter temperatura (Slika 3). NO_3^- je glavni substrat za začetek denitrifikacije, kar pomeni, da naraščanje njegove koncentracije pozitivno vpliva na denitrifikacijo. Večanje njegove koncentracije vodi predvsem v smer nepopolne denitrifikacije, ki povečuje N_2O:N_2 razmerje (Zaman in sod., 2007). Denitrifikacija je heterotrofen proces zaradi česar ima nanjo pozitiven vpliv topni organski ogljik (Saari in sod., 2009). Senbayram in sod (2012) so sicer odkrili, da visoka koncentracija topnega organskega ogljika v kombinaciji z nizko koncentracijo NO_3^- vpliva na nastanek večjega deleža N_2 glede na N_2O. Zaradi anaerobne narave procesa potrebuje denitrifikacija razmere brez kisika, ki so idealno zagotovoljene pri 80 % WFPS (Kool et al, 2010). Temperatura ima vpliv na kinetiko reakcij, difuzijo plinov in mineralizacijo organske snovi v tleh. Idealna temperatura za denitrifikacijo je okrog 30 ºC, čeprav le-ta poteka v razponu od 0 do 75 ºC (Saggar in sod., 2013).

Ekološko in kovencionalno kmetijstvo se razlikujeta predvsem v načinu in količini gnojenja (ekološko – organsko; konvencionalno – kombinirano organsko in mineralno), ter

v načinu zatiranja škodljivcev ter plevelov (ekološko – dovoljeno biotično zatiranje in sredstva na osnovi bakra, druga fitofarmacevtska sredstva niso dovoljena; konvencionalno – vsa registrirana fitofarmacevtska sredstva so dovoljena). Ekološko kmetijstvo ima s tem posledično manjše vnose energije, kar ponavadi vodi do manjših pridelkov.

Gnojenje je eden izmed agrotehničnih ukrepov, ki najbolj prispeva k emisijam N_2O saj z njim dodamo tlem dušik, ki ga potem porabljajo rastline (bolj zaželjeno) in mikroorganizmi (manj zaželjeno). Poraba dušika s strani mikroorganizmov se najbolj pogosto izrazi v nastajanju N_2O v procesu denitrifikacije. Na emisije N_2O in na z njim povezane procese najbolj vplivajo čas, količina in način gnojenja (Shcherback in sod., 2014; Velthof in sod., 2013). Ugotovljeno je namreč bilo, da podpovršinska vdelava (5 cm globine) poveča emisije N_2O pri isti količini mineralnega ali organskega gnojila. Namen gnojenja je predvsem zagotoviti potrebna hranila za rast rastlin. Ko je presežena potrebna količina, emisije N_2O začnejo naraščati eksponentno. Zaradi tega je za boljšo izrabo gnojil potrebno sezonsko količino gnojila razdeliti v 2-3 gnojenja. Čas gnojenja je pri tem pomemben, saj ga je za čim večjo učinkovitost potrebno prilagoditi vegetaciji in razmeram v tleh. Zanimivo je, da vrsta dušikovega gnojila ne vpliva na emisije N_2O. Smith in sod. (2012) so v študiji gnojenja v ozimni pšenici namreč pokazali, da različna mineralna gnojila, v katerih je dušik prisoten v različnih oblikah (nitrat, amonij in urea), nimajo vpliva na emisije N_2O. Nadalje, Louro in sod. (2015) so pokazali, da tudi različna vrsta gnojila t.j. mineralna v primerjavi z organskimi nima vpliva na kumulativne emisije N_2O v kolikor je količina dodanega N ha^{-1} tudi enaka. Posledično so večje emisije v konvencionalnih sistemih kmetovanja v primerjavi z ekološkim, kjer se gnoji z organskimi gnojili, ponavadi rezultat večjih odmerkov gnojila (več kg N ha^{-1}) v konvencionalnih sistemih (Skinner in sod., 2014).

Drug način vnosa hranil v tla, poleg gnojenja, je vdelava rastlinskih ostankov, na primer predposevkov. Pokazano je bilo, da je vnos rastlinskih ostankov z širokim C:N razmerjem celo znižal N_2O emisije, verjetno zaradi imobilizacije N (Baggs in sod., 2000). Huang in sod. (2004) pa so odkrili pozitivno povezavo med N_2O emisijami in z ozkim C:N razmerjem.

Naša študija je ena izmed prvih, ki poleg vpliva kmetijskega sistema preučuje tudi zgodovino preposevkov na emisije N_2O in mikrobno združbo. V raziskavi, kjer so preučevali vpliv zgodovine zaplevljenosti, je bilo namreč pokazano, da le-ta vpliva na mikrobno združbo in potencialno denitrifikacijo. Bolj zaplevljena polja so imela večji potencial za denitrifikacijo, kar je bilo pojasnjeno z zmanjšano uporabo herbicidov (Gulden in sod., 2015). Bolj natančnih študij s tega področja ni, so pa Philippot in sod. (2013) dobro povzeli učinek rizosfere, ki igra eno izmed ključnih vlog pri ekologiji in procesih mikrobov.

Cilj magistrske naloge je bil ugotoviti vpliv predposevka (oljna ogrščica, soja) in različnih kmetijskih sistemov (konvencionalni, ekološki) na emisije N_2O in dinamiko denitricifirajoče mikrobne združbe pri pridelavi ozimne pšenice. Postavili smo naslednje hipoteze:

- večje N_2O emisije pričakujemo v konvencionalnem sistemu zaradi večjih gnojilnih odmerkov N kot v ekološkem kmetijstvu;
- večje emisije N_2O pričakujemo pri predposevku soje zaradi ožjega C:N razmerja rastlinskih ostankov kot pri oljni ogrščici;
- organski ogljik dodan z gnojenjem bo povečal emisije v ekološkem sistemu pridelave;
- pričakujemo vpliv vsebnosti vode v tleh na denitrifikacijo;
- številčnost in transkripcijska aktivnost N_2O reducentov (*nosZ* in *nosZ II*) bo najbolj zaznavna v ekološkem sistemu zaradi majhnih gnojilnih odmerkov N in dodanega topnega organskega ogljika;
- razmerje transkriptov *nir*:*nos* bo v pozitivni zvezi z emisijami N_2O.

METODE DELA

Raziskavo smo izvedli na dolgoletnem DOK poskusu v Therwilu v Švici, kjer vzporedno potekajo 4 različni kmetijski sistemi in 1 negativna kontrola: CONMIN (konvencionalni sistem samo z mineralnim gnojenjem), CONFYM (konvencionalni sistem z uporabo mineralnih in organskih gnojil), BIOORG (ekološki sistem), BIODYN (biodinamični sistem) in NOFERT – negativna kontrola (biodinamični sistem brez gnojenja) (Mader in sod., 2012, Mayer in sod., 2015). Vsak sistem kmetovanja poteka v 4ih ponovitvah. Vsaka ponovitev je razdeljena na 3 polja, na katerih zaporedno poteka isti kolobar. V letu 2015 je bila na obravnavanjih naše raziskave posejana ozimna pšenica (*Triticum aestivum L.*), pred tem pa dva različna predposevka (ločeni parceli znotraj polja): oljna ogrščica in soja. Izbrane parametre v naši raziskavi smo spremljali 21 dni, osredotočili smo se le na kmetijska sistema BIOORG in CONFYM sistema, ki se razlikujeta v odmerkih in vrsti gnojil (BIOORG – organsko gnojenje, 36 kg N ha^{-1}; CONFYM – mineralno gnojenje, 50 kg N ha^{-1} (Preglednica 1). V času tik pred in po prvem gnojenju ozimne pšenice smo v rednih časovnih intervalih vzorčili emisije plinov, ter odvzeli vzorce tal (Preglednica 2). Vzorce plinov za analizo N_2O smo pridobili z metodo statične komore (Hutchinson in Mosier, 1981), talne vzorce za fizikalno-kemijske in molekularne analize pa smo odvzeli s sondo premera 2 cm v globini 0-12 cm 8-10x na poskusno polje, jih homogenizirani in del vzorca shranili na 4 ºC (fizikalno-kemijske analize) in del na - 80 ºC (molekularne analize). Vzorčenje je potekalo vsakič ob istem času (med 9. in 11. uro CET). Komore za vzorčenje plinov so predstavljene na Slika 4.

Plin N_2O smo določali s plinskim kromatografom (Agilent Technologies, 7890A, Santa Clara, CA, USA) opremljenim z ECD (electron capture detector) detektorjem. Uporabili smo standardne mešanice plinov (N_2O, CO_2, CH_4) za izdelavo umeritvene krivulje in na podlagi le-te so bile s pomočjo programske opreme Agilent ChemStation Revision C.01.04 določene koncentracije N_2O v vzorcih. Podatke smo obdelali z R programsko opremo (verzija 3.0.2 – 2013-09-25). Za izračun tokov N_2O smo uporabili programski paket HMR (verzija 0.3.1), ki pripiše najbolj ustrezno funkcijo (Hutchinson in Mosier nelinearno funkcijo, robustno regresijsko funkcijo ali linearno regresijsko funkcijo) dejanski krivulji N_2O emisij (Slika 5).

Fizikalno-kemijske lastnosti tal, kot so volumska gostota, pH, ter vsebnosti NO_3^-, NH_4^+, topnega organskega ogljika (DOC) in vode v tleh so bili določeni po standardiziranih in validiranih postopkih.

Za namene molekularnih analiz smo s kitom RNA PowerSoil Total RNA Isolation Kit (MoBio, RNA PowerSoil® Total RNA Isolation Kit cat.: 12866-25, Carlsbad, CA, USA) iz vzorcev tal sočasno ekstrahirali DNA in RNA. Za določanje učinkovitosti ekstrakcije smo uporabili dodatek internega standarda ($6.32 \cdot 10^9$ kopij za DNA in $2.35 \cdot 10^{11}$kopij za RNA vzorce). Koncentracija ekstraktov DNA in RNA smo pomerili s fluoremetrom Qubit (Invitrogen, Qubit 1.0, Carlsbad, CA, USA). Za zaznavanje aktivnosti transkripcije mikroorganizmov smo prepisali izolirano RNA v cDNA s kitom QuantiTect Reverse Transcription Kit (Quiagen, QuantiTect Reverse Transcription Kit, Venlo, Netherlands) po navodilih proizvajalca. Predhodno smo odstranili inhibitorje s kitom (Zymo Research, OneStep PCR Inhibitor Removal Kit, Irvine, CA, USA). Neposredno pred transkripcijo smo preverili odsotnost genomske DNA s kvantitativno verižno reakcijo s polimerazo (qPCR) za 16S DNA. Za demonstracijo efektov različnih kmetijskih sistemov in predposevkov na ključne gene denitrifikacije (*nirK*, *nirS*, *nosZ*, *nosZ II*) smo uporabili kvantitativno verižno reakcijo s polimerazo (qPCR). Oligonukleotidni začetniki, točni pogoji qPCR reakcij in izvor standardov so opisani v Preglednica 3,Preglednica 4 inPreglednica 5. Standardno krivuljo za vsako reakcijo smo konstruirali z desetkratno redčitveno vrsto. V vsaki qPCR reakciji je bilo uporabljenih 7 redčitev v 2 ponovitvah. Surove podatke s qPCR naprave smo analizirali z LinReg algoritmom, kjer je učinkovitost vsake reakcije določena posamično (Ruijter in sod., 2009). Število kopij vsake reakcije smo izračunali iz koeficienta naklona standardne krivulje in baznega cikla po enačbi (eq. 7). Število kopij posameznih vzorcev je bilo normalizirano na gram suhih tal. Zanesljivost reakcij je bila preverjena z učinkovitostjo reakcij in R^2 (Preglednica 6) ter vizualno z gelsko elektroforezo (Slika 6).

Statistično analizo smo izvedli s SPSS statistično programsko opremo (IBM, SPSS Statistics 22, Armonk, NY). Vsaka časovna točka in vsak merjeni parameter je bil testiran z analizo variance (ANOVA). Kjer so bile dokazane statistično značilne interakcije smo izvedli Duncanov Post-Hoc test.

REZULTATI

Na poskusnih poljih DOK v Švici smo spremljali emisije N_2O in dinamiko denitricifirajoče mikrobne združbe (*nirK*, *nirS*, *nosZ*, in *nosZ II*) v odvisnosti od kmetijskega sistema (BIOORG, CONFYM) in predposevka (soja, oljna ogrščica) 19 dni po gnojenju.

Na vsebnost vode v tleh, ki smo jo prikazali z deležem por napolnjenih z vodo (water filled pore space – WFPS) (Slika 7), so najbolj vplivale padavine (6. dan poskusa). Tako smo lahko ločili dve obdobji, 60-75 % v prvem in od 75-85 % WFPS v drugem (Slika 7). Vsebnost vode v tleh je bila pred padavinami manjša kot po padavinah v obeh sistemih. Če sistema medsebojno primerjamo, pa ugotovimo statistično značilno večjo vsebnost vode v tleh BIOORG sistema (70 % v primerjavi s 66 % v CONFYM) v prvem obdobju, kar je posledica dodatka tekočine pri gnojenju z gnojnico v ekološkem sistemu. Vodno-zračne razmere so bile ugodne za nitrifikacijo, ki je bila v BIOORG sistemu tudi opažena (Slika 10 a). Po padavinah (6. dan po gnojenju), torej v drugem obdobju, pa je bila vsebnost vode v tleh enaka v obeh sistemih in je znašala 80 % +/- 5 %, kar je optimalna vrednost za denitrifikacijo (Kool in sod., 2010).

Emisije N_2O so sledile dinamiki vsebnosti vode v tleh. V našem poskusu (Slika 9) smo detektirali majhne N_2O emisije, ki pa so v območju emisij primerljivih študij z zimsko pšenico (20-50 $\mu g\ m^2\ h^{-1}$). Emisije so začele naraščati v drugem obdobju poskusa (po padavinah 6. dne) (Slika 7), v katerem je bil vpliv predposevka tudi statistično značilen. Večje dnevne emisije smo izmerili pri soji v primerjavi z oljno ogrščico. Vpliv kmetijskega sistema je bil manj opazen, namreč le pri dveh vzorčenjih so bile emisije večje v konvencionalnem (CONFYM) kot v ekološkem (BIOORG) sistemu. Kumulativne emisije N_2O kažejo statistično značilen učinek predposevka, ne pa tudi vpliva kmetijskega sistema (Slika 8).

Vir emisij so bile povečane vsebnosti nitrata (NO_3^-) in amonija (NH_4^+) v tleh (Slika 10), ki so povezane z gnojenjem (Preglednica 1). V konvencionalnem sistemu smo opazili začetno povečanje vsebnosti NO_3^- (~ 30 mg N kg suhih tal^{-1}) in NH_4^+ (~ 15 mg N kg suhih tal^{-1}) takoj po gnojenju, kar smo pričakovali, saj sta obe dušikovi spojini del mineralnega gnojila (amonijev nitrat). Oba NH_4^+ in NO_3^- spojini sta bila porabljena v talnih procesih, saj so se vsebnosti v času zmanjšale. Na začetku so bile standardne napake večje, kar priča še o nepopolni razporeditvi mineralnih oblik N. Pri ekološkem sistemu se je vsebnost NH_4^+ (~ 10 mg N kg suhih tal^{-1}) povečala po gnojenju, nato pa se je postopoma zmanjšala, vsebnost NO_3^- (~ 7,5 mg N kg suhih tal^{-1}) pa se je počasi povečala. To lahko priča o nitrifikaciji, za katero je bila ugodna vsebnost vode v tleh (70 % WFPS) v prvem obdobju poskusa (Slika 7). Zgodovina različnih predposevkov ni vplivala na različne vsebnosti NO_3^- in NH_4^+ v tleh. Opazen pa je vpliv kmetijskega sistema, in sicer je bila vsebnost NO_3^- v konvencionalnem sistemu skozi celoten poskus statistično značilno večja kot v ekološkem. V ekološkem sistemu namreč dušik ni bil dodan v obliki NO_3^- (ampak NH_4^+) in

domnevamo lahko, da je ves NO_3^-, ki je bil prisoten, nastal iz NH_4^+. Pri vsebnosti NH_4^+ v tleh ni bilo nobenih statistično značilnih razlik na nivoju sistema.

Z organskim gnojilom (gnojnico) v BIOORG sistemu smo tlem dodali organsko snov (Corg), organsko vezan dušik in NH_4^+. V prvem obdobju poskusa, takoj po gnojenju, so se zato statistično značilno povečale vsebnosti topnega organskega ogljika (DOC) (~ 63 mg C kg suhih tal^{-1}) v ekološkem (BIOORG) sistemu (Slika 11). V drugem obdobju (po 7ih dneh) pa statistično značilnih razlik v vsebnosti DOC med sistemoma nismo več zaznali. Domnevamo, da se je DOC porabil v talnih procesih, zato so vsebnosti padle na začetno vrednost (~ 50 mg C kg suhih tal^{-1}). Vpliva predposevka nismo zaznali.

Drug način vnosa hranil ter posledično substrata za denitrifikacijo v tla je poleg gnojenja zadelava rastlinskih ostankov. Izračun C:N razmerja rastlinskih ostankov vključenih v preučevana obravnavanja DOK (Preglednica 7) je pokazal statistično značilne razlike na nivoju kmetijskega sistema in predposevka. V osnovi je bilo statistično značilno širše razmerje pri oljni ogrščici (~126 v ekološkem in ~ 94 v konvencionalnem) ter v BIOORG sistemu. Statistično značilno najožje C:N razmerje ostankov smo opazili pri soji v obeh kmetijskih sistemih (70 v ekološkem in 62 v konvencionalnem).

Pri pH-ju in organski snovi v tleh (SOC) nismo zaznali statistično značilnih razlik na nivoju kmetijskega sistema in predposevka (Preglednica 7).

Za namen vrednotenja dinamike mikrobne združbe po gnojenju smo z verižno reakcijo s polimerazo (qPCR) kvantificirali gene celotne populacije (16S) in gene, ki sodelujejo pri denitrifikaciji (*nirS*, *nirK*, *nosZ*, *nosZ II*). Analizirali smo 4 časovne točke: pred deževnim obdobjem, začetek, naraščanje ter padec vrha emisij N_2O (Slika 9). Detektirali smo številčnost genov (DNA nivo – Slika 13) in transkriptov (RNA nivo – Slika 14) bakterij sposobnih denitrifikacije. V namen lažje interpretacije rezultatov smo rezultate *nirS* in *nirK* združili v enotno skupino *nir*, saj obe služita isti funkciji (Philippot, 2002). Pri *nosZ* in *nosZ II* števila kopij nismo združili saj je *nosZ II* presegal *nosZ* pojavnost za 1,5 velikostnega reda in bi se izgubil pomen *nosZ*. To je bilo opaženo že prej in priča o velikosti mikrobne populacije z *nosZ II*, ki je bila šele nedavno odkrita (Jones in sod., 2014). *nir* skupina je bila načeloma bolj številčna od *nosZ*, kar je v skladu s prej objavljenimi študijami (Kandeler in sod., 2006). Za vse tri genske skupine (*nir*, *nosZ* in *nosZ II*) lahko rečemo, da so bile bolj številčno zastopane v konvencionalnem sistemu: zastopanost *nir* je bila v območju okrog $3{,}0 \cdot 10^8$ kopij g suhih tal^{-1} (konvencionalni sistem) in $2{,}3 \cdot 10^8$ kopij g suhih tal^{-1} (ekološki sistem); zastopanost *nosZ* je bila okrog $2{,}8 \cdot 10^8$ kopij g suhih tal^{-1} (konvencionalni sistem) in $1{,}5 \cdot 10^8$ kopij g suhih tal^{-1} (ekološki sistem); zastopanost *nosZ II* je bila okrog $7{,}0 \cdot 10^9$ kopij g suhih tal^{-1} (konvencionalni sistem) in $4{,}5 \cdot 10^9$ kopij g suhih tal^{-1} (ekološki sistem) Spremembe v razponu posameznih funkcionalnih genov so bile majhne skozi vse štiri časovne točke. Na razmerje med *nir* in *nosZ* kmetijski sistem in predposevek nista imela vpliva. Pri vseh genih smo detektirali

največje vrednosti pri oljni ogrščici v konvencionalnem sistemu in najmanjše pri predposevkih v ekološkem sistemu. Celotna bakterijska združba (16S rRNA) je izražala podobno dinamiko kot preučevani denitricifirajoči geni (Slika 12). Takšen trend pa ni sovpadal z detektiranimi emisijami N_2O (Slika 9).

Transkripcija denitrifikatorjev je pokazala večjo dinamiko kot zastopanost te bakterijske združbe. Opazimo lahko primerljiv porast števila transkriptov za *nir* in *nosZ* v rangu od 1 do 1,5 velikostnega razreda pri treh od štirih tretmajev (oljna ogrščica v konvencionalnem sistemu, soja v obeh sistemih) (iz ~$1{,}5 \cdot 10^4$ na ~$2{,}0 \cdot 10^6$), ki sovpada s povečano vsebnostjo vode v tleh (Slika 7) in emisijami N_2O (Slika 9). Večje razlike smo ugotovili pri *nir* skupini, in sicer je bila transkripcija *nir* v treh od štirih časovnih točkah večja v konvencionalnem sistemu in v eni od štirih časovnih točk pri soji. Kmetijski sistem in vrsta predposevka nista vplivala na vrednosti *nosZ* transkriptov. Transkriptov *nosZ II* nismo detektirali, za kar lahko obstajata dva razloga: neobstojnost tako velikega fragmenta mRNA (~ 690-720 bp) oz. inhibirana transkripcija. Prvi razlog se zdi manj verjeten, saj smo detektirali transkripte drugega fragmenta s podobno dolžino.

Korelacija med emisijami N_2O in razmerjem *nir*:*nos* genov ter genskih transkriptov denitrifikatorjev v nobenem od naših primerov ni bila statistično značilna (Preglednica 8). Največji koeficient korelacije smo določili pri razmerju transkriptov za sojo v ekološkem sistemu. Korelacijski koeficienti so bili splošno večji pri korelaciji emisij N_2O z razmerjem *nir*:*nos* transkriptov (RNA), k čemur je verjetno pripomoglo manjše število pridobljenih podatkov pri le-teh.

DISKUSIJA

Namen naše raziskave je bil ugotoviti vpliv kmetijskega sistema (ekološki – BIOORG, konvencionalni – CONFYM) in različnih predposevkov (soja, oljna ogrščica) na emisije N_2O in dinamiko denitricifirajoče mikrobne združbe. Značilne razlike med obravnavanji smo določili pri vsebnosti vode in nitratov v tleh, transkriptih denitricifirajočih genov (*nir*, *nosZ*) bakterijske združbe, ter emisijah N_2O.

Dnevne emisije N_2O (Slika 9) so bile v naši študiji majhne, kar je v skladu z drugimi študijami upoštevajoč odmerke N (Smith in sod., 2012). V konvencionalnem sistemu smo ugotovili statistično značilno večje dnevne (Slika 9), ne pa tudi kumulativnih emisij (Slika 8), v primerjavi z ekološkim. Kar se sklada z ugotovitvami drugih študij, kjer so izmerili podobne emisije N_2O pri gnojenju z različnimi mineralnimi in organskimi gnojili, v kolikor je količina dušika ha^{-1} enaka (Smith in sod., 2012; Louro in sod., 2015). Sklepamo lahko, da so večje dnevne emisije v konvencionalnem sistemu posledica povečane vsebnosti NO_3^- v tleh, dodanega z mineralnim gnojenjem (Slika 10).

Ugotovili smo vpliv predposevka pri pridelavi pšenice na N_2O emisije. Dnevne emisije so bile statistično značilno večje pri soji (Slika 9) kot pri oljni ogrščici, kar se je odrazilo tudi

pri kumulativnih emisijah (Slika 8). Prvi možni vzrok za večje emisije je lahko ožje C:N razmerje pri soji. Domnevno se je na polju oljne ogrščice sprostilo več dušika v tla in je bil posledično na voljo za denitrifikacijo (Huang in sod., 2004). Pri širših C:N razmerjih pa lahko pride do nasprotnega učinka: sproščanja ogljika in imobilizacije dušika v tleh (Baggs in sod., 2000). Razmerja ostankov v naši študiji so bila v razponu 61-70 za sojo in 94-126 za oljno ogrščico. Obe C:N razmerji sta nad mejo (41), ki sta jo Vigil in Kissel (1991) določila za izenačitev procesov sproščanja in imobilizacije N. V naši študiji smo emisije spremljali sicer 7 mesecev po vdelavi v rastlinskih ostankov v tla, ko so prej omenjeni efekti že izgubili vpliv. To smo potrdili z merjenjem vsebnosti NO_3^- (Slika 10) in topnega organskega ogljika (DOC) (Slika 11) v tleh, ki se med predposevkoma nista značilno razlikovala. Iz tega lahko sklepamo, da razlike v C:N razmerju niso ključno vplivale na razlike v emisijah N_2O. Drugi možni vzrok za večje emisije pri soji bi lahko bil vpliv rizosfere preteklih predposevkov. Gulden in sod. (2015) so na primer pokazali, da imajo predposevki skupaj v kombinaciji z zgodovino zatiranja plevelov vpliv na denitrifikacijski potencial, ampak točnega mehanizma za to niso odkrili. V našem primeru torej lahko govorimo o podobnem vplivu predposevka na N_2O emisije, ampak točnega razloga zanj ne poznamo.

Dokazano je bilo, da je denitrifikacija kot heterotrofen proces stimulirana z dodatkom topnega organskega ogljika (Saari in sod., 2009). Pri velikih vsebnostih DOC pride do popolne denitrifikacije (N_2 kot končni produkt) in s tem zmanjšanja N_2O:N_2 razmerja (Huang in sod., 2004; Senbayram in sod., 2012). Vsebnosti DOC so bile v tleh našega poskusa majhne(~ 63 mg C kg suhih tal^{-1}), zato smo predpostavili, da bo dodani DOC stimuliral nepopolno denitrifikacijo in s tem N_2O emisije v BIOORG sistemu. Povečanja emisij v BIOORG nismo opazili, prav tako so bile dnevne emisije manjše ali enake emisijam v CONFYM sistemu, ki organskega ogljika ni dobil. To lahko razložimo na 2 načina: (i) emisije v BIOORG sistemu so sicer bile povečane, ampak jih nismo zaznali, ker so bile oddane v obliki N_2 (potekla je popolna denitrifikacija) ali (ii) dejansko ni prišlo do večjih emisij. Zadnja razlaga je bolj verjetna, saj smo v BIOORG sistemu izmerili majhne vsebnosti NO_3^- v tleh (~ 7,5 mg N kg suhih tal^{-1}) (Slika 11).

Vsebnost vode v tleh je imela pričakovano velik vpliv na potek procesov v tleh in emisij v našem poskusu. V prvem obdobju poskusa z vsebnostjo vode v tleh okrog 60-70 % WFPS je opazna nitrifikacija (Slika 11), kasneje v drugem obdobju pri 80 % WFPS, kjer pride do vrha emisij N_2O, pa denitrifikacija (Slika 9). Verjetno je povečana vsebnost vode v tleh prispevala k mobilizaciji hranil in posledično k vzpostavitvi anaerobnih razmer (potrebnih za denitrifikacijo) zaradi povečane respiracije. Pričakovan vpliv na denitrifikacijsko združbo je bil bolj opazen pri aktivnosti (Slika 14) kot pri potencialu (Slika 13) denitricifirajočih funkcionalnih genov (*nirK*, *nirS*, *nosZ*, *nosZ II*). Povečanje števila transkriptov (*nir* in *nosZ*) je sovpadalo s povečanjem vsebnosti vode v tleh pri treh od štirih obravnavanj (pri oljni ogrščici v CONFYM sistemu ter soji v obeh sistemih) (Slika 14).

V molekularni biologiji se velikokrat srečujemo z vprašanjem ali sta struktura in funkcija bakterijske združbe povezani. Philippot in sod. (2011) so v poskusu po eni strani dokazali povečanje emisij N_2O z večanjem deleža *nir*, po drugi strani pa razmerje N_2O:(N_2O+N_2) ni koreliralo z razmerjem (*nirK*+*nirS*):*nosZ*. Struktura bakterijske družbe torej do določene mere vpliva na funkcijo. V naši študiji smo hoteli vzpostaviti povezavo med molekularno-biološkimi (številčnost genov in transkriptov genov) in fizikalno-kemijskimi parametri (vsebnost NO^{3-} in DOC v tleh, N_2O emisije). V ekološkem (BIOORG) sistemu smo tako pričakovali večji vpliv N_2O reducentov (*nosZ*), saj so bili pogoji z majhno vsebnostjo NO_3^- in razpoložljivim DOC ustrezni za popolno denitrifikacijo in s tem redukcijo N_2O do N_2 (Senbayram in sod., 2012). Naša hipoteza ni bila potrjena, saj je bil v dveh od štirih datumov vzorčenja *nosZ* bolj zastopan v konvencionalnem sistemu, v ostalih dveh časovnih točkah pa ni bilo razlik (Slika 13). Številčnost *nosZ* transkriptov ni izkazovala nobenih razlik med kmetijskima sistemoma. Sklepamo lahko, da dinamika denitricifirajoče združbe na genskem nivoju v našem poskusu ni imela ključnega vpliva na emisije N_2O. Centralna dogma molekularne biologije pravi, da je za vsako izvedeno reakcijo potreben encim zapisan na DNA nivoju, nato prepisan v mRNA in na koncu preveden v aminokislinsko zaporedje. Regulacija je možna pri vsakem koraku, kar privede do veliko različnih profilov izražanja. Struktura mikrobne združbe ima torej v njenem najbolj osnovnem pomenu v vsakem primeru vpliv na njeno funkcijo. Težje pa je dokazati, da ima vpliv na njo tudi njen posamezen del (številčnost posameznega gena/ transkripta). V pregledni študiji so tako Rocca in sod. (2014) ugotovili korelacijo med zastopanostjo genov ne pa tudi transkriptov denitricifirajočih funkcionalnih genov in emisijami N_2O. Izmed vseh ekosistemov so bila obdelovalna zemljišča opredeljena kot edini habitat, kjer je bila korelacija dokazana. Na osnovi obeh zaključkov smo v naši študiji predpostavili, da bo razmerje zastopanosti funkcionalnih genov in genskih prepisov (*nir*:*nos*) pozitivno koreliralo z emisijami N_2O. V nasprotju s prej omenjeno študijo (Rocca in sod., 2014) smo dobili boljšo pozitivno povezavo pri zastopanosti transkriptov (Preglednica 8), ampak sta bili obe (zastopanost genov in transkriptov) statistično neznačilni.

Pričakovano sta imeli velik vpliv na rezultate našega poljskega poskusa prostorska in časovna heterogenost oz. kombinacija obeh, kar se je odrazilo predvsem v standardnih napakah merjenih parametrov. Če k temu dodamo še tla kot zelo raznolik matriks z mnogimi interakcijami, je toliko bolj razumljivo, zakaj so študije procesov v tleh, še posebej na nivoju molekularne ekologije, zelo zahtevne za interpretacijo. Za reševanje teh problemov v prihodnje predvidevamo dve možnosti in sicer večjo kontrolo okoljskih pogojev (vsebnost vode, NO_3^- in NH_4^+ v tleh, bolj homogena struktura tal) na poskusnem polju in bolj specifične metode zaznavanja različnih dejavnikov v tleh.

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ANNEX A

Statistical analysis of N_2O emissions through time series. Duncan‘s Post-Hoc (D) test was performed. Statistically significant differences are marked in bold.

PRILOGA A

Statistična analiza dnevnih merjenj N_2O emisij. Za razlikovanje med skupinami je bil uporabljen Duncanov Post Hoc test (D). Statistično značilne razlike so označene s krepkim tiskom.

Days after fertilization	-1			1			3			4		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	3,74	0,61		5,70	0,74		**2,39**	**0,36**	**a**	**4,11**	**0,31**	**a**
Soybean	4,08	0,44		5,68	0,48		**3,87**	**0,51**	**b**	**5,54**	**0,48**	**b**
CONFYM	3,66	0,64		**4,45**	**0,33**	**a**	**2,47**	**0,34**	**a**	4,44	0,32	
BIOORG	4,16	0,39		**6,92**	**0,53**	**b**	**3,80**	**0,54**	**b**	5,21	0,56	
Rapeseed CONFYM	3,27	1,02		4,28	0,40		2,17	0,59		4,07	0,47	
Rapeseed BIOORG	4,21	0,57		7,12	1,01		2,62	0,39		4,15	0,40	
Soybean CONFYM	4,04	0,71		4,62	0,52		2,76	0,27		4,82	0,33	
Soybean BIOORG	4,11	0,53		6,73	0,31		4,98	0,58		6,27	0,74	
Days after fertilization	5			6			7			8		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	4,22	0,39		**0,98**	**0,14**	**a**	5,25	0,59		**3,49**	**0,47**	**a**
Soybean	5,04	0,47		**7,60**	**2,09**	**b**	6,70	0,92		**15,51**	**1,61**	**b**
CONFYM	4,15	0,39		**2,40**	**0,90**	**a**	6,23	0,97		9,53	2,41	
BIOORG	5,12	0,45		**6,17**	**2,33**	**b**	5,72	0,60		9,46	2,46	
Rapeseed CONFYM	3,77	0,34		0,84	0,23		4,27	0,39	a	3,43	0,72	
Rapeseed BIOORG	4,67	0,62		1,12	0,11		6,23	0,89	ab	3,55	0,61	
Soybean CONFYM	4,52	0,65		3,97	1,40		8,19	1,32	b	15,64	2,03	
Soybean BIOORG	5,57	0,56		11,23	2,97		5,21	0,73	ab	15,38	2,50	
Days after fertilization	10			12			15			19		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	**6,06**	**1,08**	**a**	**2,85**	**0,38**	**a**	7,31	1,09		7,84	1,65	
Soybean	**30,16**	**6,64**	**b**	**38,44**	**9,71**	**b**	5,98	1,36		10,96	2,77	
CONFYM	**24,15**	**7,73**	**b**	27,43	11,46		**9,25**	**0,93**	**b**	11,98	2,53	
BIOORG	**12,07**	**3,55**	**a**	13,86	5,55		**4,04**	**0,77**	**a**	6,82	1,72	
Rapeseed CONFYM	7,67	1,68		2,86	0,49		9,08	1,44		6,85	1,10	
Rapeseed BIOORG	4,46	0,73		2,84	0,58		5,53	1,05		8,84	3,03	
Soybean CONFYM	40,63	10,03		52,00	14,94		9,41	1,16		17,12	3,33	
Soybean BIOORG	19,69	4,57		24,88	7,89		2,55	0,39		4,80	0,80	

ANNEX B

Statistical analysis of NO_3^- dynamics through time series. Duncan‘s Post-Hoc (D) test was performed. Statistically significant differences are marked in bold.

PRILOGA B

Statistična analiza dnevnih merjenj talne vsebnosti NO_3^-. Za razlikovanje med skupinami je bil uporabljen Duncanov Post Hoc test (D). Statistično značilne razlike so označene s krepkim tiskom.

Days after fertilization	-1			1			3			5		
	mean	.se	D	mean	.se	D	mean	.se	D	mean	.se	D
Rapeseed	2,54	0,25		15,47	7,37		9,67	1,50		15,96	3,17	
Soybean	2,17	0,13		16,27	6,20		13,71	2,90		12,63	2,86	
CONFYM	2,14	0,13		**28,39**	**7,32**	**b**	**17,24**	**1,94**	**b**	**21,54**	**2,21**	**b**
BIOORG	2,57	0,24		**3,35**	**0,17**	**a**	**6,14**	**0,44**	**a**	**7,05**	**0,96**	**a**
Rapeseed CONFYM	2,14	0,11		27,37	12,10		**12,99**	**1,64**	**b**	23,87	2,53	
Rapeseed BIOORG	2,95	0,39		3,57	0,29		**6,35**	**0,85**	**a**	8,06	1,61	
Soybean CONFYM	2,14	0,23		29,41	8,22		**21,49**	**1,83**	**c**	19,22	3,22	
Soybean BIOORG	2,20	0,11		3,13	0,07		**5,93**	**0,17**	**a**	6,04	0,74	

Days after fertilization	7			10			12			15		
	mean	.se	D	mean	.se	D	mean	.se	D	mean	.se	D
Rapeseed	12,67	3,67		13,47	4,13		15,16	6,22		10,92	2,87	
Soybean	11,45	2,00		11,16	1,87		8,75	2,04		8,80	2,49	
CONFYM	**17,29**	**3,24**	**b**	**17,79**	**3,64**	**b**	**17,94**	**6,02**	**b**	**15,07**	**2,79**	**b**
BIOORG	**6,83**	**0,50**	**a**	**6,84**	**0,37**	**a**	**5,97**	**0,49**	**a**	**4,65**	**0,39**	**a**
Rapeseed CONFYM	17,63	6,42		19,25	7,16		23,50	10,94		16,47	4,16	
Rapeseed BIOORG	7,72	0,64		7,69	0,24		6,82	0,67		5,37	0,55	
Soybean CONFYM	16,95	0,82		16,33	0,76		12,38	3,16		13,68	3,57	
Soybean BIOORG	5,94	0,43		6,00	0,35		5,12	0,37		3,93	0,24	

Days after fertilization	19		
	Mean	.se	D
Rapeseed	5,73	0,93	
Soybean	4,94	0,64	
CONFYM	**7,05**	**0,72**	**b**
BIOORG	**3,62**	**0,24**	**a**
Rapeseed CONFYM	7,43	1,39	
Rapeseed BIOORG	4,02	0,30	
Soybean CONFYM	6,67	0,31	
Soybean BIOORG	3,22	0,24	

ANNEX C

Statistical analysis of NH_4^+ dynamics through time series. Duncan‘s Post-Hoc (D) test was performed. Statistically significant differences are marked in bold.

PRILOGA C

Statistična analiza dnevnih merjenj talnih vsebnosti NH_4^+. Za razlikovanje med skupinami je bil uporabljen Duncanov Post Hoc test (D). Statistično značilne razlike so označene s krepkim tiskom.

Days after fertilization	-1			1			3			5		
	mean	.se	D	mean	.se	D	mean	.se	D	mean	.se	D
Rapeseed	1,18	0,08		13,50	4,15		5,68	0,79		7,66	1,12	
Soybean	0,97	0,12		11,64	3,01		9,53	1,34		5,96	1,62	
CONFYM	0,98	0,10		16,02	4,75		8,28	1,51		8,47	1,55	
BIOORG	1,16	0,11		9,12	0,97		6,93	0,97		5,15	0,98	
Rapeseed CONFYM	1,07	0,12		15,93	8,04		**4,33**	**0,77**	**a**	9,05	1,13	
Rapeseed BIOORG	1,29	0,09		11,08	1,14		**7,03**	**0,99**	**ab**	6,26	1,66	
Soybean CONFYM	0,90	0,14		16,10	5,07		**12,23**	**0,86**	**b**	7,89	2,86	
Soybean BIOORG	1,04	0,19		7,17	0,74		**6,82**	**1,66**	**ab**	4,03	0,69	

Days after fertilization	7			10			12			15		
	mean	.se	D	mean	.se	D	mean	.se	D	mean	.se	D
Rapeseed	4,66	1,08		4,03	1,20		8,77	4,42		6,37	3,42	
Soybean	4,20	0,71		3,88	0,48		2,61	0,50		3,89	1,21	
CONFYM	4,93	1,21		4,59	1,23		9,23	4,35		**8,81**	**3,18**	**b**
BIOORG	3,93	0,40		3,33	0,21		2,09	0,29		**1,45**	**0,19**	**a**
Rapeseed CONFYM	4,71	2,13		4,85	2,29		15,50	7,44		11,46	5,81	
Rapeseed BIOORG	4,62	0,38		3,22	0,37		2,04	0,34		1,27	0,22	
Soybean CONFYM	5,16	1,14		4,33	0,89		2,95	0,74		6,15	1,79	
Soybean BIOORG	3,24	0,50		3,44	0,20		2,16	0,50		1,63	0,29	

Days after fertilization	19		
	Mean	.se	D
Rapeseed	5,61	2,26	
Soybean	3,08	0,80	
CONFYM	6,26	2,13	
BIOORG	2,44	0,84	
Rapeseed CONFYM	7,67	4,03	
Rapeseed BIOORG	3,56	1,47	
Soybean CONFYM	4,84	1,00	
Soybean BIOORG	1,32	0,16	

ANNEX D

Statistical analysis of WFPS dynamics through time series. Duncan‘s Post-Hoc (D) test was performed. Statistically significant differences are marked in bold.

PRILOGA D

Statistična analiza dnevnih merjenj talnih vrednosti poljske kapacitete (WFPS). Za razlikovanje med skupinami je bil uporabljen Duncanov Post Hoc test (D). Statistično značilne razlike so označene s krepkim tiskom.

Days after fertilization	-1			1			3			5		
	mean	.se	D	mean	.se	D	mean	.se	D	mean	.se	D
Rapeseed	64,55	2,11		67,26	2,66		68,32	2,18		62,21	1,70	
Soybean	63,79	2,11		67,98	2,34		67,72	1,84		63,12	1,58	
CONFYM	61,23	1,26		**61,61**	**1,33**	**a**	**64,30**	**1,15**	**a**	**59,40**	**1,24**	**a**
BIOORG	67,11	2,28		**73,63**	**1,33**	**b**	**71,74**	**1,84**	**b**	**65,93**	**1,11**	**b**
Rapeseed CONFYM	61,11	1,78		60,47	1,28		64,79	1,42		58,09	1,18	
Rapeseed BIOORG	67,99	2,96		74,05	1,91		71,85	3,28		66,33	1,28	
Soybean CONFYM	61,34	1,79		62,75	2,20		63,81	1,78		60,71	1,96	
Soybean BIOORG	66,24	3,40		73,21	1,82		71,63	1,65		65,53	1,79	

Days after fertilization	7			10			12			15		
	mean	.se	D	mean	.se	D	mean	.se	D	mean	.se	D
Rapeseed	80,45	1,64		80,27	2,21		80,87	2,63		82,78	1,88	
Soybean	78,39	1,93		76,74	1,99		77,59	2,01		78,92	1,93	
CONFYM	77,22	1,43		76,92	1,67		77,09	2,04		78,88	1,32	
BIOORG	81,62	1,85		80,09	2,49		81,37	2,52		82,81	2,34	
Rapeseed CONFYM	77,82	1,29		79,56	2,02		78,31	2,93		80,50	1,51	
Rapeseed BIOORG	83,08	2,37		80,98	3,90		83,43	3,98		85,06	3,04	
Soybean CONFYM	76,63	2,51		74,27	1,90		75,87	2,71		77,27	1,85	
Soybean BIOORG	80,16	2,65		79,21	3,02		79,31	2,72		80,56	3,19	

Days after fertilization	19		
	Mean	.se	D
Rapeseed	76,89	2,17	
Soybean	74,59	1,95	
CONFYM	73,95	1,60	
BIOORG	77,53	2,34	
Rapeseed CONFYM	75,53	2,53	
Rapeseed BIOORG	78,25	3,40	
Soybean CONFYM	72,37	1,61	
Soybean BIOORG	76,81	3,19	

ANNEX E

Statistical analysis of DOC dynamics through time series. Duncan‘s Post-Hoc (D) test were performed. Statistically significant differences are marked in bold.

PRILOGA E

Statistična analiza dnevnih merjenj talnih vrednosti raztopljenega organskega ogljika (DOC). Za razlikovanje med skupinami je bil uporabljen Duncanov Post Hoc test (D). Statistično značilne razlike so označene s krepkim tiskom.

Days after fertilization	-1			1			3			5		
	mean	.se	D	mean	.se	D	mean	.se	D	mean	.se	D
Rapeseed	55,03	3,38		59,25	3,44		53,33	3,50		49,09	3,45	
Soybean	51,15	4,22		58,05	3,75		54,83	4,95		53,32	3,26	
CONFYM	47,65	1,64		**50,32**	**2,71**	**a**	46,41	3,57		46,92	2,93	
BIOORG	58,53	4,48		**66,99**	**1,11**	**b**	61,75	3,07		55,49	3,24	
Rapeseed CONFYM	46,92	2,14		50,17	1,66		46,68	3,60		46,95	5,83	
Rapeseed BIOORG	63,15	2,86		68,34	1,80		59,98	3,74		51,22	3,36	
Soybean CONFYM	48,37	2,44		50,47	5,17		46,13	6,17		46,89	0,56	
Soybean BIOORG	53,92	7,84		65,64	0,89		63,53	4,70		59,75	4,65	

Days after fertilization	7			10			12			15		
	mean	.se	D	mean	.se	D	mean	.se	D	mean	.se	D
Rapeseed	**52,26**	**1,72**	**b**	47,88	0,95		56,90	3,47		57,25	3,16	
Soybean	**46,12**	**1,44**	**a**	51,40	2,60		51,54	1,21		54,69	1,39	
CONFYM	**46,28**	**1,88**	**a**	**47,00**	**0,94**	**a**	52,65	2,06		53,26	2,34	
BIOORG	**52,10**	**1,32**	**b**	**52,28**	**2,41**	**b**	55,79	3,23		58,68	2,23	
Rapeseed CONFYM	49,15	2,49		**48,44**	**0,78**	**ab**	53,72	4,05		53,08	4,02	
Rapeseed BIOORG	55,37	0,89		**47,32**	**1,68**	**a**	60,08	5,18		61,42	3,88	
Soybean CONFYM	43,41	1,94		**45,56**	**1,38**	**a**	51,58	0,29		53,44	2,41	
Soybean BIOORG	48,83	0,92		**57,24**	**2,86**	**b**	51,50	2,40		55,94	1,06	

Days after fertilization	19		
	Mean	.se	D
Rapeseed	52,57	1,69	
Soybean	50,18	0,74	
CONFYM	50,60	1,66	
BIOORG	52,16	0,93	
Rapeseed CONFYM	52,11	2,97	
Rapeseed BIOORG	53,04	1,59	
Soybean CONFYM	49,09	1,05	
Soybean BIOORG	51,28	0,72	

ANNEX F

Statistical analysis of cumulative N_2O emissions, crop residue C: N ratio and pH. Duncan‘s Post-Hoc (D) test was performed. Statistically significant differences are marked in bold.

PRILOGA F

Statistična analiza merjenj izračuna kumulativnih emisij N_2O, C: N razmerja rastlinskih ostankov in izmerjenega pH. Za razlikovanje med skupinami je bil uporabljen Duncanov Post Hoc test (D). Statistično značilne razlike so označene s krepkim tiskom.

	Cumulative emissions			Crop residue C: N ratio			pH		
	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	**99,04**	**6,72**	**a**	**110,41**	**5,92**	**b**	5,68	0,05	
Soybean	**268,65**	**47,11**	**b**	**65,74**	**1,94**	**a**	5,65	0,06	
CONFYM	221,00	55,82		**78,33**	**6,07**	**a**	5,66	0,06	
BIOORG	146,69	24,52		**97,82**	**10,15**	**b**	5,67	0,05	
Rapeseed CONFYM	**101,17**	**11,41**	**a**	**94,81**	**3,02**	**b**	5,74	0,05	
Rapeseed BIOORG	**96,91**	**6,95**	**a**	**126,01**	**3,03**	**c**	5,61	0,07	
Soybean CONFYM	**340,82**	**71,79**	**b**	**61,85**	**1,56**	**a**	5,58	0,09	
Soybean BIOORG	**196,48**	**33,44**	**b**	**69,63**	**2,25**	**a**	5,73	0,06	

ANNEX G

Statistical analysis of functional gene abundances. Duncan‘s Post-Hoc (D) test was performed. Statistically significant differences are marked in bold.

PRILOGA G

Statistična analiza kvantifikacije funkcionalnih genov. Za razlikovanje med skupinami je bil uporabljen Duncanov Post Hoc test (D). Statistično značilne razlike so označene s krepkim tiskom.

	Days after fertilization											
nir	5			7			10			15		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	4,45E+08	6,29E+07		2,64E+08	6,39E+07		3,19E+08	4,13E+07		**2,85E+08**	**3,83E+07**	**a**
Soybean	3,29E+08	4,80E+07		3,10E+08	2,60E+07		2,87E+08	2,47E+07		**4,62E+08**	**4,58E+07**	**b**
CONFYM	4,77E+08	5,74E+07		**3,48E+08**	**3,00E+07**	**b**	3,27E+08	4,02E+07		3,94E+08	5,08E+07	
BIOORG	3,15E+08	4,96E+07		**2,06E+08**	**5,62E+07**	**a**	2,80E+08	2,49E+07		3,57E+08	5,40E+07	
Rapeseed CONFYM	5,63E+08	2,09E+07		**4,01E+08**	**2,95E+07**	**b**	**4,12E+08**	**4,00E+07**	**b**	3,16E+08	4,90E+07	
Rapeseed BIOORG	3,26E+08	9,15E+07		**8,14E+07**	**3,53E+07**	**a**	**2,27E+08**	**3,07E+07**	**a**	2,61E+08	5,30E+07	
Soybean CONFYM	3,61E+08	9,73E+07		**2,94E+08**	**3,61E+07**	**b**	**2,42E+08**	**3,58E+07**	**a**	4,73E+08	6,18E+07	
Soybean BIOORG	3,05E+08	3,73E+07		**3,30E+08**	**3,35E+07**	**b**	**3,33E+08**	**1,20E+07**	**a**	4,54E+08	6,51E+07	
nosZ	5			7			10			15		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	**3,07E+08**	**4,90E+07**	**b**	1,93E+08	4,47E+07		2,33E+08	3,39E+07		2,70E+08	4,94E+07	
Soybean	**2,34E+08**	**2,53E+07**	**a**	2,07E+08	1,05E+07		2,49E+08	1,54E+07		3,74E+08	3,08E+07	
CONFYM	**3,56E+08**	**4,43E+07**	**b**	2,15E+08	2,67E+07		**2,80E+08**	**2,05E+07**	**b**	3,81E+08	4,37E+07	
BIOORG	**2,00E+08**	**1,66E+07**	**a**	1,77E+08	3,36E+07		**2,02E+08**	**2,48E+07**	**a**	2,64E+08	3,60E+07	
Rapeseed CONFYM	**4,42E+08**	**2,80E+06**	**b**	**2,45E+08**	**4,88E+07**	**b**	**3,27E+08**	**9,03E+06**	**c**	3,61E+08	7,19E+07	
Rapeseed BIOORG	**1,72E+08**	**2,11E+07**	**a**	**8,70E+07**	**1,06E+07**	**a**	**1,39E+08**	**9,15E+06**	**a**	1,80E+08	2,25E+07	
Soybean CONFYM	**2,41E+08**	**5,45E+07**	**a**	**1,85E+08**	**2,82E+06**	**b**	**2,32E+08**	**2,19E+07**	**b**	4,01E+08	4,76E+07	
Soybean BIOORG	**2,28E+08**	**1,63E+07**	**a**	**2,37E+08**	**8,19E+06**	**b**	**2,66E+08**	**1,82E+07**	**b**	3,47E+08	3,43E+07	

Continued

Continuation of annex G:

nosZ II	5			7			10			15		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	5,69E+09	9,57E+08		5,42E+09	1,17E+09		5,87E+09	1,01E+09		6,11E+09	9,31E+08	
Soybean	6,88E+09	6,47E+08		5,82E+09	4,04E+08		4,82E+09	3,20E+08		8,15E+09	5,23E+08	
CONFYM	6,98E+09	8,27E+08		**6,90E+09**	**5,57E+08**	**b**	**6,49E+09**	**8,19E+08**	**b**	7,48E+09	8,88E+08	
BIOORG	5,61E+09	8,29E+08		**3,92E+09**	**8,45E+08**	**a**	**4,20E+09**	**4,31E+08**	**a**	6,69E+09	8,24E+08	
Rapeseed CONFYM	7,26E+09	1,22E+09		**7,99E+09**	**4,05E+08**	**c**	**8,48E+09**	**6,44E+08**	**b**	6,87E+09	1,39E+09	
Rapeseed BIOORG	4,13E+09	9,76E+08		**1,99E+09**	**5,80E+08**	**a**	**3,27E+09**	**4,94E+08**	**a**	5,34E+09	1,12E+09	
Soybean CONFYM	6,59E+09	1,00E+09		**5,80E+09**	**6,87E+08**	**b**	**4,51E+09**	**5,48E+08**	**a**	8,29E+09	7,01E+08	
Soybean BIOORG	7,10E+09	8,31E+08		**5,84E+09**	**2,22E+08**	**b**	**5,14E+09**	**2,45E+08**	**a**	8,04E+09	7,45E+08	

16S	5			7			10			15		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	1,10E+10	1,45E+09		**6,98E+09**	**1,56E+09**	**a**	8,16E+09	9,23E+08		9,95E+09	1,64E+09	
Soybean	9,34E+09	1,15E+09		**1,00E+10**	**7,72E+08**	**b**	8,79E+09	7,54E+08		1,46E+10	1,89E+09	
CONFYM	1,19E+10	1,34E+09		**9,62E+09**	**5,13E+08**	**b**	8,66E+09	8,29E+08		1,46E+10	2,17E+09	
BIOORG	8,75E+09	1,15E+09		**6,99E+09**	**1,97E+09**	**a**	8,29E+09	8,66E+08		9,96E+09	1,25E+09	
Rapeseed CONFYM	1,37E+10	1,09E+09		**1,03E+10**	**7,22E+08**	**b**	**1,02E+10**	**9,75E+08**	**b**	1,18E+10	2,33E+09	
Rapeseed BIOORG	8,28E+09	1,90E+09		**2,54E+09**	**8,89E+08**	**a**	**6,16E+09**	**6,81E+08**	**a**	8,06E+09	1,88E+09	
Soybean CONFYM	9,51E+09	2,10E+09		**8,94E+09**	**5,45E+08**	**b**	**7,15E+09**	**8,20E+08**	**a**	1,74E+10	3,08E+09	
Soybean BIOORG	9,22E+09	1,25E+09		**1,14E+10**	**1,23E+09**	**b**	**1,04E+10**	**5,19E+08**	**b**	1,19E+10	9,44E+08	

ANNEX H

Statistical analysis of functional gene transcripts. Only genes where transcripts were detected are presented. Duncan‘s Post-Hoc (D) test was performed. Statistically significant differences are marked in bold.

PRILOGA H

Statistična analiza kvantifikacije transkriptov funkcionalnih genov. Za razlikovanje med skupinami je bil uporabljen Duncanov Post Hoc test (D). Statistično značilne razlike so označene s krepkim tiskom.

	Days after fertilization											
nir	5			7			10			15		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	1,24E+05	3,59E+04		**1,34E+05**	**7,20E+04**	**a**	7,68E+05	3,74E+05		6,39E+05	2,37E+05	
Soybean	6,86E+04	1,87E+04		**7,67E+05**	**5,23E+05**	**b**	3,45E+05	1,14E+05		1,97E+05	8,00E+04	
CONFYM	**1,29E+05**	**3,35E+04**	**b**	1,31E+05	7,17E+04		**9,35E+05**	**3,53E+05**	**b**	**7,02E+05**	**2,29E+05**	**b**
BIOORG	**6,34E+04**	**2,09E+04**	**a**	7,69E+05	5,23E+05		**1,78E+05**	**5,83E+04**	**a**	**1,34E+05**	**4,58E+04**	**a**
Rapeseed CONFYM	1,41E+05	6,58E+04		**1,89E+05**	**1,35E+05**	**a**	**1,47E+06**	**5,56E+05**	**b**	1,11E+06	3,32E+05	
Rapeseed BIOORG	1,07E+05	2,59E+04		**7,85E+04**	**3,06E+04**	**a**	**6,31E+04**	**3,28E+04**	**a**	1,65E+05	4,34E+04	
Soybean CONFYM	1,17E+05	9,37E+03		**7,40E+04**	**2,47E+04**	**a**	**3,96E+05**	**2,12E+05**	**b**	2,91E+05	1,23E+05	
Soybean BIOORG	2,02E+04	1,18E+04		**1,46E+06**	**9,24E+05**	**b**	**2,94E+05**	**7,67E+04**	**b**	1,03E+05	7,76E+04	
nosZ	5			7			10			15		
	Mean	.se	D	Mean	.se	D	Mean	.se	D	Mean	.se	D
Rapeseed	2,88E+04	7,84E+03		5,33E+04	2,87E+04		5,44E+05	2,72E+05		6,92E+05	4,45E+05	
Soybean	2,36E+04	6,16E+03		4,17E+05	3,44E+05		4,30E+05	2,98E+05		1,05E+06	5,76E+05	
CONFYM	1,59E+04	4,49E+03		5,80E+04	3,12E+04		8,25E+05	3,22E+05		8,73E+05	4,20E+05	
BIOORG	3,46E+04	6,84E+03		3,51E+05	2,94E+05		1,02E+05	5,49E+04		8,35E+05	6,15E+05	
Rapeseed CONFYM	1,92E+04	9,43E+03		7,49E+04	5,26E+04		9,24E+05	3,77E+05		1,03E+06	6,02E+05	
Rapeseed BIOORG	3,36E+04	9,94E+03		3,16E+04	1,70E+04		3,68E+04	2,57E+04		2,02E+04	4,89E+03	
Soybean CONFYM	1,42E+04	4,59E+03		3,54E+04	1,03E+04		6,93E+05	5,49E+05		5,65E+05	2,63E+05	
Soybean BIOORG	3,60E+04	8,86E+03		9,90E+05	6,84E+05		1,67E+05	9,26E+04		1,38E+06	8,96E+05	

ANNEX I

Mena temperature of the soil during the experiment.

PRILOGA I

Povprečna temperatura tal med potekom poskusa

Date	Days after fertilization	Temperature [ºC]
18.3.2015	-1	7,2
20.3.2015	1	7,3
22.3.2015	3	7,1
23.3.2015	4	7,2
24.3.2015	5	7,4
25.3.2015	6	8,1
26.3.2015	7	7,3
27.3.2015	8	8,5
29.3.2015	10	9,8
31.3.2015	12	10,8
3.4.2015	15	10,5
7.4.2015	19	11,0