UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY
ACADEMIC STUDY IN MICROBIOLOGY

Eva WEBER

AMMONIUM SOURCES FOR ARCHAEAL AND BACTERIAL AMMONIA OXIDISERS

M. SC. THESIS

Master Study Programmes: Field Microbiology

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VIRI AMONIAKA ZA AMONIAK-OKSIDIRAJOČE ARHEJE IN BAKTERIJE

MAGISTRSKO DELO

Magistrski študij - 2. stopnja Mikrobiologija

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Advisor: Prof. Dr. Ines Mandić Mulec

Co-advisor: Prof. Dr. Jim I. Prosser

Reviewer: Assist. Prof. Dr. Blaž Stres

Committee for evaluation and defence of Master Sc. thesis (komisija za oceno in zagovor):

Chairman (predsednica):	Prof. Dr. Romana Marinšek Logar University of Ljubljana, Biotechnical Faculty, Department of Animal Science
Member (član):	Prof. Dr. Ines Mandić Mulec University of Ljubljana, Biotechnical Faculty, Depatment of Food Science and Technology
Member (član):	Prof. Dr. Jim I. Prosser University of Aberdeen, School of Biological Sciences
Member (član):	Assist. Prof. Dr. Blaž Stres University of Ljubljana, Biotechnical Faculty, Department of Animal Science

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AB

Until recently bacteria were thought to be the only organisms able to oxidise ammonia to nitrate, but the recent discovery of ammonia-oxidising archaea changed this belief. Furthermore recent evidence indicated that ammonia oxidising archaea (AOA) in acidic forest soil prefer organic sources of ammonium over inorganic sources. The aim of this thesis was to determine the influence of organic and mineral sources of ammonium on nitrification kinetics of AOA and ammonia oxidising bacteria (AOB) in two peat soils: an acidic forest peat soil (Bog) and an agricultural soil with neutral pH (Bevke). Nitrification was monitored in soil microcosms amended only once with glutamate and ammonium sulphate. A second experiment was performed in neutral soil microcosms which were repeatedly amended with glutamate or ammonium sulphate. After qPCR, DGGE and colorimetric analyses it was concluded that in acidic soil, AOA are in control of the nitrification and prefer an organic source of ammonium, but different sources of ammonium did not affect the growth or structure of AOA, in contrast to previously published data (Levičnik-Höfferle *et al.*, 2012). In the neutral soil repeated amendment of organic source resulted in a faster nitrification rate along with a trend of increased AOB *amoA* abundance. Different sources of ammonium had no detectable effect on AOA or AOB communities in the neutral soil.

KLJUČNA DOKUMENTACIJSKA INFORMACIJA

ŠD	Du2
DK	UDK 579.266: 631.461: 582.233: 577.2.083 (043)=111
KG	arheje/bakterije/mikrobne združbe/amoniak-oksidirajoče bakterije/amoniak-oksidirajoče arheje/nitrifikacija/amoniak/mikrokozmi/mikrobiologija tal/kisla tla/pH nevtralna tla/qPCR/DGGE/pretočni spektrofotometer
AV	WEBER, Eva
SA	MANDIĆ MULEC, Ines (mentor)/PROSSER, Jim I. (somentor)/STRES, Blaž (recenzent)
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JI	en/sl
AI	

Do nedavnega je prevladovalo mnenje, da so le AOB zmožne nitrifikacije, a pred kratkim so dokazali, da tudi arheje lahko oksidirajo amoniak in tako vršijo prvo stopnjo nitrifikacije. Poleg tega so nedavno pokazali, da v kislih gozdnih (šotnih) tleh AOA preferenčno oksidirajo organski viri amonijskega dušika pred anorganskimi viri. Cilj te magistrske naloge je bil preučiti vpliv organskega (glutamat) in anorganskega (amonijev sulfat) vira amoniaka na nitrifikacijsko hitrost ter na številčnost in sestavo AOA in AOB v talnih mikrokozmih. Kot referenčna tla smo uporabili kisla gozdna (Bog), ter nevtralno karbonatno polžarico s kmetijske površine (tla Bevke). V poskusu smo uporabili talne mikrokozme, katerim smo dodali vir amonijskega dušika bodisi dodali enkrat na začetku poskusa ali v drugem eksperimentu večkrat med samim poskusom. Na osnovi kolorimetričnih testov, qPCR in DGGE analiz smo zaključili, da so v kislih tleh AOA tiste, ki kontrolirajo nitrifikacijo in tudi preferenčno izrabljajo organski vir amonijaka, vendar slednji ni vplival na rast ali strukturo AOA, kar je je bilo v nasprotju z objavljenimi rezultati (Levičnik-Höfferle in sod., 2012). V nevtralnih kmetijskih tleh je večkratno dodajanje organskega vira amonijaka spodbudilo hitrost nitrifikacije, opažen je bil tudi trend povečanja števila AOB *amoA* genov. Različni viri amonijaka niso vplivali na strukturo AOA ali AOB v nevtralnih tleh.

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ABBRIVATIONS AND SYMBOLS

16S	Component of a small subunit of prokaryotic ribosomes
AMO	Enzyme ammonia monooxygenase
amoCAB	Ammonia monooxygenase encoding genes
AOA	Ammonia-oxidising archaea
AOB	Ammonia-oxidising bacteria
BOG	Acidic forest peat soil
C:N	Ratio between carbon and nitrogen
DGGE	Denaturing gradient gel electrophoresis
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
N	Elemental nitrogen
N_2O	Chemical symbol for nitrous oxide
NH_3	Chemical symbol for ammonia
NH_4^+	Chemical symbol for ammonium
NH_4^+-N	In the text as a concentration of ammonium nitrogen
NO_2^-	Chemical symbol for nitrite
NO_3^-	Chemical symbol for nitrate
$(NO_2^-+NO_3^-)$-N	In the text as a total of nitrite and nitrate nitrogen
qPCR	Quantitative Real-time polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
WHC	Water holding capacity

1 INTRODUCTION

Ammonia oxidation is the first step of nitrification and an important process within the nitrogen cycle performed by microorganisms. Understanding ammonia oxidation is important as a cause of environmental pollution and in agriculture, but the first question that must be answered is who is in control of ammonia oxidation?

AOB were considered to be the only organisms involved in oxidation of ammonia to nitrite (Belser, 1979; Prosser, 1989) until the recent discovery that AOA could also oxidise ammonia (Könneke *et al.*, 2005; Treusch *et al.*, 2005) and are often more abundant in soil than AOB (Leininger *et al.*, 2006; Prosser in Nicol, 2008). Research also showed that in the environments with low ammonia concentration such as unfertilised soil (Leininger *et al.*, 2006) and soil with low pH (Stopnišek *et al.*, 2010*)*, where ammonia is released in soil from mineralisation of organic matter, AOA may have a selective advantage over AOB. In addition, Levičnik-Höfferle *et al.* (2012) showed that in soil with low pH, where AOA are more abundant, nitrification and ammonia oxidiser growth are stimulated by organic sources of ammonia, while inorganic ammonia has no effect.

Although archaeal ammonia oxidation in soil with low pH is quite well researched, little is known of the relative activities of AOA and AOB, and the influence of different sources of ammonia, in soil with neutral pH, where both of the communities are equally represented. This was investigated using two soils from Ljubljana marsh. The marsh is one of the biggest of its kind in Slovenia with an area of approximately 150 km^2. Ljubljana marsh has various sediments and a water table that is very close to the surface. It has been declared as a Regional park with legal protection. This and its rich diversity of soil conditions make it ideally suited to investigation of soil microbial community structure and activity (Strokovne podlage za ustanovitev Krajinskega parka Ljubljansko barje, 2007; Kraigher *et al.*, 2006).

1.1 HYPOTHESIS AND OBJECTIVES

The main objective of this Master's thesis was to determine the effect of different sources of ammonium on ammonia oxidiser communities in soil with acid and neutral pH values. The study was based on the following objectives:

- In soil with low pH, where only AOA are present, organic sources of ammonia are preferred and result in a higher nitrification rate, faster growth, greater transcriptional activity of AOA and changes in AOA community structure.
- In soil with neutral pH, where both AOA and AOB are present:
    - Amendment with inorganic ammonium will increase AOB growth and activity and change AOB community structure, but will not affect the AOA community.
    - Amendment with mineralisable organic nitrogen will stimulate AOA growth and activity, and change AOA community structure, but will not influence AOB.

2 LITERATURE REVIEW

2.1 NITROGEN CYCLE AND NITRIFICATION

Nitrogen is an important element for all living organisms, since it is a component of nucleic acids and proteins. Around 78% of all available nitrogen is in the atmosphere and therefore unavailable for biological activity. Other large pools of nitrogen can be found in soil in various forms as it is transformed in the nitrogen cycle, mainly through microbial activity. The cycle itself has five major reactions in which nitrogen compounds are oxidised or reduced. They are shown below in figure 1.

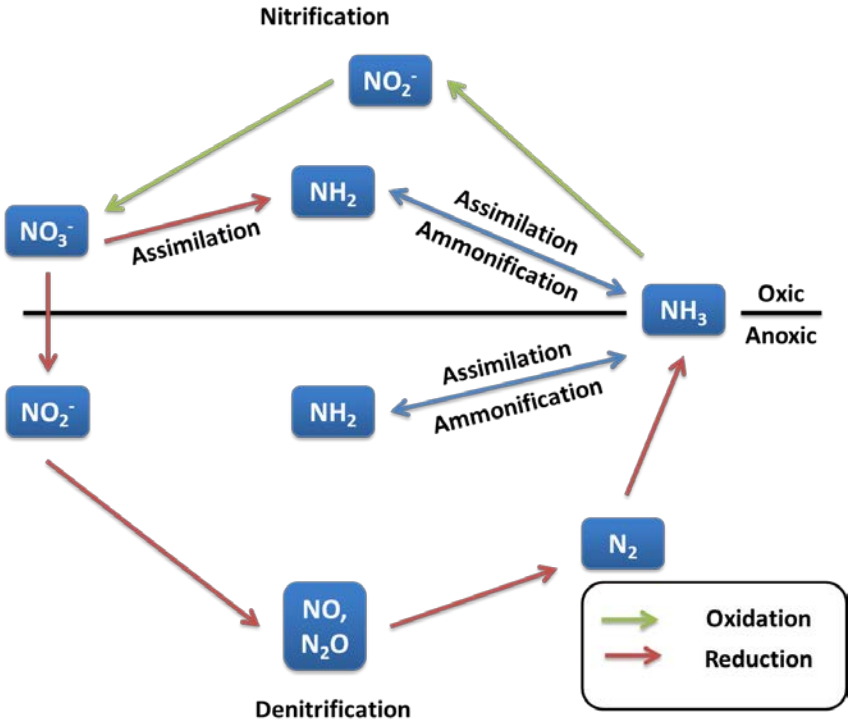


Figure 1: Major processes in the soil nitrogen cycle (Madigan and Martinko, 2006)

Slika 1: Shema kroženja dušika v tleh s predstavljenimi glavnimi reakcijami (Madigan in Martinko, 2006)

Nitrification consists of two processes. In the first process, ammonia oxidised to nitrite. For AOB, this is achieved through conversion of ammonia to hydroxylamine, by the enzyme ammonia monooxygenase (AMO), and subsequent conversion to nitrite, by hydroxylamine oxidoreductase:

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \qquad \ldots(1)$$

$NH_2OH + H_2O \rightarrow NO_2 + 5H^+ + 4e^-$ …(2)

In the second reaction, nitrite is oxidised further to nitrate (NO_3^-) by the enzyme nitrite oxidoreductase:

$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^-$ …(3)

AOA also possess ammonia monooxygenase but genome analysis provides no evidence for genes homologous to hydroxylamine oxidoreductase (Walker *et al.*, 2010). This suggests a different pathway for ammonia oxidation by AOA that does not involve hydroxylamine as an intermediate. Details of this metabolic pathway in AOA are currently not characterised.

In natural environments, ammonia oxidisers can be detected by amplification of 16S rRNA genes or genes encoding AMO (Rotthauwe *et al.*, 1997). AMO is encoded by three genes, *amoCAB*. *amoA* and *amoB* genes encode subunits A and B, while *amoC* gene encodes a third enzyme subunit that is more stable than the other two (Arp *et al.*, 2002). Rotthauwe *et al.* (1997) suggested targeting the *amoA* gene for detection of ammonia oxidisers. This became a leading molecular tool for detecting AOB and AOA for its specificity, good resolution and for detecting functional property of microorganism instead of phylogenetic one.

The substrate for nitrification is ammonia (NH_3), rather than ammonium (NH_4^+). NH_3 is uncharged and is therefore not bound to negatively charged soil particles. Although ammonia can be leached from soil, or lost through volatilisation, ammonia and ammonium (NH_4^+) are in equilibrium. Ammonium is positively charged, can bind to soil particles and is available to both plants and microorganisms as a source of nitrogen (Suzuki *et al.*, 1974). The product of nitrification, nitrate, is negatively charged and not adsorbed by soil particles (Figure 1). Although it can also provide a source of nitrogen to plants, it is lost from soil through leaching and can also act as a substrate for denitrification, through which it is converted into nitrous oxide (N_2O), a greenhouse gas with a negative effect on the environment, or nitrogen gas. Research on nitrification is therefore crucial for agriculture, as it is important to manage fertiliser loss, and for the environmental reasons, as nitrous oxide is an atmospheric pollutant, and nitrate pollutes groundwater. If soil is fertilised for a long period of time, the C:N ratio in soil can change with increased inorganic N, therefore it is important to determine how much of N fertilisers can be applied for an

environmentally safe agriculture (Madigan and Martinko, 2006; Kowalchuk and Stephen, 2001; Raun *et al.*, 1998).

2.1.1 Nitrification and pollution

Humans have influenced the natural nitrogen cycle by adding significant amounts of reactive-N to various ecosystems, through fertiliser addition and atmospheric nitrogen deposition. Soil nitrifiers convert fertiliser ammonium to nitrate and, even though plants can assimilate nitrate as well as ammonium, much of the nitrate leaches into groundwater or is converted to nitrogen or greenhouse gases, NO and N_2O. A solution to this problem is in controlled nitrification. Unsupervised addition of fertilisers generates a rapid nitrifying soil (Subbarao *et al.*, 2012). As a result a large amount of effort has been made to regulate nitrification in soil in order to prevent fertiliser loss. This includes development of a range of nitrification inhibitors. Acetylene was the first nitrification inhibitor to be found and was followed by others such as mechanism-based inhibitors, S compounds and heterocyclic compounds used in soil and are industrially made (McCarty, 1999).

Another more recent solution to controlling nitrification is use of biological inhibitors of nitrification. These are compounds released by plants that inhibit nitrifying microorganisms. For example, Subbarao *et al.* (2006) discovered a nitrification inhibitor that is produced by roots of *Brachiaria humidicola* plant. They used a bioluminescence assay to evaluate whether different plants can produce biological nitrification inhibitors but as this utilised only one test organism, *N. europaea*, it is still unconfirmed whether biological nitrification inhibitors are suitable for other nitrifying microorganisms.

Nitrification can also be inhibited by atmospheric pollutants, e.g. heavy metals and hydrocarbons. These pollutants have a negative effect on nitrification, reducing nitrification rates in polluted soils (Christensen *et al.*, 2001; Nevell and Wainwright, 1987). A possible application of this finding is that nitrification could be used as a pollution indicator (Christensen *et al.*, 2001).

2.2 AMMONIA-OXIDISING BACTERIA

AOB are obligate aerobic microorganisms even though some like *Nitrosomonas europaea* can tolerate anaerobic conditions and can even denitrify (Geets *et al.*, 2006). Their

substrate is acknowledged to be ammonia and not ammonium, whose retention in soil is greater in most soils, because of adsorption of ammonium ions to soil particles (Kowalchuk and Stephen, 2001). Some AOB species can grow in the environment with urea as a source of ammonia, even though it was thought that AOB are unable to grow with organic source of nitrogen. That is achieved by hydrolysis of urea to ammonia, which is then oxidised (Jiang *et al.*, 1999).

Initially all nitrifying bacteria were classified in a single group, belonging to the family *Nitrobacteraceae* (Watson, 1989), but classification based on 16S rRNA analysis led to separation of ammonia-oxidising bacteria and nitrite-oxidising bacteria. Autotrophic AOB were placed in three subclasses within the betaproteobacteria and gammaproteobacteria (Kowalchuk and Stephen, 2001).

Woese (1987) already divided AOB in two groups of beta-purple bacteria and gamma-purple bacteria following 16S rRNA gene sequence analysis. Stephen *et al.* (1996) further determined that *Nitrosococcus halophilus* and *Nitrosococcus oceanis* belong to the gammaproteobacteria, while *Nitrosomonas* spp. and *Nitrosospira* spp. belong to a group of betaproteobacteria. They also suggested that *N. europaea* and *Nitrosococcus mobilis* should be combined in a single phylogenetic group, based on similarities in 16S rRNA gene sequences. Altogether they divided beta-subclass AOB into 7 clusters. Purkhold *et al.* (2000) confirmed these findings with phylogenetic analysis of 16S rRNA sequence and suggested additional clusters. The difference in classification can be seen in figure 2.

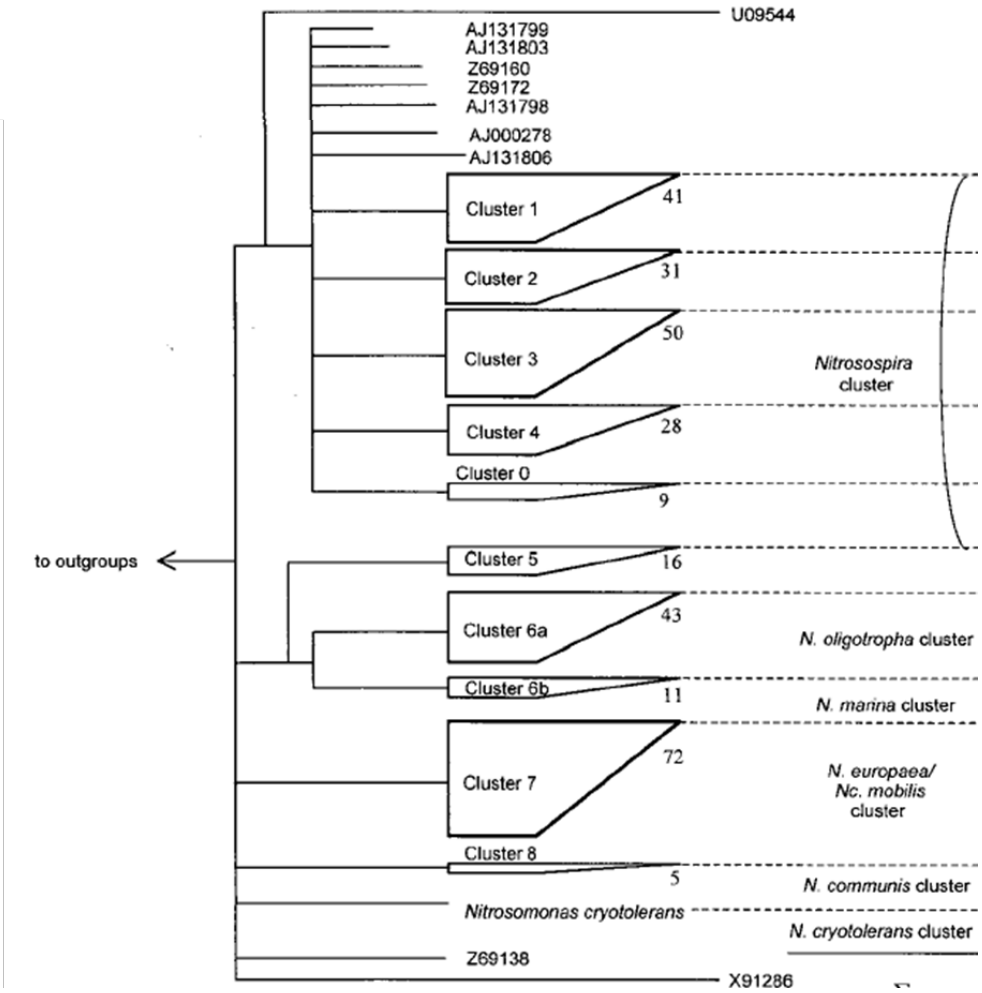


Figure 2: Classification of AOB and grouping in clusters as Purkhold *et al.* (2000) suggested. The cluster designations were adopted from Stephen *et al*. (1996) and two additional clusters were suggested

Slika 2: Klasifikacija AOB in združenje v skupke, kot predlaga Purkhold in sod. (2000). Oblikovanje v skupke je bilo povzeto po Stephen in sod. (1996) s predlaganimi dvema dodatnima skupkoma

2.3 AMMONIA-OXIDISING ARCHAEA

Even before archaea were recognised as a separate domain, it was known that their properties were not consistent with the universal description of the prokaryota domain (Table 1). Finally after Woese's proposal (Woese *et al.*, 1990) of the third domain of life, the world of science changed. Archaeobacteria were not part of bacterial domain, but were classified separately, as Archaea. There was still a belief, however, that archaea were extremophiles, since they were only found in extreme environment, such as hot springs and volcanoes (Woese *et al.*, 1990).

Table 1: Summary of major properties and differences between Bacteria, Archaea and Eukarya (Madigan and Martinko, 2006)

Preglednica 1: Glavne razlike med tremi domenami: Bacteria, Archaea in Eukarya. (Madigan in Martinko, 2006)

Characteristic	Bacteria	Archaea	Eukarya
Prokaryotic cell structure	Yes	Yes	No
DNA in covalently closed and circular form	Yes	Yes	No
Histone proteins	No	Yes	Yes
Cell wall (present muramic acid)	Yes	No	No
Membrane lipids	Ester-linked	Ether-linked	Ester-linked
Ribosome (mass)	70S	70S	80S
Introns in most genes	No	No	Yes
Transcriptional factors required	No	Yes	Yes
Promoter structure	-10 and -35 sequence	TATA box	TATA box
Initiator tRNA	Formylmethionine	Methionine	Methionine

In 1992 two research groups (DeLong, 1992; Fuhrman, 1992) showed that archaea also exist in cold seawater. Fuhrman (1992) discovered archaea in marine plankton 100 m and 500 m below the surface. They reported new sequences that were only distantly related to

those of extremophiles and suggested that the new 16S rRNA gene sequences were archaeal, even though they had never before been detected in the sea. DeLong (1992) also discovered mesophilic archaea in oxic surface coastal seawater and therefore confirmed the existence of a new archaeal phylum, the Crenarchaeota.

Following further discoveries of mesophilic Crenarchaeota, Brochier-Armanet *et al.* (2008) compared hyperthermophilic Crenarchaeota and newly discovered mesophilic archaea. Since there are differences between the two it was not appropriate to classify them under the same phylum and they proposed a third phylum called Thaumarchaeota (Figure 3).

Ochsenreiter *et al.* (2003) analysed DNA extracted from soil samples with specific primers for non-thermophilic Crenarchaeota. 16S rRNA genes were targeted and it was discovered their presence in different types of soil, where they formed a stable and abundant community. Later Kemnitz *et al.* (2007) confirmed the presence of archaea in a range of mesophilic environments. Further studies of the archaea:bacteria ratio in soil samples demonstrated a higher percentage of bacterial 16S rRNA genes, but a significant and stable proportion of archaeal sequences. Within the crenarchaeota, the most abundant fell within the 1.1c group in the studied soil, but the numbers decreased with depth.

In a soil metagenome study, Treusch *et al.* (2005) identified a fosmid, 54d9, from a library prepared from sandy soil ecosystem. With 16S rRNA analysis it was determined that the fragment was affiliated with 1.1b group of Crenarchaeota and also contained *amoAB* genes but not amoC; *amoA*-like genes were found at even higher abundance in the soil after amendment with ammonia. Similar genes were also found in an environmental library from the sea plankton. In conclusion this study suggests that mesophilic archaea are present in terrestrial and marine environments and are capable of oxidising ammonia.

Könneke *et al.* (2005) then isolated an aerobic autotrophic ammonia-oxidising archaea again in marine samples. This discovery shook the scientific world as it was thought that only bacteria were capable of oxidising ammonia. After comparing archaeal and bacterial *amoA* genes it was discovered that they differed in structure, but were sufficiently similar to conclude that AOA have an important role in nitrogen cycle (Nicol and Schleper, 2006).

Since then AOA have been discovered in many different environments, but it seems that they are mainly found in soil and marine samples. In soil samples Leininger *et al.* (2006)

determined AOA and AOB *amoA* gene abundance and concentration of archaea specific lipids in soil. The results were fascinating as they showed that AOA are more abundant than AOB in soil and the ratio of AOA to AOB increased with depth and in non-fertilised soil (Figure 4).

The statement that AOA are more abundant than AOB in some soils triggered the question: Who contributes more to the nitrification process; AOA or AOB?

Figure 3: Archaeal tree of life from Woese's proposition to Brochier-Armanet's recent classification (Brochier-Armanet *et al.*, 2008)

Slika 3: Filogenetsko drevo arhej od prvotne oblike, ki jo je predlagal Woese, do končnega Brochier-Armanetovega predloga (Brochier-Armanet in sod., 2008)

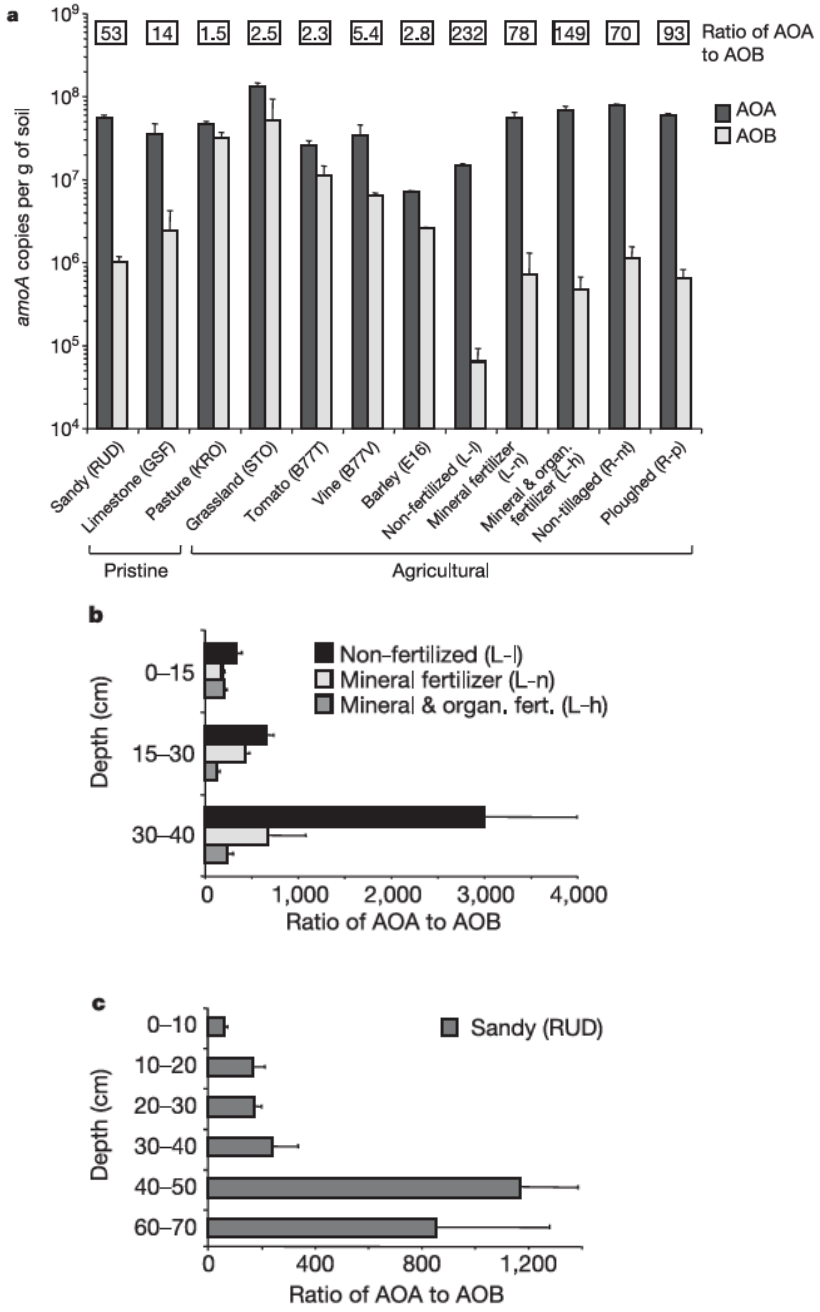


Figure 4: Abundance of AOA and AOB amoA genes in all sampled soils (a). Ratio of AOA to AOB with increasing depth (b,c) (Leininger *et al.*, 2006)

Slika 4: Številčnost AOA in AOB *amoA* genov v vseh vzorčenih tleh (a). Razmerje AOA proti AOB z globino tal (b,c) (Leininger in sod., 2006)

2.4 WHO IS PERFORMING NITRIFICATION?

Who is performing the nitrification, and under what conditions, are the leading questions to many researchers active in the field of nitrification. After several studies measuring AOA or AOB *amoA* abundance in different types of soil it became clear that several factors might determine their relative numbers (Prosser and Nicol, 2012).

Jia and Conrad (2009) amended agricultural soil microcosms with ammonium and prevented acidification due to nitrification by liming, ensuring that soil remained at neutral pH. AOA and AOB *amoA* gene abundance was measured and community structure was determined by denaturing gradient gel electrophoresis (DGGE). Even though AOA outnumbered AOB, the latter were more active and performed most of the nitrification following addition of ammonium. This was demonstrated by inhibiting nitrification by adding acetylene to microcosms and observing nitrification kinetics. Acetylene is an inhibitor of nitrification and therefore inhibits growth and transcriptional activity of the community that controls nitrification. In this study acetylene affected only AOB and not AOA. The conclusion was that AOA were not significantly important in ammonia oxidation in the examined agricultural soil.

In contrast, Gubry-Rangin *et al.* (2010) demonstrated the importance of AOA in an acidic agricultural soil. Again relative abundance of *amoA* gene was determined for both AOA and AOB and community structure was observed using DGGE. The soil used was a Scottish acidic agricultural soil with pH 4.5 and 6. To confirm which community controls nitrification, microcosms were amended with acetylene as described before. Observing only nitrification kinetics brought no conclusion, as the rates were high for both soils in the absence of acetylene and low in its presence. However, analysis of population growth showed that AOA grew faster than AOB. In the presence of acetylene, growth of AOA decreased significantly, as well as transcriptional activity, but acetylene had no significant influence on the AOB community. This study therefore provided strong evidence that AOA contributed significantly to nitrification in this acidic soil.

These studies suggested that pH may influence the relative contributions of AOA and AOB to nitrification. Nicol *et al.* (2008) discovered that community structure of both AOA and AOB differed in acidic and neutral soil plots (Figure 5). They also determined *amoA* gene

abundance for both communities and while AOA had greater growth and activity than AOB, an influence of pH was observed. With increasing pH from 4.9 to 7.5, AOA growth and activity decreased, while an increase was observed for AOB (Figure 6). They also mixed sampled soil with adjusted pH and it was concluded that the community controlling nitrification in its native soil was the most successful at the pH that it was adapted to. These results suggested distinct ecological niches for AOA and AOB. Tourna *et al.* (2008) showed that temperature is another factor influencing AOA communities. It had an effect on nitrification kinetics and community structure. Interestingly only AOA community changed with increasing temperature from 10 °C to 30 °C, and this change does not influence AOB community.

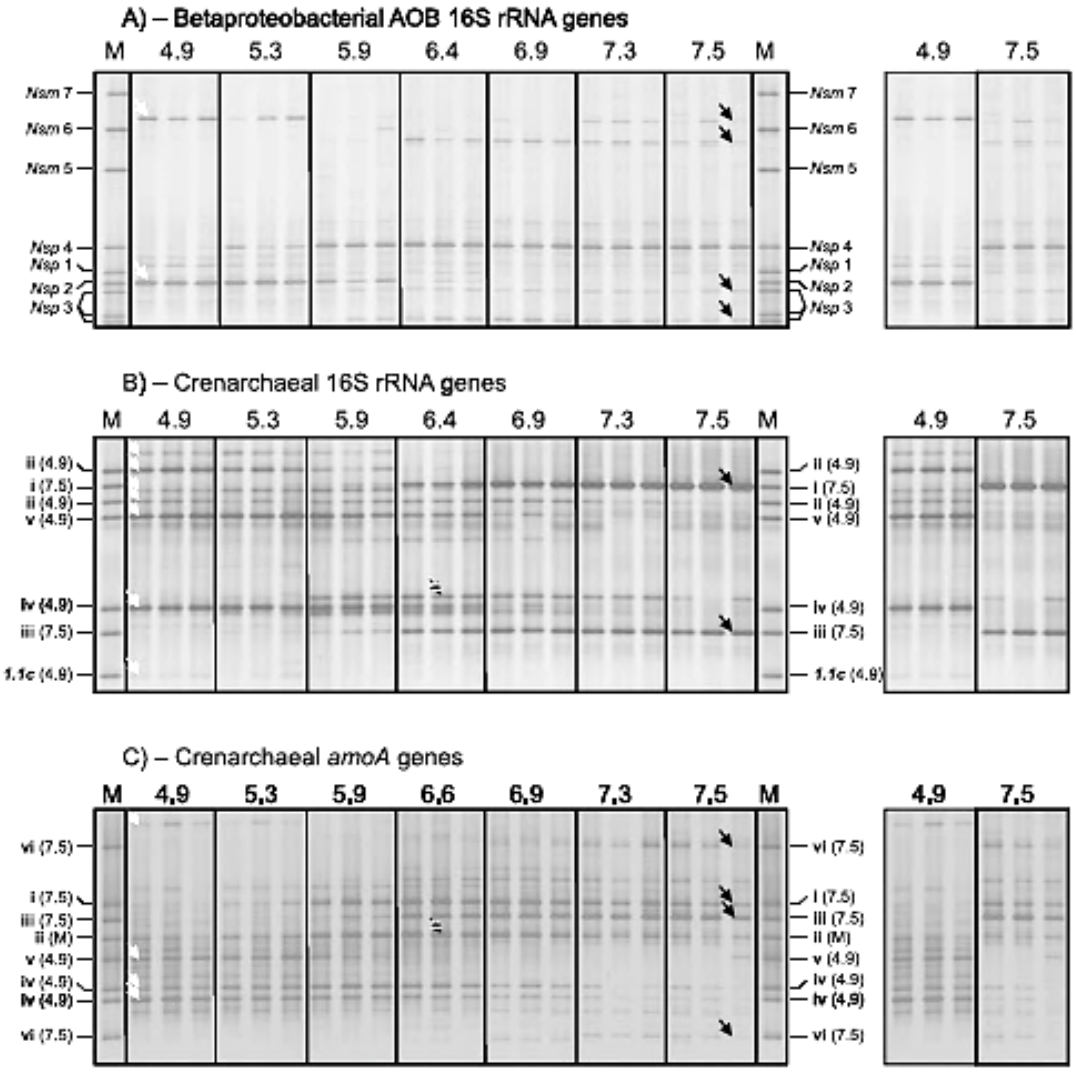


Figure 5: Structural changes in AOA and AOB community as shown by Nicol *et al.* (2008). Statistically significant changes in community can be seen from the gels with increasing pH

Slika 5: Strukturne spremembe združb AOA in AOB, kot so prikazali Nicol in sod. (2008). Statistično značilne spremembe so opazne z višanjem pH vrednosti

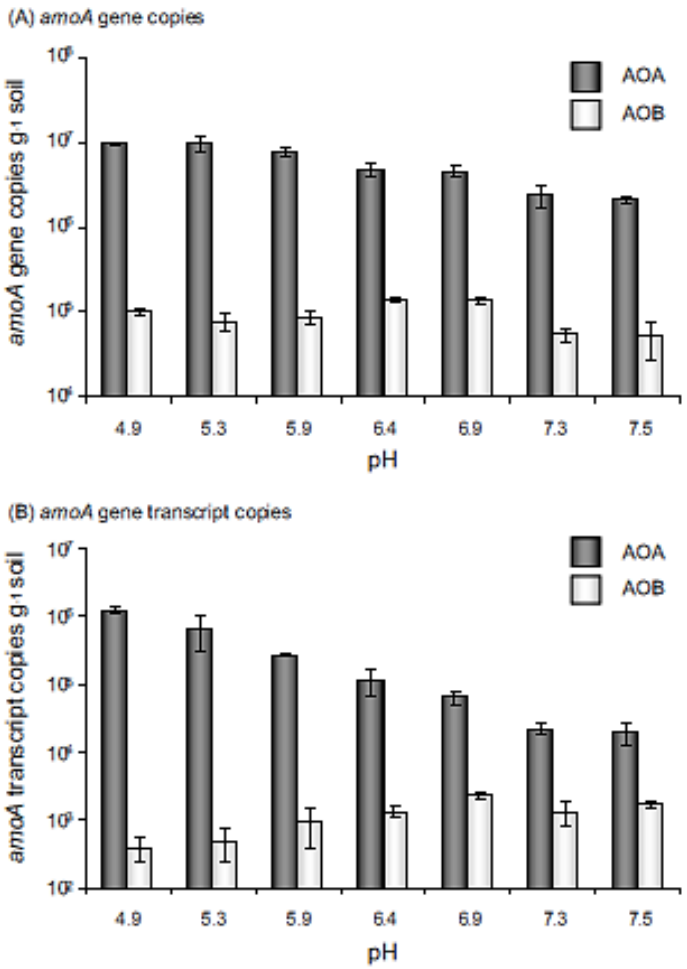


Figure 6: Overall abundance of AOA and AOB *amoA* genes (A) and transcripts (B). Decreasing abundance in the AOA community can be observed with increased pH and increasing abundance in the AOB community (Nicol *et al.*, 2008)

Slika 6: Številčnost AOA in AOB *amoA* genov (A), ter njihovih transkriptov (B) s spreminjanjem pH. Z zviševanjem pH vrednosti se zmanjša številčnosti amoA gena in transkripta gena pri AOA združbi. AOB združba se z zviševanjem pH obnaša ravno obratno (Nicol in sod., 2008)

There is another important factor that can determine whether AOA or AOB will control nitrification. Because of their different physiologies they can adjust to different circumstances. Stopnišek *et al.* (2010) studied acidic forest peat soil with pH 4.1 that has low ammonia concentration, but high potential for acquiring substrate produced through mineralisation. AOB *amoA* genes could not be detected, suggesting a selective advantage of AOA over AOB in this soil. AOA *amoA* genes increased in abundance during nitrification, but amendment of microcosms with ammonia had no influence on growth, nitrification rate or AOA community structure. This suggests that AOA in acidic soil get

their source of nitrogen through mineralisation and do not respond to added inorganic source of nitrogen.

A subsequent study by Levičnik-Höfferle *et al.* (2012) tested the hypothesis that AOA prefer organic source of nitrogen using the soil investigated by Stopnišek *et al.* (2010). Levičnik-Höfferle *et al.* (2012) tested the influence of organic and inorganic source of nitrogen in this soil. Nitrification rate was stimulated by addition of an organic source of nitrogen, but did not differ from the control (microcosms amended with water) when amended with inorganic nitrogen. Increased *amoA* gene abundance was observed in microcosms amended with organic nitrogen, but did not change with the amendment of inorganic source of nitrogen. This research showed the importance of source of nitrogen for ammonia oxidisers.

In conclusion, these studies indicate that no single factor determines whether AOA or AOB control nitrification in soil. Every study was dealing with different physiology and large diversity. This makes it difficult to predict conditions under which the different communities will predominate, but one factor is clear. All the studies showed adaptation of AOA to pH lower than 5.5, but not AOB. This makes it clear that only AOA can oxidise ammonia under this conditions (Prosser and Nicol, 2012).

3 MATERIALS AND METHODS

3.1 MATERIALS

Table 2: Molecular materials and suppliers

Preglednica 2: Materiali za molekularne metode in proizvajalci

Material	Supplier
General laboratory materials	BDH-Merc, Leicestershire, UK Fisher Scientific, Leicestershire, UK Oxoid, Hampshire, UK Sigma-Aldrich, Dorset, UK
Agarose (molecular grade) BIOPRO DNA polymerase Deoxyribonucleotides (dATP, dCTP, dGTP and dTTP) Hyperladder I DNA size and mass ladder	Bioline Ltd., London, UK
NucleoSpin Extract II (DNA purification kit)	Macherey-Nagel, Düren, Germany
Quiagen® RNA/DNA Purification kit Quantitect SYBR Green PCR kit Quantifast SYBR Green PCR kit	Quiagen, West Sussex, UK
Oligonucleotides	Thermo Electron Coporation, Ulm, Germany
MO-BIO Powersoil DNA Isolation kit	Carlsbad, CA USA
GelBond PAG film for polyacrylamide gels	Amersham Bioscineces AB, Uppsala, Sweden

Table 3: Electrophoresis and DGGE stock solutions and buffers

Preglednica 3: Založne raztopine in pufri za elektroforezo in DGGE

Solution or buffer	Ingredients for the solution or buffer
TAE 50×	242 g Tris; 57.1 ml Glacial acetic acid;100 ml 0.5 M EDTA (pH 8.0); dH_2O to 1 l
6x loading buffer	0.25% Bromophenol blue; 0.25% Xylene cyanol; 30% Glycerol
Acrylamide solution 0% denaturant	2 ml 50×TAE; 20 ml 40% 37:1 acrylamide-bisacrylamide; dH_2O to 100 ml
Acrylamide solution 80% denaturant	2 ml 50×TAE; 20 ml 40% 37:1 acrylamide-bisacrylamide; 33.6 g Urea; 32 ml Formamide; dH_2O to 100 ml
Fixing solution (for DGGE)	100 ml Ethanol; 5 ml Glacial acetic acid; 859 ml dH_2O
Staining solution (for DGGE)	0.3 g $AgNO_3$; 300 ml dH_2O
Developing solution (for DGGE)	6 g NaOH; 3 ml 40% Formaldehyde; 200 ml dH_2O

3.2 METHODS

3.2.1 Soil sampling

Two different soils were used for this experiment. The first soil (termed Bog soil or acidic soil) is a forest peat soil with low pH (4.3), high organic content and high C:N ratio (Stopnišek *et al*., 2010). The second soil (Bevke or neutral soil) is carbonated snail soil that is a residue of sediment that has been exposed to higher layers due to digging of the drainage canals. Addition of the alkaline snail soil over the peat soil resulted in mixing of both soil, an increase in soil pH (7.5) and a decrease in organic content (Štrubelj, 2010). A different approach was used in sampling because of these structural differences between two soils

3.2.1.1 Acidic soil (Bog)

Soil with low pH was collected in Kozlar's deciduous forest; coordinates for sampling site are 45°59'34.68"N and 14°30'8.60"E (refer to figure 7). Soil was collected from 3 sampling sites, each separated by 5 m. On each sampling site 5 random samples were collected from the upper 20 cm of the soil layer using a corer. The soil was then sieved through an 8 mm sieve instead through 4 mm sieve due to high organic content and consequently heavy clumping. Approximately 4 kg of soil was collected.

a) b)

Figure 7: Sampling site for the acidic soil marked with an arrow (a) (Google Earth, 2012) and the photograph of acidic soil (b).

Slika 7: Mesto vzorčenja za kisla tla označeno s puščico v Kozlarjevem gozdu (a) (Google Earth, 2012) in slika kislih tal (b).

3.2.1.2 Neutral soil

Agricultural soil with neutral pH was collected in Bevke, from an unvegetated field in December 2011, and from the same field, but with vegetation, in May 2012. The coordinates for the sampling site are 46° 0'5.04"N and 14°22'1.50"E (refer to figure 8). Each time the soil was collected on 3 randomly chosen sampling sites. The upper 10 cm of soil was collected, as described above, since this type of soil accumulated at the upper layers of the field. 4 kg was collected and sieved through 4 mm sieve.

a) b)

Figure 8: Sampling site for neutral soil marked with an arrow (a) (Google Earth, 2012) and the photograph of neutral soil (b).
Slika 8: Mesto vzorčenja za nevtralna tla označeno s puščico, pri vasi Bevke (a) (Google Earth, 2012) in slika nevtralnih tal (b).

3.2.2 Determining the moisture content

Moisture content was determined to enable calculation of soil characteristics per g of dry soil. It was determined for experiment with single amendment by drying at 60 °C for three days. Moisture content was calculated as:

$$\text{Moisture content} = \text{mg}(H_2O)/\text{mg}(\text{dry soil}) \quad \ldots(5)$$

% Moisture content for neutral soil was 58.8% and for acidic soil it was 53.2%.

For experiment with repeated amendment, percentage of moisture content was measured using a Moisture Analyser Balance–Ohaus MB45 (Thomson Science) and was 35% moisture content.

3.2.3 Soil microcosms

3.2.3.1 Solutions used for the amendment of microcosms

a) Experiment with single amendment using acidic and neutral soil. Glutamate, ammonium sulphate or water were added to the microcosms at day 0
    a. Ammonium sulphate and glutamate (150 µg N g^{-1} dry soil) solutions for neutral soil (1 ml was added to each microcosm)
    b. Ammonium sulphate and glutamate (150 µg N g^{-1} dry soil) solutions for acidic soil (3 ml was added to each microcosm)
b) Experiment with repeated amendment using neutral soil, that was amended with glutamate, ammonium sulphate or water at day 0, 3, 6, 11, 16, 18 and 21
    a. Ammonium sulphate and glutamate (100 µg N g^{-1} dry soil) solutions for continuous amendment (1 ml was added to each microcosm)[1]

Ammonium sulphate and glutamate were chosen as previously described by other publications (Stopnišek *et al.*, 2010; Levičnik-Höfferle *et al.*, 2012).

[1] 1 ml of solutions was added only on day 0. For continuous amendment loss of moisture was determined and the solutions were prepared accordingly. Concentration of solutions stayed the same (100 µg N g^{-1} dry soil).

3.2.3.2 Experiment with single amendment

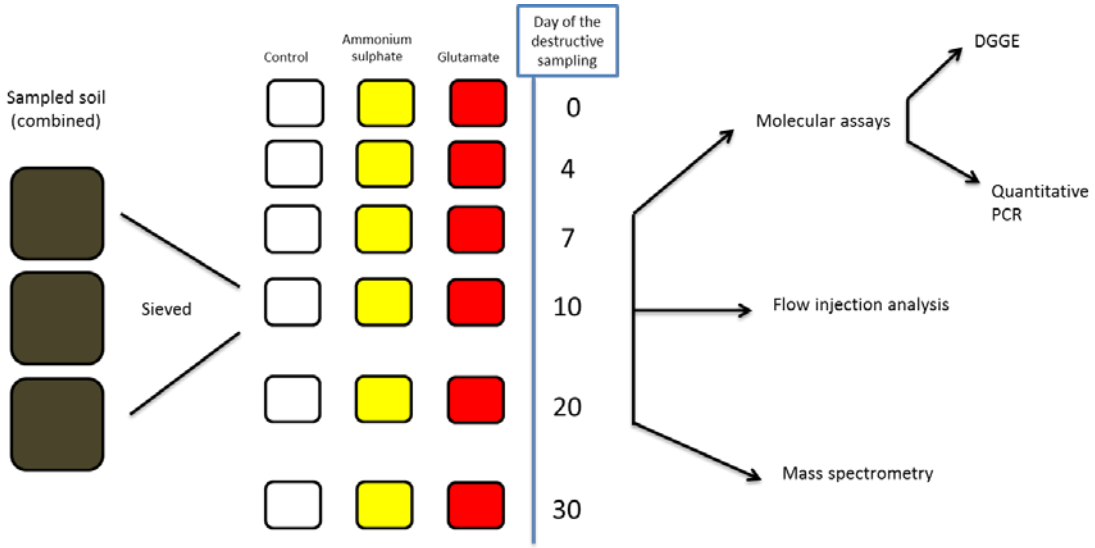


Figure 9: Scheme of the outline of the microcosm experiment with single amendment. On day 0; soil microcosms with 150 µg N g^{-1} dry soil of ammonium sulphate or glutamate. In control microcosms, water was added instead. White squares represent control, yellow squares represent ammonium sulphate microcosms and red squares represent glutamate microcosms. Large brown squares represent sampled soil. Each square represents three repetitions. Microcosms were sampled destructively on days 0, 4, 7, 10, 20 and 30. The same outline was used for acidic and neutral soils.

Slika 9: Shematski prikaz mikrokozemskega eksperimenta z enkratnim dodajanjem dodatkov. Rjavi kvadrati predstavljajo tla, ki smo jih vzorčili. Beli kvadrati predstavljajo kotrolo, kjer smo dodali deionizirano vodo; z rumeno barvo so označeni mikrokozmi, kjer smo dodali 150 µg N g^{-1} suhih tal amonijevega sulfata; rdeča barva pa označuje mikrokozme kjer smo dodali 150 µg N g^{-1} suhih tal glutamata. Dodatke smo v mikrokozme dodali ob dnevu 0. Vsak kvadrat predstavlja tri ponovitve. Uporabili smo destruktivno vzorčenje ob dnevih 0, 4, 7, 10, 20 in 30. Enaka struktura poskusa velja tako za kisla tla, kot tudi za nevtralna tla.

For the first experiment, the microcosms consisted of glass bottles, with rubber caps, containing 30 g of soil and were amended either with ammonium sulphate or glutamate, to the final concentration of 150 µg N g^{-1} dry soil. In control microcosms, water was added instead. Microcosms were weighted throughout the experiment to control the water evaporation. The appropriate amount of distilled water was replaced then in all microcosms. Microcosms were destructively sampled after incubation at 28 °C for 0 (immediately after amendment), 4, 7, 10, 20 and 30 days. On each sampling day 10 g of soil was used for KCl extraction, 5 g was used for pH measurement and remaining soil was stored in plastic bags at -80 °C.

3.2.3.3 Experiment with repeated amendment

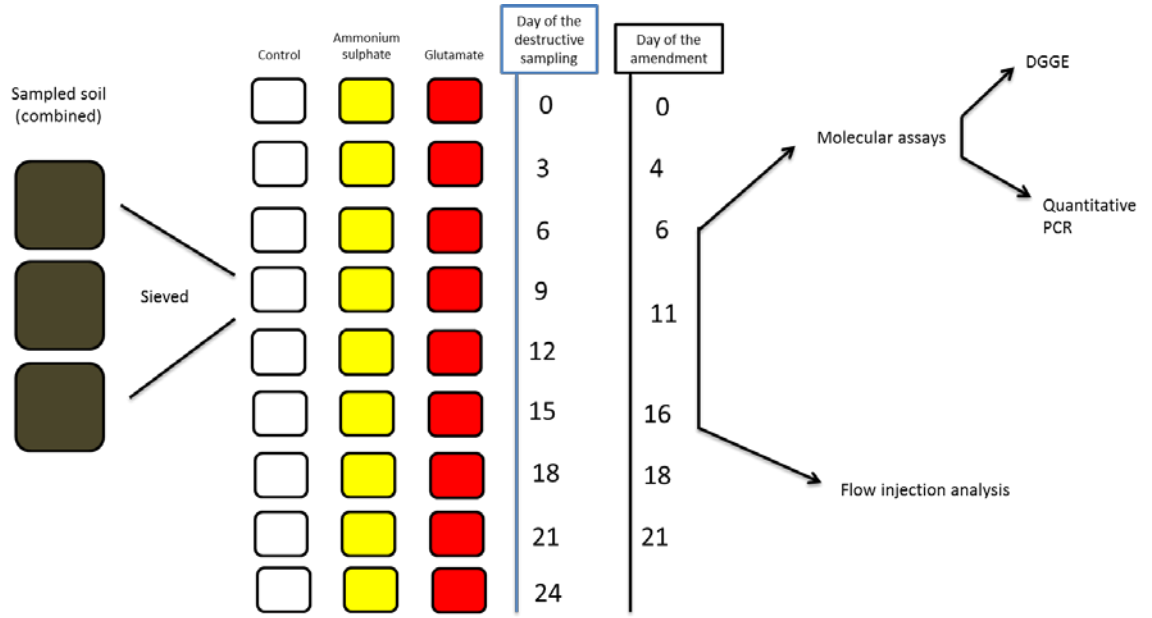


Figure 10: Scheme of the outline of the microcosm experiment with repeated amendment with 100 µg N g^{-1} dry soil, to ammonium sulphate and glutamate microcosms on days 0, 4, 6, 11, 16, 18 and 21. In control microcosms, water was added instead. White squares represent control, yellow squares represent ammonium sulphate microcosms and red squares represent glutamate microcosms. Large brown squares represent sampled soil. Each square represents three repetitions. Microcosms were destructively sampled on days 0, 3, 6, 9, 12, 15, 18, 21 and 24. Only neutral soil was used in this experiment.

Slika 10: Shematski prikaz mikrokozemskega poskusa s ponavljajočim dodajanjem amonijevih spojin. Rjavi kvadrati predstavljajo vzorčena tla. Beli kvadrati predstavljajo kontrolo, kjer smo dodali deionizirano vodo; z rumeno bravo so označeni mikrokozmi, kjer smo dodali amonijev sulfat; rdeča barva pa označuje mikrokozme kjer smo dodali glutamat. Dodatke smo mikrokozmom dodajali ob dnevih 0, 4, 6, 11, 16, 18 in 21. Dodali smo 100 µg N g^{-1} suhih tal. Vsak kvadratek predstavlja tri ponovitve. Ob dnevih 0, 3, 6, 9, 12, 15, 18, 21 in 24 smo uporabili destruktivno vzorčenje. Pri tem poskusu smo uporabili le nevtralna tla.

For the second experiment, microcosms consisted of glass bottles, with loose fitting caps, containing 15 g of neutral soil and were amended with ammonium sulphate or glutamate to the final concentration of 100 µg N g^{-1} dry soil. Microcosms were amended with an additional 100 µg µg N g^{-1} dry soil on days 0, 4, 6, 11, 16, 18 and 21. Because of evaporation, after each destructive sampling all of the remaining microcosms were weighted and adequate amount of distilled water (with source of ammonia for ammonium sulphate and glutamate microcosms) was replaced. Microcosms were destructively sampled after incubation at 28 °C for 0, 3, 6, 9,12,15,18, 21, and 24 days; sampled soil was

placed into plastic bags and stored at -20 °C. KCl extraction and pH measurements were performed after all microcosms were sampled.

3.2.4 pH measurement

pH was measured after each destructive sampling. In the falcon tubes 10 ml of distilled H_2O was added to 5 g of the collected soil. Falcon tubes were incubated at the room temperature for 30 min. pH was then measured using InoLAB pH/Cond 720 (WTW GmbH)

3.2.5 Nitrate and ammonium measurements

Nitrate and ammonium concentrations in KCl soil extracts were determined colorimetrically using flow injection analysis (FIA star 5000 Analyzer, Foss Tecator). To 10 g of soil, 50 ml of 2M KCl (Fisher, analytical gradient) was added and placed on a rotator (Rotator drive STR4, Stuart) for 1 hour. After 15 min centrifugation at 3000 × g, supernatant was collected and analysed. Standards in range from 0.1 µM to 5 µM were used and samples for nitrification measurements were diluted in ratio 1:11. Nitrification rate was calculated separately for each experiment by linear regression of increases and decreases in nitrate + nitrite and ammonium concentration, respectively.

3.2.6 Molecular assays

3.2.6.1 DNA extraction

DNA was extracted from 0.25 g of soil, using MO-BIO PowerSoil DNA isolation kit. The vortexing step was modified by fixing samples to the Vortex genie 2 (Scientific Industries) and samples were vortexed for 15 min on speed 8. DNA was eluted with 100 µl of elution buffer and later aliquoted into 4 eppendorf tubes. The concentration of DNA was then measured spectrophotometrically with NanoDrop (Thermo Scientific). Tubes were stored at -20 °C and -80 °C.

3.2.6.2 Quantitative real-time PCR (qPCR)

Standards for qPCR were prepared from DNA extracted from pure cultures; *Nitrosospira multiformis* for AOB and *Nitrosotalea devanaterra* for AOA. For AOB assays, the targeted

fragment was amplified using PCR program B (Table 6) with primers AOB-*amo*C-F and AOB-*amo*B-R. For AOA nested PCR programs A and C were used (Table 5); with Ndev-amo-f1, Ndev-amo-f2 and Ndev-amo-r10 sets of primers. DNA for standards was diluted to 10^9 copies per qPCR reaction and dilutions of standards from 10^8 to 10^1 copies per reaction were then made.

qPCR of archaeal *amoA* gene was performed in 25 µl volume containing 10 µl Quantitect SYBER Green Master Mix (Qiagen), 1.5 µM of each primer (CrenamoA23f and CrenamoA616r), 0.2 µM of bovine serum albumin (BSA) and 5 µl of DNA extract, as indicated in Table 8. Three replicates were used for each time point. Negative template control was used in two replicates, with cycle threshold (Ct) value higher that the Ct value of 10^1 copies of standard.

For qPCR of bacterial *amoA* genes, each reaction was performed in a 25 µl volume containing 12.5 µl Quantifast SYBER Green Master Mix (Qiagen), 0.4 µM of each primer (amoA-1F and amoA-2R), 0.2 µM of bovine serum albumin (BSA) and 5 µl of DNA extract, as indicated in Table 9. Three replicates were used for each time point. Negative template control was used in two replicates, with cycle threshold (Ct) value higher that the Ct value of 10^1 copies of standard.

All results with negative template controls were then checked with gel electrophoresis using 2% agarose gel (Bioline, molecular grade) and 100 V. A standard Hyperladder I (Bioline) was used. Gels were stained with 5% ethidium bromide and checked under the UV light (HP System, AlphaImager).

3.2.6.3 Denaturing gradient gel electrophoresis (DGGE)

DGGE analysis with DCode Universal Mutation Detection (Bio-Rad) was used to determine changes within the archaeal or bacterial communities. Specific primers with attached GC-clamp for *amoA* gene and 16S rRNA gene were used to increase the separation of bands in the gel. For 16S rRNA gene, a nested PCR strategy was used and for archaeal *amoA* gene, primers without GC-clamp were used (Table 6; Table 7). DGGE for bacterial *amoA* gene was not performed. All the primers used for DGGE are described in Table 4.

Gels were made using the method described previously by McCaig *et al.* (2001). They contained 8% polyacrylamide that was polymerised with addition of 0.1% ammonium persulfate and 0.01% TEMED solution. For 16S rRNA and *amoA* genes, 35%-75% and 15%-55% gradients of denaturant were used respectively. Gels were poured using Miniplus3 Peristaltic pump (Gilson).

For electrophoresis 7 l of 1 × TAE buffer were heated to a constant 60 °C temperature. Electrophoresis ran for 960 min and 75 V under these conditions. Afterwards the gels were placed in a fixing solution for 30 min and stained with the silver staining method (Nicol *et al.*, 2005).

Gels were then scanned with Epson GT-9600 (Epson) scanner.

3.2.6.4 PCR programs and primers

Table 4: Primers used in the experiments
Preglednica 4: Začetni oligonukleotidi, uporabljeni pri magistrski nalogi

Primers	Sequence 5' - 3'	Reference
AOB *amoA* gene-standard		
AOB-*amoC F*	GTCGTTTGGAACRGCARAGCAAA	Unpublished
AOB-*amoB R*	TCCCAGCTKCCGGTRATGTTCATCC	Unpublished
AOA *amoA* gene-standard		
Ndev-amo-f1	GTTTTCACTAGATGACTTAG	Unpublished
Ndev-amo-f2	GAAAAGAGAGGGGGTGATGATTG	Unpublished
Ndev-amo-r10	GATTTAGTCCCACTTAGACC	Unpublished
AOA *amoA* gene		
CrenamoA23f	ATGGTCTGGCTWAGACG	Tourna *et al.*, 2008
CrenamoA616r	GCCATCCATCTGTATGTCCA	Tourna *et al.*, 2008
AOB *amoA* gene		
amoA-1F	GGGGTTTCTACTGGTGGT	Rotthauwe *et al.*, 1997
amoA-2R	CCCCTCKGSAAAGCCTTCTTC	Rotthauwe *et al.*, 1997
Bacterial 16S rRNA gene		
CTO189fa	GGAGRAAAGCAGGGGATCG	Kowalchuk *et al.*, 1997
CTO189fc	GGAGGAAAGTAGGGGATCG	Kowalchuk *et al.*, 1997
CTO654r	CTAGCYTTGTAGTTTCAAACGC	Kowalchuk *et al.*, 1997
MF (Muyzer) -GC clamp	CCTACGGGAGGCAGCAG-GC clamp	Muyzer *et al.*, 1993
MR (Muyzer)	ATTACCGCGGCTGCTGG	Muyzer *et al.*, 1993
Archaeal 16S rRNA gene		
A109f	ACKGCTCAGTAACACGT	Großkopf *et al.*, 1998
1492R-GN	GYYACCTTGTTACGACTT	Nicol *et al.*, 2008
771F	ACGGTGAGGGATGAAAGCT	Ochsenreiter *et al.*, 2003
957R-GC clamp	CGGCGTTGACTCCAATTG-GC clamp	Ochsenreiter *et al.*, 2003

Y = T or C, **K** = G or T, **M** = A or C, **W** = A or T, **R** = G or A

Table 5: PCR program A
Preglednica 5: PCR program A

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	5 min	1 ×
Denaturation	95 °C	30 s	35 ×
Annealing	45 °C	30 s	
Elongation	72 °C	3 min	
Extension	72 °C	10 min	1 ×

Table 6: PCR program B
Preglednica 6: PCR program B

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	5 min	1 ×
Denaturation	94 °C	30 s	35 ×
Annealing	55 °C	30 s	
Elongation	72 °C	1 min	
Extension	72 °C	10 min	1 ×

Table 7: PCR program C
Preglednica 7: PCR program C

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	5 min	1 ×
Denaturation	94 °C	30 s	10 ×
Annealing	55 °C	30 s	
Elongation	72 °C	30 s	
Denaturation	92 °C	30 s	25 ×
Annealing	55 °C	30 s	
Elongation	72 °C	30 s	
Extension	72 °C	10 min	1 ×

Table 8: Program for qPCR of archaeal *amoA* genes
Preglednica 8: qPCR program za arhejski *amoA* gen

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	15 min	1 ×
Denaturation	94 °C	20 s	45 ×
Annealing	55 °C	30 s	
Elongation	72 °C	1 min	
Plate read	80 °C	8 s	
Extension	72 °C	10 min	1 ×

Table 9: Program for qPCR of bacterial *amoA* genes
Preglednica 9: qPCR program za bakterijski *amoA* gen

Step	Temperature	Time	Number of cycles
Initial denaturation	95 °C	5 min	1 ×
Denaturation	95 °C	15 s	45 ×
Annealing with elongation	60 °C	1 min	
Plate read	80 °C	8 s	1 ×

3.2.7 Statistical analysis

Data acquired from molecular assays was statistically analysed to determine if non-dependable variable (days of incubation and treatment) has a significant effect on dependable variable (*amoA* gene abundance). The analysis was done in IBM SPSS 20 program and two-way ANOVA statistical model was used. Normality of the data and homogeneity of variances were also checked to determine if two-way ANOVA could be performed. If not, data was first transformed and then analysed again. Bonferroni correction was also used to test the p-value.

4 RESULTS

4.1 SOIL pH MEASUREMENTS

Only in microcosms amended with ammonium sulphate, decrease in pH was detected (Figure 11; Figure 12). The overall difference in pH value could be seen as the heterogenic properties of soils.

a) b)

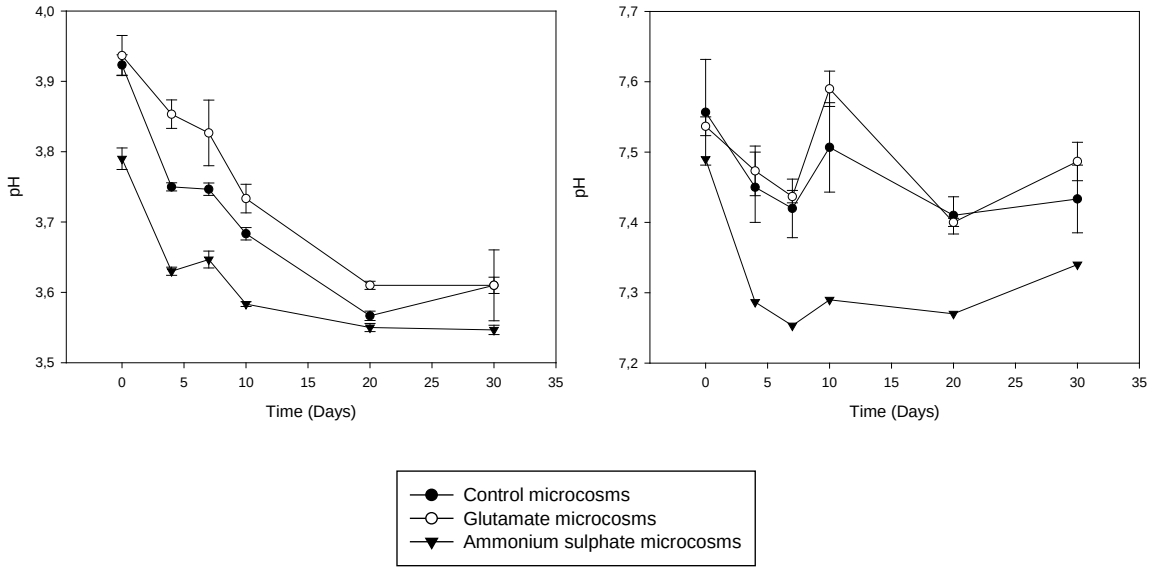


Figure 11: pH of acidic soil (a) and pH of neutral soil (b) in microcosms amended with water (●), glutamate (○) or ammonium sulphate (▼) in experiment with single amendment. Data represent the means and standard error calculated from triplicate microcosms for each treatment

Slika 11: pH meritve mikrokozmov za kisla tla pri poskusu z enkratnim dodajanjem dodatkov. Krivulje prikazujejo kontrolo (●), dodatek glutamata (○) in amonijevega sulfata (▼). Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek

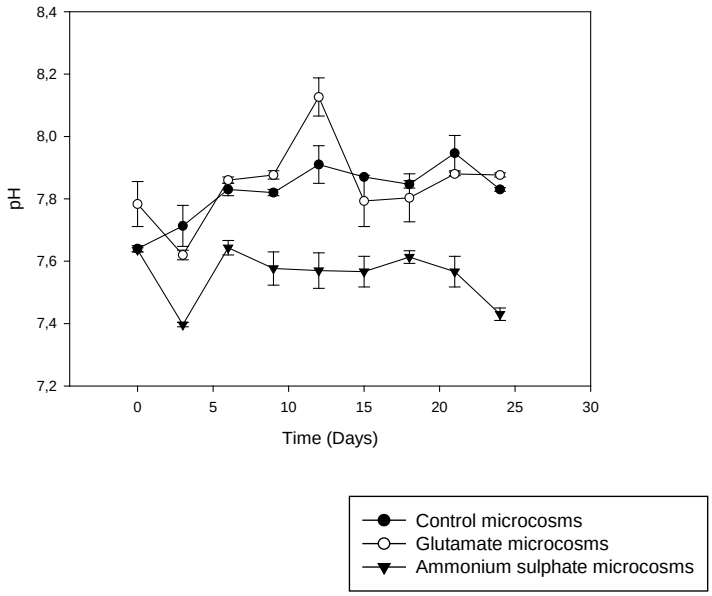


Figure 12: pH measurements in neutral soil microcosms with repeated amendment. Each line represents different amendment: control (●), glutamate (○) or ammonium sulphate (▼). Data represent the means and standard error calculated from triplicate microcosms for each treatment

Slika 12: pH meritve mikrokozmov za nevtralna tla pri poskusu z večkratnim dodajanjem dodatkov. Krivulje prikazujejo kontrolo (●), dodatek glutamata (○) in amonijevega sulfata (▼). Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek

4.2 INFLUENCE OF AMMONIUM SOURCE ON NITRIFICATION RATE

Nitrification kinetics was measured in soil microcosms amended with two sources of ammonium: glutamate and ammonium sulphate.

4.2.1 Experiment with single amendment

In this experiment, glutamate or ammonium sulphate was added to soils only once after construction of microcosms. In acidic soil microcosms amended with ammonium sulphate the concentration of mineral ammonium in soil decreased to 90 µg NH_4^+-N g^{-1} dry soil at day 4 and then increased slowly to a final concentration of 130 µg NH_4^+-N g^{-1}dry soil. A different pattern was observed for glutamate-amended microcosms where NH_4^+-N dropped to levels comparable to those in control microcosms after incubation for only 7 days (Figure 14a). Nitrification kinetic, expressed as accumulation of nitrate g^{-1} soil day^{-1} was highest (16 µg (NO_2^-+NO_3^-)-N g^{-1}dry soil day^{-1}) in microcosms amended with glutamate (Table 10).

In neutral soil microcosms ammonium decreased to concentrations comparable to those measured in control microcosms after incubation for only 4 days regardless of whether ammonium amendment was organic or inorganic (Figure 15a). The nitrification kinetics were similar for both treatments (Figure 15b), with highest nitrification speed in ammonium sulphate amended microcosms (40 µg $(NO_2^-+NO_3^-)$-N g^{-1}dry soil day^{-1}) during the first 4 days of incubation. The control microcosms showed the slowest rate 5 µg $(NO_2^-+NO_3^-)$-N g^{-1}dry soil day^{-1}. After day 4, the nitrification rate for all three microcosms was constant (8 µg $(NO_2^-+NO_3^-)$-N g^{-1}dry soil) (Table 10). Microcosms amended with ammonium sulphate had the highest concentration of $(NO_2^-+NO_3^-)$-N after 30 days, namely 270 ± 6 µg $(NO_2^-+NO_3^-)$-N g^{-1}dry soil.

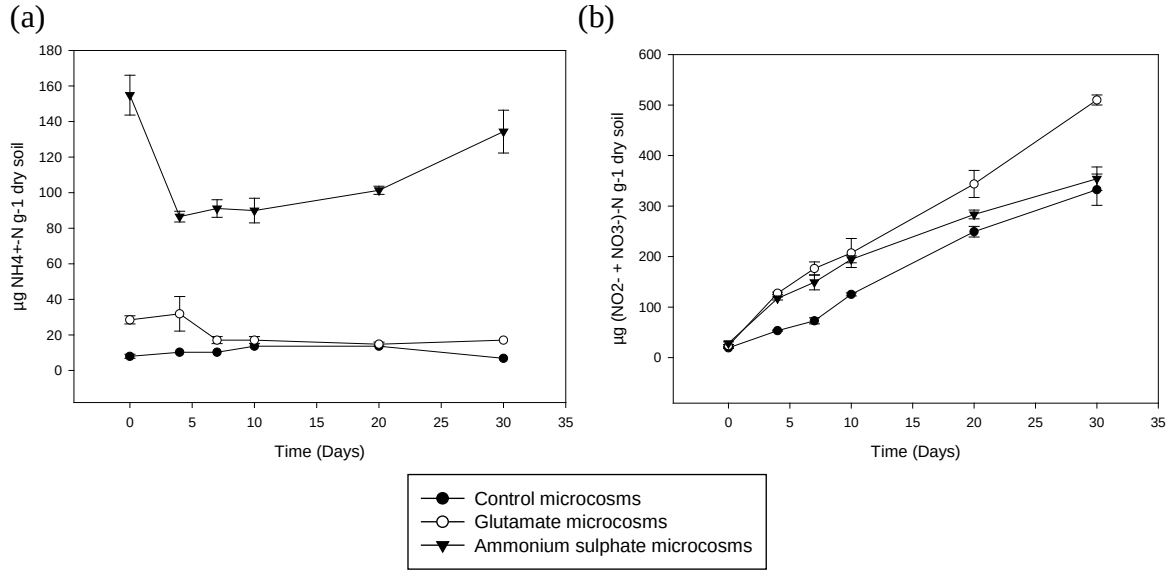


Figure 14: Accumulation of NH_4^+-N g^{-1} dry soil (a) and $(NO_2^-+NO_3^-)$-N g^{-1} dry soil (b) in experiment with single amendment during incubation of microcosms with acidic soil amended with glutamate or ammonium sulphate. Each line represents different amendment: control (●), glutamate (○) or ammonium sulphate (▼). Data represent the means and standard error calculated from triplicate microcosms for each treatment

Slika 14: Koncentracije NH_4^+-N g^{-1} suhih tal (a) in $(NO_2^-+NO_3^-)$-N g^{-1} suhih tal (b) v talnih mikrokozmih (kisla tla), obogatenih z glutamatom ali amonijevim sulfatom pri poskusu z enkratnim dodajanjem dodatkov. Krivulje prikazujejo kontrolo (●), dodatek glutamata (○) in amonijevega sulfata (▼). Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek

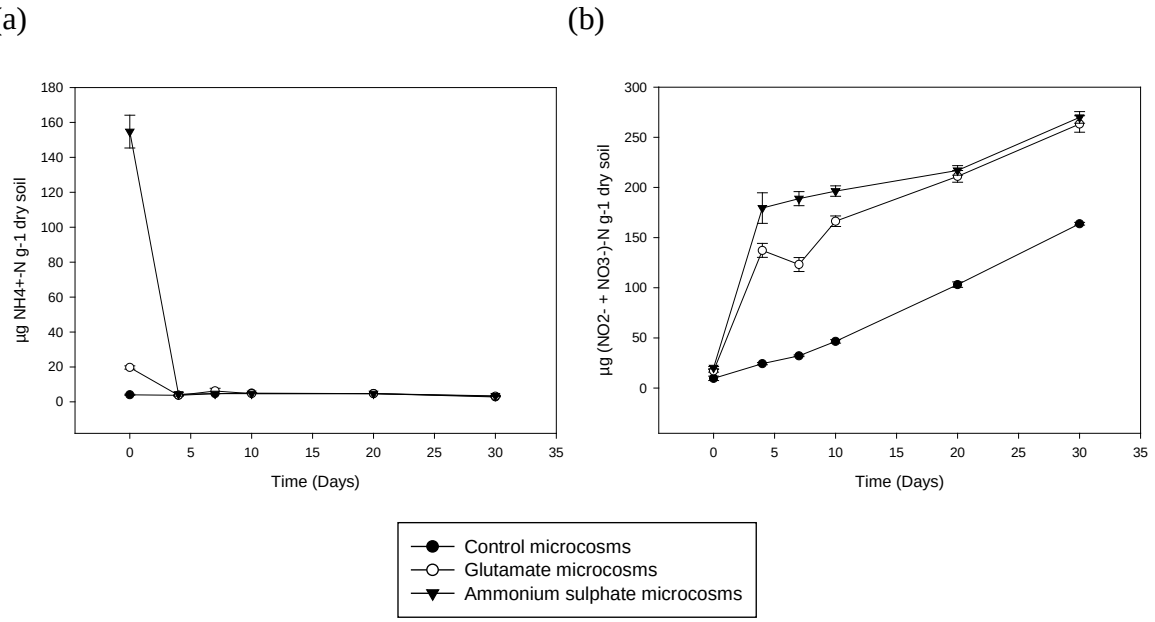


Figure 15: Accumulation of NH_4^+-N g^{-1} dry soil (a) and (NO_2^-+NO_3^-)-N g^{-1} dry soil (b) during incubation in experiment with single amendment in microcosms with neutral soil amended with glutamate or ammonium sulphate. Each line represents different amendment: control (●), glutamate (○) or ammonium sulphate (▼). Data represent the means and standard error calculated from triplicate microcosms for each treatment

Slika 15: Koncentracije $NH4^+$-N g^{-1} suhih tal (a) in (NO_2^-+NO_3^-)-N g^{-1} suhih tal (b) v talnih mikrokozmih (nevtralna tla), obogatenih z glutamatom ali amonijevim sulfatom v poskusu z enkratnim dodajanjem dodatkov. Krivulje prikazujejo kontrolo (●), dodatek glutamata (○) in amonijevega sulfata (▼). Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek

4.2.2 Experiment with repeated amendment of ammonium sources

In neutral soil, a similar response in NH_4^+- N concentration was observed in experiment with repeated amendment as in the experiment with single amendment. The nitrification rate was constant for the first 18 days with the highest nitrification rate of 35 µg (NO_2^- +NO_3^-)-N g^{-1} dry soil day^{-1} in glutamate amended microcosms. Subsequently, nitrification rate increased sharply for both glutamate and ammonium sulphate treatments and the nitrification rates were 172 µg and 197 µg (NO_2^-+NO_3^-)-N g^{-1}dry soil day^{-1} for ammonium sulphate and glutamate treatments, respectively. In contrast, nitrification rates in un-amended controls were low throughout the incubation (Table 10).

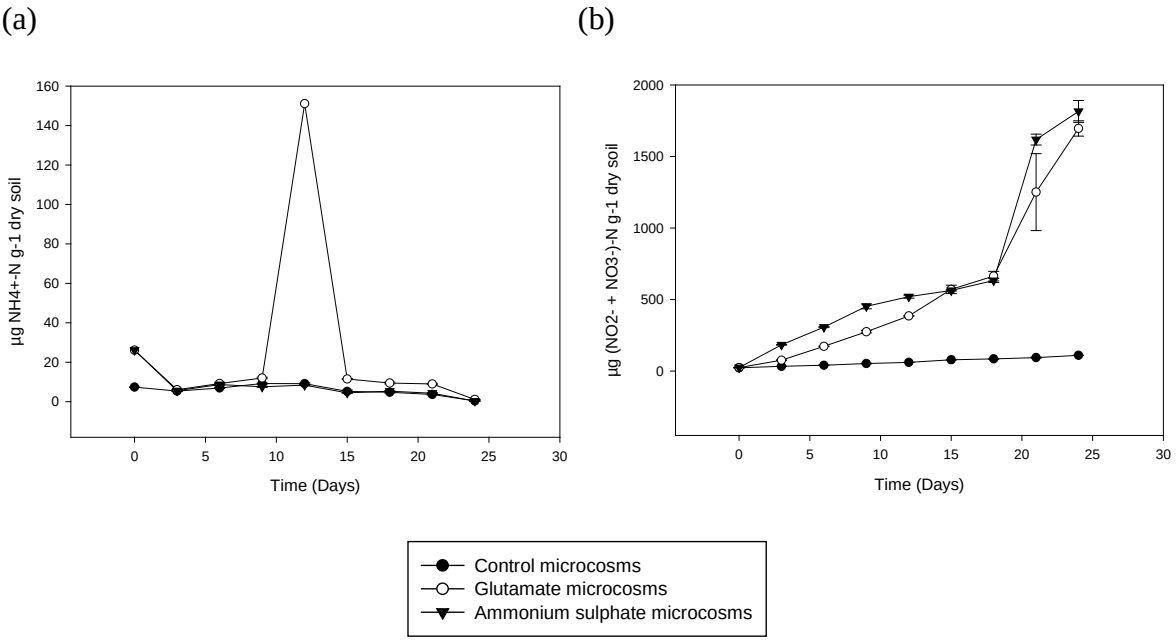


Figure 16: Accumulation of NH_4^+-N g^{-1} dry soil (a) and (NO_2^-+NO_3^-)-N g^{-1} dry soil (b) during incubation in microcosms with neutral soil that was repeatedly amended with glutamate or ammonium sulphate. Repeated amendments were done after incubation for 0, 4, 6, 11, 16, 18 and 21 days. Each line represents different amendment: control (●), glutamate (○) or ammonium sulphate (▼). Data represent the means and standard error calculated from triplicate microcosms for each treatment

Slika 16: Koncentracije NH4+-N g^{-1} suhih tal (a) in (NO_2^-+NO_3^-)-N g^{-1} suhih tal (b) v talnih mikrokozmih (nevtralna tla), obogatenih z glutamatom ali amonijevim sulfatom. Dodatki so bili dodani mikrokozmom večkrat in sicer ob dnevih 0, 4, 6, 11, 16, 18 in 21. Krivulje prikazujejo kontrolo (●), dodatek glutamata (○) in amonijevega sulfata (▼). Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek

Table 10: Nitrification rates calculated from (NO_2^-+NO_3^-)-N g^{-1} dry $soil^{-1}$ concentrations, assuming that the nitrification rate is linear

Preglednica 10: Hitrost nitrifikacije preračunana iz koncentracije (NO_2^-+NO_3^-)-N g^{-1} suhih tal^{-1}, če predpostavimo, da je hitrost nitrifikacije linearna

			Initial nitrification rate (NO_2^-+NO_3^-)-N g^{-1}dry soil day^{-1}	Late nitrification rate (NO_2^-+NO_3^-)-N g^{-1}dry soil day^{-1}	Average nitrification rate (NO_2^-+NO_3^-)-N g^{-1}dry soil day^{-1}
Experiment with repeated amendment	Neutral soil	Control	3	4	4
		Ammonium sulphate	41	197	75
		Glutamate	30	172	70
Experiment with single amendment	Neutral soil	Control	4	5	5
		Ammonium sulphate	40	3	8
		Glutamate	30	5	8
Experiment with single amendment	Acidic soil	Control	11	10	10
		Ammonium sulphate	17	8	11
		Glutamate	18	15	16

In control microcosms, the (NO_2^-+NO_3^-)-N concentration was higher in acidic soil in comparison with neutral soil in experiment with single amendment as well as in experiment with repeated amendment (Figure 13a).

In ammonium sulphate amended microcosms in experiment with single amendment, the initial nitrification rate was higher in neutral soil, but average nitrification rate was higher in acidic soil (Figure 13b; Table 10). Noticeable difference in nitrification kinetics was observed in experiment with repeated amendment. At day 6 (NO_2^-+NO_3^-)-N concentration was comparable to final concentration in experiment with single amendment (300 µg (NO_2^-+NO_3^-)-N g^{-1}dry soil). It was also noticeable, that late nitrification rate was much higher from the initial nitrification rate, which was not observed in the experiment with single amendment.

In glutamate amended microcosms in experiment with single amendment, (NO_2^-+NO_3^-)-N concentration was similar for both soils until day 10. Afterwards nitrification rate was

again higher in acidic soil than in neutral soil for both experiments. In experiment with repeated amendment, nitrification kinetics is similar to that in ammonium sulphate amended microcosms.

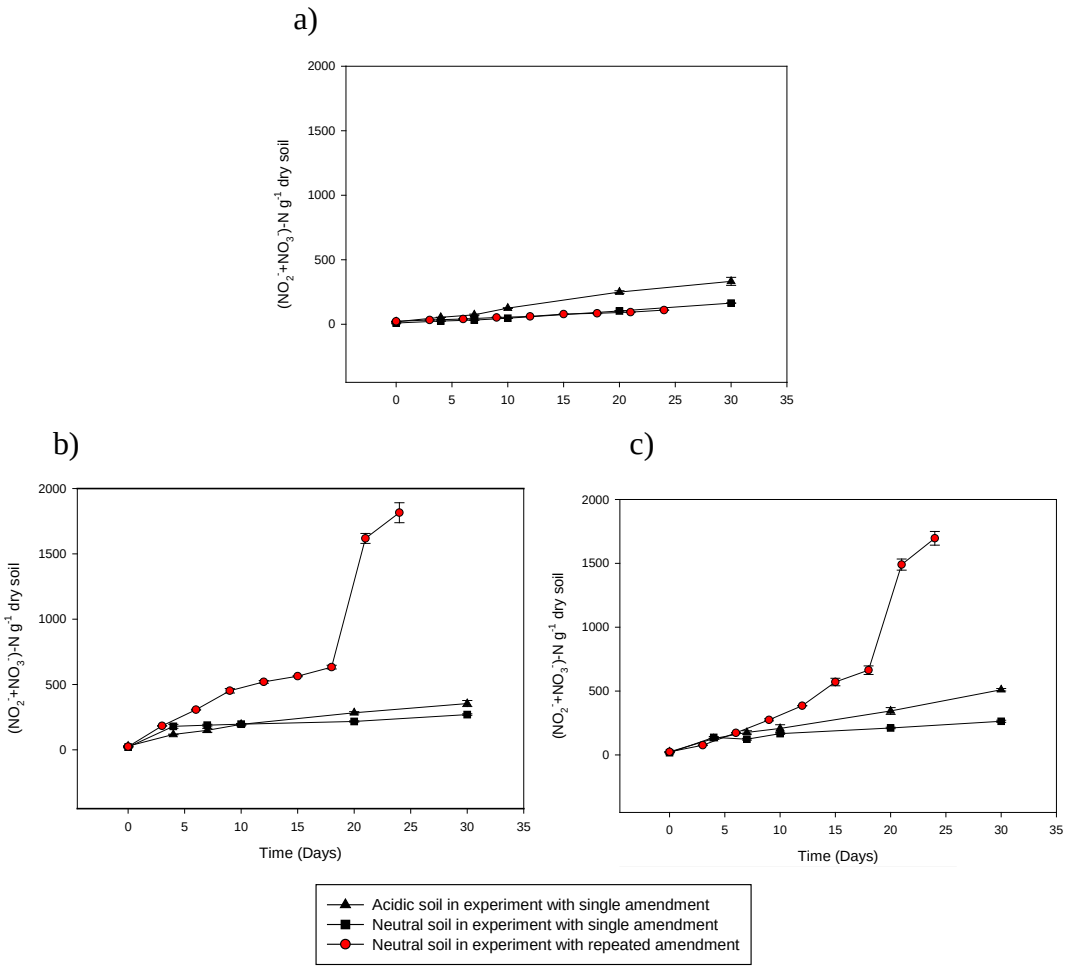


Figure 13: Accumulation of $(NO_2^-+NO_3^-)$-N g^{-1} dry soil in control microcosms (a), microcosms amended with ammonium sulphate (b) and microcosms amended with glutamate (c). Each line represents: acidic soil in experiment with single amendment (▲), neutral soil in experiment with single amendment (■) and neutral soil in experiment with repeated amendment (●). Data represent the means and standard error calculated from triplicate microcosms for each treatment

Slika 13: Koncentracije (NO2-+NO3-)-N g^{-1} suhih tal v talnih kontrolnih mikrokozmih (a), mikrokozmih z amonijevim sulfatom (b) in mikrokozmih obogatenih z glutamatom (c). Krivulje prikazujejo kisla tla v poskusu s posameznim dodajanjem dodatkov (▲), nevtralna tla pri poskusu s posameznim dodajanjem dodatkov (■) in nevtralna tla pri poskusu z večkratnim dodajanjem dodatkov (●). Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek

4.4 ANALYSIS OF *amoA* GENE ABUNDANCE IN SOIL MICROCOSMS

The abundance of the specific *amoA* genes was determined to assess whether different treatments (glutamate and ammonium sulphate) influenced the abundance of AOA and AOB.

In acidic soil, bacterial *amoA* gene was below the level of detection. In contrast abundance of AOA *amoA* was high, reaching 10^7 gene copies g^{-1} dry soil. There was no statistically significant difference between treatments and time points in AOA *amoA* gene abundance ($p > 0.05$) (if p-value was more than 0.05 it could be assumed that null hypothesis was not rejected and there was no significant effect of non-dependable variables to dependant variable), but there was a noticeable trend of decreasing AOA *amoA* gene abundance (Figure 17).

Similarly, in neutral soil there was no statistically significant difference in AOA *amoA* gene abundance between treatments and time points ($p > 0.05$) (Figure 18a). Two-way ANOVA showed no significant difference between time points and treatments in AOB *amoA* gene abundance ($p > 0.05$). Furthermore no trend of increase or decrease could be determined because of *amoA* gene abundance variation. However it is noticeable that AOB *amoA* gene abundance was ten-times higher than AOA *amoA* gene abundance.

Even though two-way ANOVA showed no statistical difference between time points and treatments ($p > 0.05$), some trend of increase could be observed in both AOA and AOB *amoA* gene abundance. However AOB *amoA* gene abundance (Figure 19b) in microcosms repeatedly amended with glutamate indicated the highest gene abundance after 24 days.

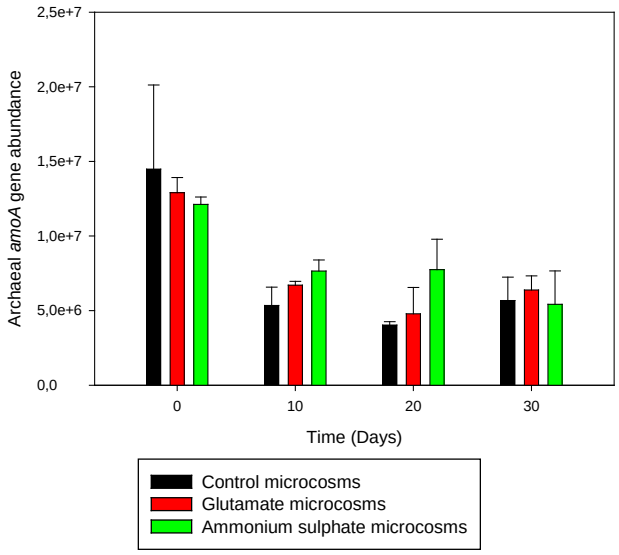


Figure 17: AOA *amoA* gene abundance in acidic soil in experiment with single amendment for microcosms, amended with glutamate (red) or ammonium sulphate (green) or unamended control microcosms (black). Data represent the means and standard error calculated from triplicate microcosms for each treatment. IBM SPSS 20 program and two-way ANOVA statistical model were performed for the statistical analysis of these results

Slika 17: Številčnost genov *amoA* AOA v talnih mikrokozmih pripravljenih s kislimi tlemi visokega barja in obogatenih z glutamatom (rdeči stolpci), amonijevim sulfatom (zeleni stolpci) in neobogatenimi kontrolni mikrokozmi (črni stolpci) v poskusu z enkratnim dodajanjem dodatkov. Posamezen stolpec predstavlja povprečno vrednost treh ponovitev. Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek

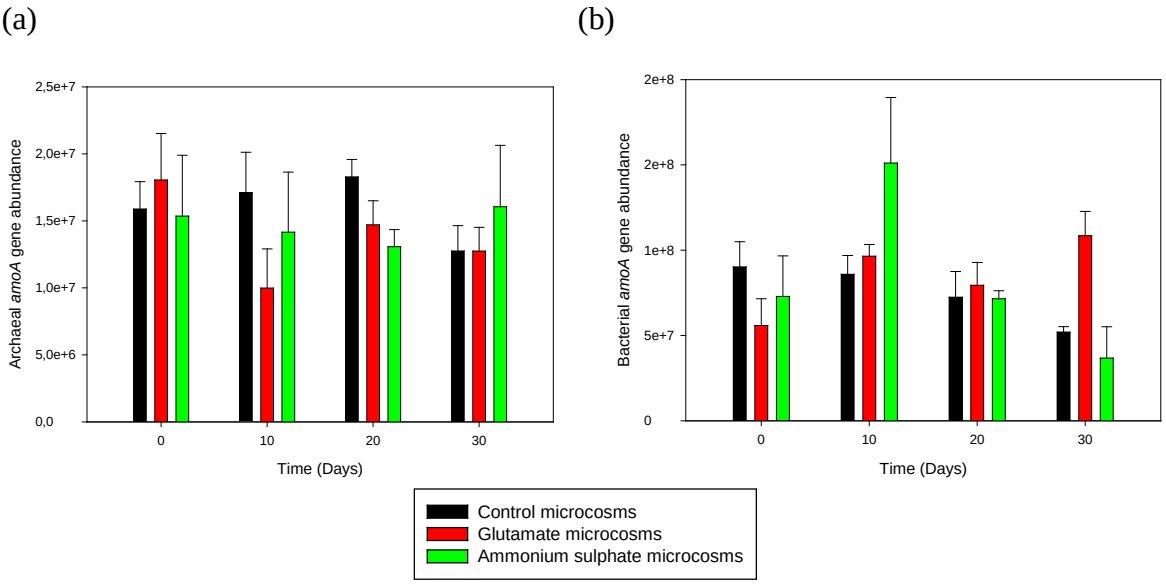


Figure 18: *amoA* gene abundance in neutral soil in experiment with single amendment for microcosms, amended with glutamate (red) or ammonium sulphate (green) or unamended control microcosms (black). Results are showed for AOA *amoA* gene abundance (a) and AOB *amoA* gene abundance (b). Data represent the means and standard error calculated from triplicate microcosms for each treatment. IBM SPSS 20 program and two-way ANOVA statistical model were performed for the statistical analysis of these results

Slika 18: Številčnost *amoA* genov v talnih mikrokozmih pripravljenih z nevtralnimi tlemi obogatenih z glutamatom (rdeči stolpci), amonijevim sulfatom (zeleni stolpci) in neobogatenimi kontrolni mikrokozmi (črni stolpci) v poskusu z enkratnim dodajanjem dodatkov. Rezultati prikazujejo število AOA *amoA* genov (a) in AOB *amoA* genov (b). Posamezen stolpec predstavlja povprečno vrednost treh ponovitev. Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek

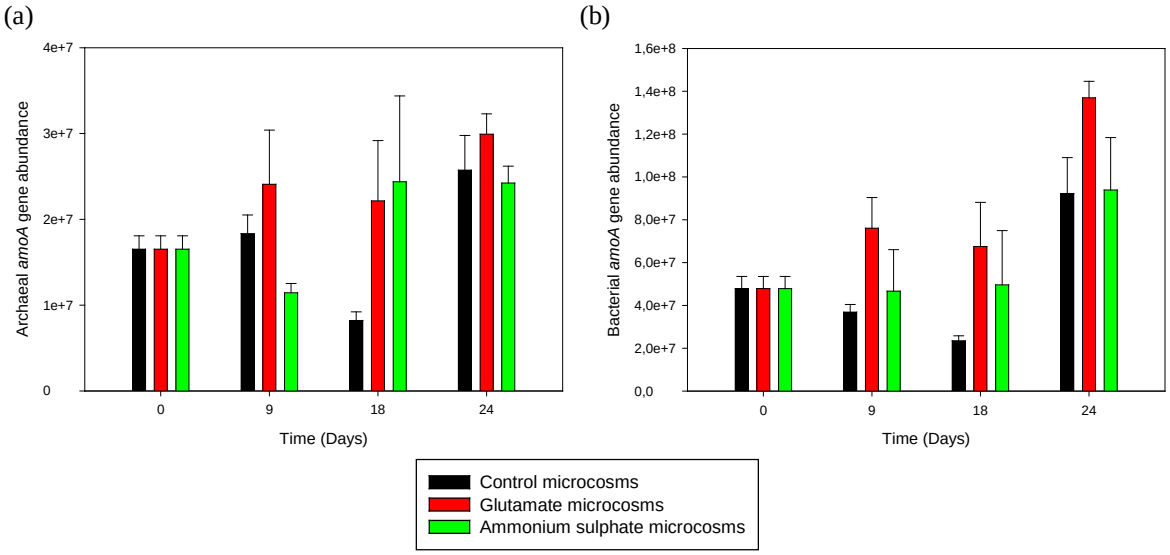


Figure 19: *amoA* gene abundance in neutral soil for microcosm experiment with repeated amendment of glutamate (red) or ammonium sulphate (green) or unamended control microcosms (black). Results are showed for AOA *amoA* gene abundance (a) and AOB amoA gene abundance (b). Data represent the means and standard error calculated from triplicate microcosms for each treatment. IBM SPSS 20 program and two-way ANOVA statistical model were performed for the statistical analysis of these results.

Slika 19: Številčnost *amoA* genov v talnih mikrokozmih pripravljenih z nevtralnimi tlemi, večkratno obogatenih z glutamatom (rdeči stolpci), amonijevim sulfatom (zeleni stolpci) in neobogatenimi kontrolni mikrokozmi (črni stolpci). Rezultati prikazujejo število AOA *amoA* genov (a) in AOB *amoA* genov (b). Posamezen stolpec predstavlja povprečno vrednost treh ponovitev. Podatki predstavljajo srednje vrednosti in standardne napake izračunane iz treh ponovitev za vsak posamezen dodatek.

4.5 DGGE ANALYSIS

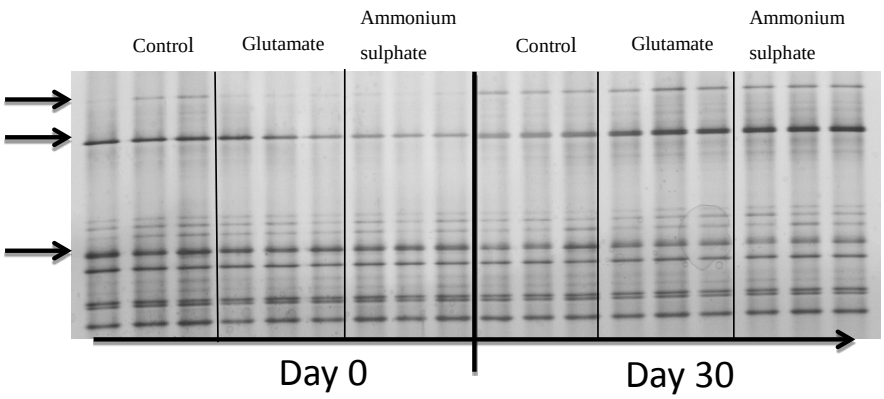
DGGE analysis was performed to determine structural changes in AOA and AOB microbial communities following amendment of soil microcosms with glutamate or ammonium sulphate.

The results indicated that in acidic soil in the experiment with single amendment communities did not respond to either amendment but some temporal changes were observed in archaeal 16S rRNA gene profiles. After incubation for 30 days, two bands showed increased relative intensity, while relative intensity of other bands decreased (figure 20a). Furthermore, changes in relative band intensity between time point 0 and 30 were also observed for AOA *amoA* gene with one band becoming almost invisible (Figure 20b).

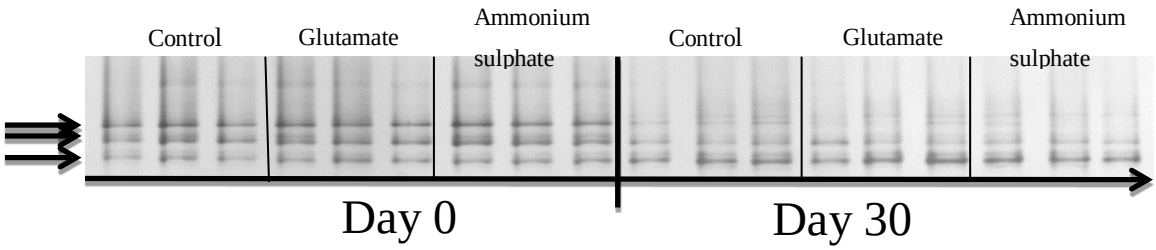
In neutral soil in the experiment with single amendment, two bands in bacterial 16S rRNA gene profiles became more intense after 30 days of incubation (Figure 20c).

There were no differences in intensity of the bands between treatments or time points in any of the DGGE gels for the experiment with repeated amendments.

a)



b)



c)

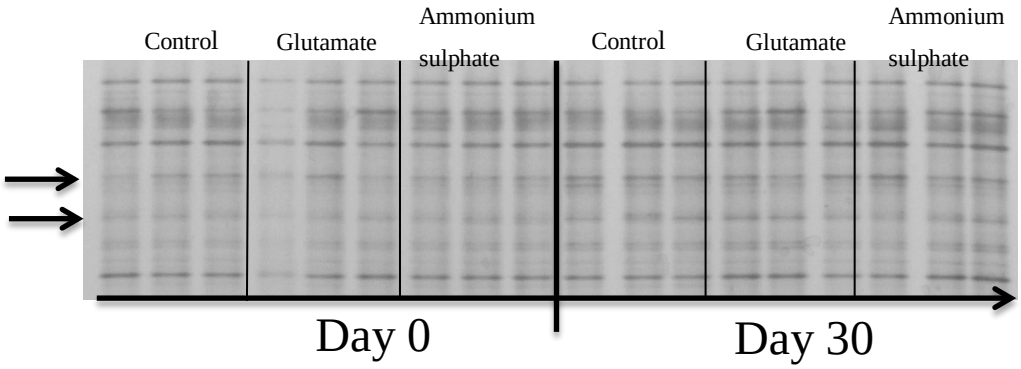


Figure 20: DGGE gels of AOA 16S rRNA gene in acidic soil microcosms (a); thaumarchaeal *amoA* gene in acidic soil microcosms (b) and bacterial 16S rRNA gene in neutral soil microcosms (c) in experiment with single amendment

Slika 20: DGGE geli mikrokozemskega poskusa z enkratnim dodajanjem dodatkov za arhejski gen za 16S rRNK v kislih tleh (a), arhejski *amoA* gen v kislih tleh (b) in bakterijski gen za 16S rRNK v nevtralnih tleh (c)

5 DISCUSSION

The aim of this study was to determine if AOA and AOB communities respond differently to the addition of different sources of ammonium in two different types of soil. Acidic forest soil (Bog) was used as a reference soil to address the response of AOA to different ammonium sources. This soil was dominated by AOA, but AOB were below detectable levels (Stopnišek *et al.*, 2010). In addition, nitrification was for the first time addressed in a neutral agricultural soil (Bevke) that included AOA and AOB. Here the responses of AOA and AOB to different sources of ammonium were explored.

5.1 ACIDIC SOIL (BOG)

I was predicted that AOA were dominant ammonia oxidisers in acidic forest soil and that an organic source of ammonium was a preferred source of ammonium and that this would result in a higher nitrification rate, faster growth and changes in AOA community.

5.1.1 Activity in acidic soil

Nitrification rates were determined from measurements of (NO_2^-+NO_3^-)-N concentration in microcosms. Levičnik-Höfferle *et al.* (2012) showed that nitrification rate was highest when acidic forest soil was amended with organic sources of ammonium and inorganic ammonium did not influence the nitrification rate. A similar observation was obtained in this study when total nitrate-N was evaluated at the end of incubation and it showed that glutamate was the preferred source of ammonium for nitrification. However, in contrast to previous findings (Levičnik-Höfferle *et al.*, 2012), inorganic ammonium was also nitrified. This could be due to a changed community of nitrifiers in this soil as compared to the soil examined by Levičnik-Höfferle *et al.* (2012) one year earlier. Stroo *et al.* (1986) found heterotrophic fungi in acidic soil that were involved in a nitrification process. Alternatively different groups of AOA could have been enriched in this soil during the preceding year, leading to slightly different findings. It was also observed that in control microcosms, nitrification rate was higher in acidic soil than in neutral soil. This could be due to higher organic content in acidic soil which could be mineralised and therefore provide a substrate for nitrification.

5.1.2 Community structure in acidic soil

AOB could not be detected in the acidic forest soil by PCR but AOA were abundant and these results suggested that AOA may be in control of nitrification in this soil. This is in agreement with results of Stopnišek *et al.* (2010) and Levičnik-Höfferle *et al.* (2012) who also found AOA dominant and AOB undetectable by PCR in the same soil. As previously found (Stopnišek *et al.,* 2010), the growth of archaea was also very slow in acidic soil. Furthermore in this experiment a trend of decrease in growth could be observed.

There were some changes in community structure over incubation period, but not as a consequence of the amendment by different sources of ammonium.

Based on these finding it could be concluded that AOA are probably controlling the nitrification in acidic soils and prefer an organic source of ammonium. Since increase in nitrification kinetics could be observed, this suggested an upregulation of nitrification activity in AOA despite slow growth. To further study this it was suggested to prolong the incubation period and test the transcriptional activity.

5.2 NEUTRAL SOIL (BEVKE)

Preliminary studies (Levičnik-Höfferle unpublished) indicated that AOA and AOB are both present in this neutral agricultural soil; therefore it was predicted that both organic and inorganic source of ammonium would affect nitrification rate, but the growth and the community structure of AOA would be affected by organic source, while AOB would respond to inorganic source of ammonium.

5.2.1 Nitrification activity in neutral soil

In both experiments in neutral soil, nitrification rates were similar following amendment with organic and inorganic sources of ammonium. This suggests that there was no preference in using mineral or organic ammonium source for nitrification in the neutral soil, as predicted. There were some unusual occurrences in nitrification kinetics in the second experiment. An unusual occurrence was observed after day 18, when a tenfold increase in nitrification rate was determined in microcosms amended with organic and inorganic sources of ammonium (Figure 16b) reaching almost 2000 µg (NO_2^-+NO_3^-)-N g^{-1} dry soil. Repeated amendments led to cumulative addition of 700 µg NH_4^+-N g^{-1} dry soil

and the rest should have come from mineralisation. This could not be explained and it has not been reported before, even though some studies also observed a similar final concentration of measured (NO_2^-+NO_3^-)-N in soil heavily exposed to sheep urine (Webster *et al.*, 2005). However, it was possible that repeated N-amendment influences soil mineralisation rates and this should be explored in the future.

5.2.2 Community structure in neutral soil

In the first experiment, with single amendment of ammonium, no changes in AOA or AOB *amoA* gene abundance were observed; some changes were observed in archaeal community structure but only as a consequence of incubation, and not in connection with the source of ammonium. It was possible that insufficient ammonium was added to support detectable growth of AOA and AOB in the first experiment; therefore the second experiment was performed with repeated amendment. Again, no statistically significant change in AOA or AOB *amoA* gene abundance could be observed due to short incubation of microcosms. However, some trends were observed in *amoA* gene abundance in experiment with repeated amendments. In AOA *amoA* gene abundance there was a trend of overall increase in growth, which suggested that repeated amendment of the source of ammonia affected overall growth in both ammonium sulphate and glutamate amended microcosms.

Similarly trend of increase in AOB *amoA* gene abundance was observed at the end of the experiment in neutral soil microcosms amended with organic source of ammonium. This could not be found in previous publications (Jiang *et al.*, 1999), suggesting that AOB cannot nitrify organic source of ammonium. However, there was a high possibility that in neutral soil, microorganisms performing mineralisation coexist with AOB and therefore provide the AOB inorganic source of ammonium to oxidise.

In the experiment with repeated amendment, no changes in AOA or AOB community structure were observed.

In conclusion we could see a trend of change in gene abundance, but this alone cannot explain a dramatic increase in nitrification activity observed from the day 18 of incubation. Since it was known before that ammonia oxidisers had slow growth (Stopnišek *et al.*, 2010), this indicated a sharp increase in nitrification activity with retained abundance of

AOA and AOB during the incubation. Because no significant difference in gene abundance occurred during the 30 day incubation, it could be assumed that the incubation period was too short. In future experiments it would probably be better to use longer incubation period with special focus on trends of changes in gene abundance and with an additional assay for transcriptional activity.

6 CONCLUSIONS

The following conclusions were made in this study;

In acidic forest soil (Bog):

- Organic source of ammonium increased nitrification rate
    - Ammonia from inorganic source of ammonium was also nitrified, but in lower rates than organic source of ammonium
- AOA were controlling nitrification and prefered an organic source of ammonium
- Neither organic or inorganic sources of ammonium affected AOA community structure

In neutral agricultural soil (Bevke):

- Both organic and inorganic source of ammonium stimulated similar nitrification rate to similar extents
- Repeated amendment of ammonium sources stimulated nitrification kinetics
- Amendment of organic or inorganic source of ammonium did not affect changes of AOA or AOB community structure

These experiments could be repeated with acetylene which discriminates the autotrophic and heterotrophic ammonia-oxidizing organisms, since only autotrophic are sensitive to acetylene (Offre *et al.,* 2009). Transcriptional activity should also be checked since abundance of AOA and AOB *amoA* genes did not provide enough specific results to conclude if the community is changing in numbers because of nitrification or any other reason. It is also suggested that instead of ammonium sulphate, ammonium chloride is used, as it does not cause changes in pH.

7 SUMMARY

Ammonia oxidation is the first step in the nitrification process and a component of the global nitrogen cycle. Woese *et al.* (1990) proposed the third domain called "Archaea" but they were first considered as extremophiles and nitrification was still thought to be performed only by Bacteria. Bacterial nitrification is therefore more studied and understood and it is known that AOB oxidise ammonia using two enzymes, ammonia monooxygenase and hydroxylamine oxidoreductase. There are a few studies of ammonia oxidation by AOA, but the detailed mechanism is not known, and there is no genomic evidence for hydroxylamine oxidoreductase (Walker *et al.,* 2010; Arp *et al.,* 2002)

In the 1990s, research focused on classification of AOB, which belong to the beta and gamma-proteobacteria. Even though archaea were discovered by that time, it was initially thought that they are only extremophiles. Analysis of 16S rRNA genes indicated their presence in mesophilic environments at abundances that were stable and comparable to that of other prokaryotes. Ammonia oxidising archaea were discovered in 2005 in an aquatic ecosystem and after that, studies on AOA and AOB communities in soil and other environments began in hopes to understand and determine their ecological niches (Purkhold *et al.,* 2000; Stephen *et al.,* 1999; Ochsenreiter *et al.,* 2003; Könneke *et al.,* 2005)

Recent studies suggested that AOA have a selective advantage over AOB because of the ability to survive and nitrify ammonia derived from mineralised organic matter in acidic soil. Subsequent study also showed that AOA in acidic soil prefer organic sources of ammonia over inorganic ammonium, as nitrification rates in microcosms with amended inorganic source of nitrogen were the same as in control microcosms. This raised the question of whether organic and inorganic sources of ammonia have differential effects on AOA and AOB communities. (Stopnišek *et al.,* 2010; Levičnik-Höfferle *et al.,* 2012)

This question provided the basis for this Master's thesis as its main objectives were to test whether an organic source of ammonia influenced AOA and inorganic ammonium influenced the AOB community. This was investigated in an acidic soil and a pH-neutral agricultural soil. Both AOA and AOB were present at high numbers in the neutral soil, enabling assessment of the effects of different sources of ammonia on these communities

and how effects were manifested. The acidic forest peat soil (bog) was used as a reference. Microcosms were constructed, consisting of sieved soil in glass bottles, and were amended with 150 µg N g^{-1} dry soil of organic source of ammonium, glutamate; inorganic ammonium, ammonium sulphate and sterile distilled water as a control. Microcosms were incubated at 28°C for 30 days and microcosms were destructively sampled after incubation for 0, 4, 7, 10, 20 and 30 days. A portion of each soil sample was used for colorimetric analysis of NH_4^+-N and (NO_2^-+NO_3^-)-N after extraction with 2M KCl. The remaining soil was used for DNA extraction, qPCR quantification of *amoA* abundance and DGGE analysis.

The second experiment was performed with neutral soil (Bevke) and included repeated amendment of the microcosms with glutamate, ammonium sulphate or distilled water (control microcosms) in total 7 times. Microcosms were incubated at 28 °C for 24 days and were sampled destructively after incubation for 0, 3, 6, 9, 12, 15, 18, 21 and 24 days. Microcosms were amended with 100 µg N g^{-1} dry soil after incubation for 0, 4, 6, 11, 16, 18 and 21 days. Samples were analysed as described above.

In acidic forest soil, the nitrification rates were greatest in soil amended with the organic source of ammonium and addition of inorganic ammonium did not influence nitrification rate. A similar observation was obtained when nitrate-N was evaluated at the end of incubation and glutamate was the preferred source of ammonia for nitrification. However, in contrast to previous findings (Levičnik-Höfferle *et al.*, 2012), inorganic ammonium was also nitrified.

AOB could not be detected in the acidic forest soil by PCR, but AOA were abundant, suggesting that AOA may be in control of nitrification in this soil. There were some changes in community structure over time, but not as a consequence of the amendment by different sources of nitrogen. Based on these finding it could be concluded, that AOA are control nitrification in acidic soils and prefer an organic source of ammonium.

In neutral agricultural soil (Bevke) in both experiments, inorganic and organic sources of ammonium were oxidised at similar rates. This suggests that there was no preference in using mineral or organic ammonium source for nitrification in neutral soil.

In the first experiment with single amendment of source of ammonia, no changes in AOA or AOB *amoA* gene abundance were observed. Relatively minor temporal changes in AOA community structure were not related to the source of ammonium. Because of the possibility that insufficient source of ammonium was added to support detectable growth of AOA and AOB in the first experiment the second experiment was performed with repeated amendment. Even though no statistically significant change in AOA or AOB *amoA* gene abundance could be observed, there was a trend of overall increase in gene abundance. Interestingly a trend of increase in AOB *amoA* gene abundance was observed at the end of the experiment in microcosms amended with organic source of ammonium, which was not seen in any publications before.

In conclusion in neutral soil, repeated amendment of ammonium sources stimulates nitrification kinetics. Since no change in AOA or AOB abundance was observed, changes in nitrification kinetics could be induced by upregulation of nitrification activity in ammonia nitrifiers. Nevertheless it could be concluded that different source of ammonia does not have an effect on AOA or AOB communities.

Because no significant difference occurred during the 30 day incubation, it could be assumed that incubation period was too short. In future experiments it would be probably better to use longer incubation period with special focus on trends of changes in gene abundance and with an additional assay for transcriptional activity.

7.1 POVZETEK

Oksidacija amoniaka je prvi korak nitrifikacije, ki je del večjega sistema-kroženja dušika. Pri kroženju so najbolj pomembni mikroorganizmi in tako je tudi pri nitrifikaciji. Še do nedavnega je prevladovalo mnenje, da so nitrifikatorji le bakterije, pred kratkim pa so pokazali, da so tudi arheje pomembne pri oksidaciji amonijaka. Pri bakterijski oksidaciji sodelujeta 2 encima, amoniak monooksigenaza in hidroksilamin oksidoreduktaza, ki postopno oksidirata amonijak do nitrita. Walker in sod. so leta 2010 v svoji raziskavi pokazali, da se amoniak monooksigenaza nahaja tudi pri amoniak-oksidirajočih arhejah (AOA). Ta encim je kodiran na operonu *amoCAB*, podobno kot pri bakterijah (Arp in sod., 2002) vendar se encim razlikuje od bakterijskega. Poleg tega pri AOA še niso dokazali prisotnosti intermediata hidroksilamina.

Amoniak je plin in je brez naboja. Zato se ne veže na talne delce. Vendar v okolju amonijak prehaja v ionsko obliko (amonij) ki se zaradi svojega pozitivnega naboja veže na koloide v tleh in je tako manj mobilen. Na drugi strani pa je produkt nitrifikacije nitrat, ki je negativno nabit in se ne more vezati na negativno nabite talne delce ter je zato visoko mobilen in se spira v podtalnico. Poleg tega ga absorbirajo rastline kot vir dušika ali pa med denitrifikacijo v plinasto obliko.

V zadnjem času človek z nenadzorovanim gnojenjem tal le še pripomore k povečani nitrifikaciji v tleh in posledično k onesnaževanju okolja. Tako je preučevanje nitrifikacije in razumevanje sistema ključno za izboljšanje strategij gnojenja v kmetijstvu (Suzuki in sod., 1974; Kowalchuk in Stephen, 2001; Raun in sod., 1998). V uporabi so sintetični ali biološki zaviralci nitrifikacije, vendar so na tem področju potrebne dodatne raziskave še posebej v povezavi z AOA (Subbarao in sod., 2012; McCarty in sod., 1999).

AOB so aerobi, nekateri med njimi (*Nitrosomonas europeae)* pa ob zmanjšani vsebnosti kisika lahko vršijo tudi denitrifikacijo (Geets in sod., 2006). AOB uvrščamo med gama-proteobakterije (npr. *Nitrosococcus)* in beta-proteobakterije (npr. *Nitrosomonas* ter *Nitrosospira*) (Stephen in sod., 1996; Purkhold in sod., 2002).

Arheje najdemo v zelo raznolikih ekosistemih. Sprva je prevladovalo mnenje, da so značilne le za ekstremna okolja, vendar so kasnejše raziskave pokazale, da so prisotne tudi v drugih okoljih (na primer tla). Amonij oksidirajoče arheje so odkrili leta 2005 v vodnem ekosistemu in v nadaljevanju so primerjalne študije AOA in AOB v tleh in ostalih okoljih pripeljale do boljšega razumevanje ekoloških niš obeh skupin (Purkhold in sod., 2000; Stephen in sod., 1999; Ochsenreiter in sod., 2003; Könneke in sod., 2005)

Jia in Conrad (2009) sta pokazala, da čeprav so AOA v tleh bolj številčne, so AOB bolj aktivne. Nasprotno pa so Gubry-Rangin in sod. (2010) in Stopnišek in sod. (2010) pokazali da so AOA bolj aktivne in bolj številčne od AOB v kislih tleh in da so v kislih tleh AOA odgovorne za nitrifikacijo. Poleg tega so ugotovili, da z višanjem talnega pH upada število AOA in narašča število AOB (Nikol in sod., 2008), kar pomeni, da bi lahko bil pH pomemben dejavnik ki vpliva na ekološko nišo organizmov, ki oksidirajo amonij. V šotnih kislih tleh z visoko stopnjo nitrifikacije dodatek mineralnega amonijaka ni pospešil

nitrifikacije in večina dodanega amonijaka je ostala neporabljena. Ti rezultati so bili osnova za predpostavko, da AOA preferenčno izrabljajo organski vir amoniaka (Stopnišek in sod., 2010). Dodajanje organskega in anorganskega vira amoniaka v kisla šotna tla je potrdila to predpostavko in dodatek organskega vira amonijaka v tla je spodbudil rast AOA. Slednja je bila inhibirana v prisotnosti acetilena (Levičnik-Höfferle in sod., 2012). Vprašanje, ki se odpira ob teh rezultatih je, ali ima različen vir amoniaka različen vpliv na AOA in AOB združbe v tleh. To vprašanje smo si zastavili tudi v okviru te magistrske naloge.

Cilji magistrske naloge so:

- določiti preferenčni vir amoniaka (organski in anorganski) za prvo stopnjo nitrifikacije v tleh z nevtralnim pH v primerjavi s kislimi tlemi
- določiti vpliv vira amoniaka (anorganski/organski) na hitrost nitrifikacije v kislih in nevtralnih šotnih tleh
- določiti vpliv organskega in anorganskega vira amoniaka na številčnost in strukturo AOA in AOB v kislih šotnih tleh in tleh z nevtralnim pH

Pri poskusu smo uporabili dva različna tipa tal: kisla šotna tla nizkega barja (referenčna tla), imenovana Bog; ter nevtralno karbonatno polžarico, imenovano Bevke, v katerih smo predhodno zaznali tako AOA in AOB predstavnike. Tla smo presejali skozi 4 mm velika sita in jih zatehtali v stekleničke. Tako smo ustvarili laboratorijske sisteme (mikrokozme), ki smo jim nato ob začetku poskusa dodali mineralni ali organski vir amoniaka (150 µg N g^{-1} suhih tal). Za organski vir amoniaka je bil uporabljen glutamat, za anorganski vir pa amonijev sulfat. V kontrolne mikrokozme smo dodali deionizirano vodo. Mikrokozme smo inkubirali 30 dni pri 28 °C. Destruktivno vzorčenje je potekalo ob dnevih 0, 4, 7, 10, 20 in 30, kjer smo del tal iz mikrokozmov porabili za spektrofotometrično določanje sprememb v koncentraciji NH_4^+-N in (NO_2^-+NO_3^-)-N med inkubacijo tal, del tal pa za izolacijo DNA. Izolirano DNA smo uporabili za kvantitativno določevanje števila AOA in AOB *amoA* genov (z metodo qPCR), ter za določanje sprememb v strukturi AOA in AOB združb (z metodo DGGE).

Poleg zgoraj navedenega poskusa z enkratno bremenitvijo smo izvedli tudi poskus z večkratnim dodajanjem glutamata in amonijevega sulfata mikrokozmom. Tla smo pri tem poskusu inkubirali 24 dni pod enakimi pogoji kot pri prvem poskusu, destruktivno vzorčenje pa je tokrat potekalo ob dnevih 0, 3, 6, 9, 12, 15, 18, 21 in 24. Ob dnevih 0, 4, 6, 11, 16, 18 in 21 smo dodali 100 N g^{-1} suhih tal. Ob destruktivnem vzorčenju mikrokozmov smo tla uporabili za spektrofotometrično določanje sprememb v koncentraciji NH_4^+-N in (NO_2^-+NO_3^-)-N in za izolacijo DNA za enake analize, kot smo jih izvedli pri enkratnem bremenjenju mikrokozmov z dušikom (qPCR in DGGE) za AOA in AOB.

Domnevali smo, da v kislih šotnih tleh nizkega barja prevladujejo AOA in uporabljajo organski vir amoniaka kot preferenčni vir amoniaka. To bi se kazalo v hitrejši nitrifikaciji in rasti AOA, ter v strukturnih spremembah AOA združbe.

V poskusu z enkratnim dodajanjem vira amonijaka kislim tlem opazimo upad mineralnega amonija po štirih dneh v mikrokozmih z dodanim amonijevim sulfatom in nato ponoven porast do končne koncentracije 130 µg NH_4^+-N g^{-1} suhih tal.

Drugačna kinetika je bila opažena pri mikrokozmih z dodanim glutamatom, kjer se je koncentracija NH_4^+-N izenačila s tisto v kontrolnih mikrokozmih v sedmih dneh.

O nitrifikacijski kinetiki smo sklepali iz podatkov o koncentraciji (NO_2^-+NO_3^-)-N v mikrokozmih. Levičnik-Höfferle in sod. (2012) so v svojem poskusu opazili hitrejšo nitrifikacijo v mikrokozmih z dodanim organskim virom amoniaka. V mikrokozmih z dodanim anorganskim virom amoniaka pa ni prišlo do vidnih sprememb v nitrifikacijski kinetiki v primerjavi s kontrolnimi mikrokozmi. Do podobnih zaključkov smo prišli tudi v našem poskusu z dodanim organskim virom amoniaka, a smo v nasprotju s prejšnjimi raziskavami (Levičnik-Höfferle in sod., 2012) opazili, da je v mikrokozmih z dodanim amonijevim sulfatom nitrifikacija hitrejša kot v kontrolnih mikrokozmih.

Dobljeni rezultat je lahko posledica spremembe v združbi nitrifikatorjev, kot navaja tudi Levičnik-Höfferle in sod. (2012). Poleg tega so Stroo in sod. (1986) našli heterotrofne glive v kislih tleh, ki so bile sposobne nitrifikacije, zato bi lahko bile tudi te odgovorne za nitrifikacijo anorganskega vira amoniaka v kislih šotnih tleh. Poleg tega se tla lahko v času spreminjajo in bi se lahko tla obogatila z različno skupino AOA, ki preferenčno izrabljajo

mineralni vir amonijaka tudi v kislih tleh. Vseeno pa je zanimivo da dodajanje amonijevega sulfata pripeljalo do kopičenja amoniaka v tleh mikrokozmov, kar kaže na to, da se le majhen delež anorganskega vira amoniaka nitrificira. Preostanek se lahko tesno veže na organsko snov v tleh in zatorej postane nedostopen nitrifikatorjem.

V kislih šotnih tleh nizkega barja so prevladovale AOA, AOB pa so bile pod mejo detekcije, zato lahko sklepamo, da AOA vodijo nitrifikacijo v teh tleh. To je v skladu s prejšnjimi raziskavami, kjer so bile AOB tudi pod mejo detekcije (Stopnišek in sod. 2010; Levičnik-Höfferle in sod. 2012). Med inkubacijo mikrokozmov je prišlo tudi manjših sprememb v sestavi AOA in AOB, a teh ne moremo pripisati vplivu različnega vira amoniaka, saj se spremembe dogajajo skozi inkubacijo.

Na osnovi predhodnih študij (Levičnik-Höfferle, neobjavljeno), s katerimi so pokazali prisotnost AOA in AOB v nevtralni karbonatni polžarici, smo predvideli vpliv organske ali anorganske oblike vira amonija na stopnjo nitrifikacije. Predpostavljali smo tudi, da bo imel organski vir amonijaka večji učinek na rast in strukturo združbe AOA, združba AOB pa naj bi se bolj odzvala na spremembo anorganskega vira dušika.

Iz pregleda rezultatov lahko sklepamo, da med poskusoma ni prišlo do prednostne porabe enega izmed virov amonija, saj se stopnje nitrifikacije v nevtralni prsti glede na organski ali anorganski vir amonija, ne razlikujejo bistveno.

V mikrokozmih z organskim in anorganskim virom amonijaka pride po 18. dnevu do kar desetkratnega porasta stopnje nitrifikacije (2000 µg $(NO_2^-+NO_3^-)$-N g^{-1} suhih tal). Zaradi zaporednega dodajanja vira amonija je prišlo do akumulacije 700 µg NH_4^+-N g^{-1} suhih tal, ostanek pa se je nakopičil najverjetneje kot rezultat mineralizacije. Čeprav so v predhodnih študijah dobili primerljive končne koncentracije $(NO_2^-+NO_3^-)$-N tudi v drugačnih tleh (Webster in sod., 2005), razlaga pojava še vedno ni znana. Zaznaven vpliv na stopnjo mineralizacije bi lahko imel zaporedno dodan vir dušika, vendar gre zagotovo za zanimivo a še neodgovorjeno vprašanje, ki ga velja raziskati v prihodnosti.

V prvem poskusu, kjer smo talnim mikrokozmom le enkrat dodali dodatke amonijskega dušika, nismo opazili nikakršnih sprememb v številu *amoA* genov ne pri AOA, niti pri AOB. Zato smo sklepali, da morda nismo dodali dovolj vira amonijskega dušika, da bi

opazili rast združb. Posledično smo nato izvedli drugi poskus, kjer smo vir amoniaka mikrokozmom dodali večkrat (sedem-krat).

Po večkratnem dodajanju virov amonijskega dušika zopet ni prišlo do statistično značilnih sprememb ne pri AOA, niti pri AOB *amoA* številu genov. Vseeno pa se opazi trend naraščanja števila genov. Tako pri AOA *amoA* število genov narašča tako pri mikrokozmih z dodanim amonijevim sulfatom, kot pri tistih z dodanim glutamatom. To sicer kaže na to, da vir amoniaka ne vpliva na AOA združbo. Trend višanja števila *amoA* genov se vidi tudi pri AOB, a je to najbolj opazno pri mikrokozmih z dodanim organskim virom amoniaka. To je sicer v nasprotju z dosedanjimi raziskavami (Jiang in sod., 1999), kjer so predlagali, da AOB niso zmožne nitrificirati organskega vira amoniaka. Vseeno pa je velika verjetnost, da so v nevtralnih tleh prisotni mikroorganizmi, ki mineralizirajo organski vir amoniaka in ga tako naredijo dostopnega AOB.

V poskusu s ponavljajočim dodajanjem amoniaka nismo opazili strukturnih sprememb v AOA in AOB združbah.

Ker pri poskusu nismo zaznali nobenih statistično značilnih sprememb v spremembi števila genov, sklepamo, da je bil čas inkubacije prekratek, saj je znano, da AOA in AOB rastejo zelo počasi (Stopnišek in sod., 2010). Zato bi bilo v nadaljnje smiselno ta čas podaljšati in upoštevati trende, ki smo jih opazili pri spreminjanju števila *amoA* genov. Pri poskusu smo sicer opazili pospešeno nitrifikacijsko kinetiko, kar skupaj z ostalimi rezultati kaže na to, da se je AOA in AOB povečala nitrifikacijska aktivnost, kljub temu, da njihovo število ostaja nespremenjeno.

V okviru te magistrske naloge smo prišli do sledečih sklepov:

V kislih šotnih tleh (Bog):

- Organski vir amoniaka povzroči hitrejšo nitrifikacijo
    - Anorganski vir amoniaka se prav tako nitrificira, a počasneje kot organski vir amoniaka
- AOA vodijo nitrifikacijo, kjer je organski vir amoniaka preferenčni
- Organski in anorganski vir amoniaka ne vplivata na strukturo AOA združbe

V nevtralni karbonatni polžarici (Bevke):

- Tako organski kot anorganski vir amoniaka spodbudita podobno nitrifikacijsko kinetiko
- Večkratno dodajanje organskega vira amoniaka spodbudi nitrifikacijsko kinetiko
- Dodajanje organskega ali anorganskega vira amoniaka ne vpliva na spremembo AOA ali AOB

Za bolj jasne in zanesljive podatke bi bilo potrebno poskus ponoviti in dodati mikrokozemski poskus z acetilenom, s katerim bi lahko ločili med avtotrofnimi in heterotrofnimi oksidatorji amoniaka. Prav tako bi bilo smiselno preveriti transkripcijsko aktivnost, saj le število AOA in AOB *amoA* genov ne zagotovi dovolj jasnih rezultatov. Tako ne vemo ali se številčnost združbe spreminja zaradi dodanega vira amoniaka ali zaradi katerih drugih dejavnikov. Priporočljivo bi bilo tudi nadomestiti amonijev sulfat z amonijevim kloridom, ki ne vpliva na pH mikrokozmov.

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