Impact of plant elicitors on ammonia oxidizing root and rhizosphere communities in winter wheat

Växt-elicitorers påverkan på ammoniak oxiderande mikrobsamhällen från höstvetes rötter och rhizosfär

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Master Thesis in Soil Science • (30 hp)
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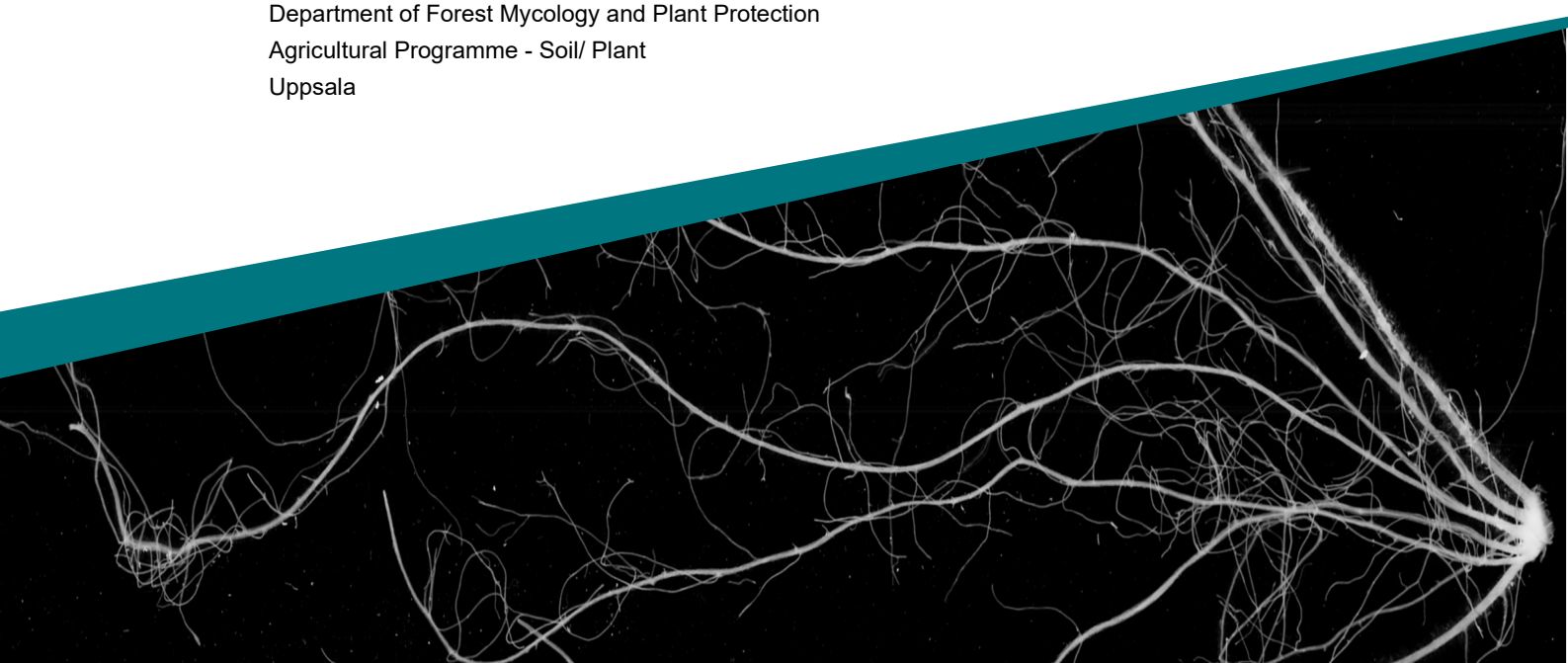

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Abstract

Nitrogen is one of the essential nutrients for plants. Nitrogen is taken up as ammonium (NH_4^+) or nitrate (NO_3^-) from roots. If not taken up by the plants, nitrate can be lost to the atmosphere by denitrification which contributes to the global warming. Nitrate can leach out in aquatic systems, this can cause eutrophication, thus it's important to save the nitrogen in soil. Nitrate can be added as fertilizer or produced by a microbial process called nitrification. The first step of nitrification is the oxidation of ammonia, done by ammonia oxidizing archaea, ammonia oxidizing bacteria and comammox (*Nitrospira*). This first step is also the rate limiting step of this process.

Plant elicitors such as cis-jasmone, methyl jasmonate and methyl salicylate are used in pest management but there are also indications that they trigger the production of secondary metabolites that can be found in root exudates and inhibit ammonia oxidizers. However, our knowledge on their effect on soil microbes remains limited.

The hypothesis was that treating winter wheat with elicitors will alter the soil ammonia-oxidizing communities without negatively affecting plant performance. The first aim was to determine the abundance, composition and activity of the ammonia oxidizing root and rhizosphere communities. The second aim was to measure plant traits both above- and below ground. The third aim was to assess effects of ammonium availability of the elicitor treated plants.

The elicitor treatments showed most effects in the fertilized pots. The elicitors had most significant effects on the ammonia-oxidizing root communities compared to the rhizosphere communities. Methyl jasmonate showed most significant effects and methyl salicylate showed second most significant effects of both decreased microbial abundance and changed community composition. In both cases the plant traits were positively affected except for the shoot biomass. However, it is possible that this effect is temporary and that a longer growth period would allow an increase in the shoot biomass. Cis-jasmone showed no significant effect on microbial abundance but a trend of changed composition. Cis-jasmone positively affected below-ground plant performance.

Methyl jasmonate seems to be the most promising elicitor and methyl salicylate is the second promising elicitor. Cis-jasmone is still interesting, showing positive effects on plant performance although limited effect on microbial abundance. Fertilizer as ammonium chloride increase the effects of the elicitor treatments limited to the roots microbial community. We should continue use cis-jasmone, methyl jasmonate and methyl salicylate to see how they can be used in practical agriculture and in different crops.

Keywords: ammonia oxidizing archaea, ammonia oxidizing bacteria, comammox, elicitors, ammonia oxidation inhibitors, cis-jasmone, methyl jasmonate, methyl salicylate

Sammanfattning

Kväve är ett essentiellt näringsämne för växter. Kväve tas upp som ammonium (NH_4^+) eller nitrat (NO_3^-) genom växtrötterna. Nitrat är lättrörligt i jorden, om nitrat inte utnyttjas av växter kan det försvinna till atmosfären genom denitrifikationen, detta bidrar till den globala uppvärmningen. Nitrat kan också läcka ut till vattendrag och orsakar övergödning, det är därför viktigt att spara kvävet i marken. Nitrat kan tillföras genom gödsel eller bildas genom mikrobiella processer i marken via nitrifikationen. Ammoniakoxideringen är det första steget i nitrifikationen vilket bestämmer hasigheten av nitrifikationen. Ammoniak oxideras av ammoniak oxiderande arkéer, ammoniak oxiderande bakterier eller comammox (*Nitrospira*). Växt-elicitorer som cis-jasmonsyra, metyljasmonsyra och metylsalicylsyra inducerar försvarsmekanismer mot herbivorer och patogener. Det finns indikationer om att elicitorer kan också trigga syntes av sekundära metaboliter i rotexudatet som inhiberar ammoniakoxiderande mikrober i jorden. Kunskapen om effekten på jordmikrober är dock minimal.

Hypotesen var att elicitorer påverkar det ammoniakoxiderande mikrobsamhället som är kopplat till höstvete, utan negativ påverkan på växtens tillväxt. Det första syftet var att mäta rot- och rhizosfärsamhällets storlek, komposition och aktivitet. Det andra syftet var att mäta växtens ovan- och underjordiska delar för att undersöka växtens tillväxt av behandlingarna. Det tredje syftet var att bedöma eventuella effekter från ammoniumgödsling på elicitorbehandlade plantor.

Elicitorbehandlingarna visade mest signifikant effekt i de gödslade leden. Rotmikrobsamhället visade fler signifikanta effekter jämfört med rhizosfärmikrobsamhället.

Metyljasmonsyra visade flest signifikanta effekter och därefter metylsalicylsyra. Dessa reducerade storleken på rotmikrobsamhället, förändrade samhällets komposition, samt hade positiv påverkan på växtens tillväxt förutom skottbiomassa. Det är dock möjligt att denna effekten är temporär och efter en längre tillväxtperiod ökar skottbiomassan. Cis-jasmonsyra visade inga signifikant skillnader på mikrobsamhällets storlek, men en trend på förändrad komposition. Cis-jasmonsyra visade ändå positiv påverkan på rottillväxten.

Metyljasmonsyra är den mest lovande elicitoren och metylsalicylsyra den näst lovande elicitoren. Cis-jasmonsyra är fortfarande intressant då den visade ha positiv inverkan på växtens tillväxt men begränsad påverkan på mikrobsamhällets storlek. Gödsel som ammoniumklorid visade sig ge en förstärkt effekt på elicitorbehnadlingarna, begränsad till rotmikrobsamhället. Vi bör fortsätta använda cis-jasmonsyra, metyljasmonsyra och metylsalicylsyra för att se hur de kan användas i praktiskt jordbruk och vilken effekt de har i oliak grödor.

Popular science summary

Feed an increasing global population and at the same time reach a sustainable development of agriculture without overconsume our global resources. This is some of our future's global challenges. The growing crops in the fields need water and nutrients to harvest high yields. Nitrogen is one of the most important nutrients for plant growth and is provided to the crop as fertilizer. However, nitrogen can easily disappear from the soil if it is not taken up by plants. Globally, 50% of added fertilizer is lost and this causes environmental problems as the eutrophication of lakes and seas. But nutrients as nitrogen are also produced from a microbial process in soils called *nitrification*. The nitrification process nitrogen that is unavailable for the plant to take up (ammonia) to nitrogen that is available to take up (nitrate). Nitrification consist of two parts, the first is the ammonia oxidation coursing by ammonia oxidizers, this step is limiting the rate of nitrification. The next step is the nitrite oxidation. This master thesis has investigated one potential solution to improve the N use efficiency and decrease the nitrogen leaching out by treating wheat with *elicitors.* Elicitors are a group on non-related compounds that trigger defense responses against herbivory and pathogens in plants. There is also indication that they can trigger the plant to induce secondary metabolites in root exudate. This affect the nitrifying microbes in soil which can stop the first part of nitrification. Threating the plant with elicitors can there by both induce a plant defense and at the same time improve the nutrient use efficacy. This is a win win situation for both a sustainable plant protection and nutrient use efficiency for crops. There is more knowledge about how elicitors can be used as plant defense, and less about how elicitors affect soil microbes, thus this thesis is done.

In this thesis winter wheat was treated with three different elicitors, cis-jasmone, methyl jasmonate and methyl salicylate. The thesis aimed to determine the effects from the elicitors on ammonia oxidizing communities on wheat roots and the environment closest the roots - rhizosphere. We measured the abundance and composition of the ammonia oxidizers with molecular methods. Several plant traits were measured to evaluate the performance of the wheat treated with elicitors. Half of all treatment were fertilized with ammonium nitrogen because earlier studies have shown that the fertilizer triggered the elicitors to induce secondary metabolites in root exudate.

The results showed that the fertilized elicitor treatment showed most significant effects, most of the root community. The elicitors methyl jasmonate and methyl salicylate had the most promising results of affecting the ammonia oxidizers without negatively affecting plant performance. Cis-jasmone showed fewer effects on the microbial communities but seems to have positive effects on the development of roots. We need to expand the knowledge of how the elicitors can be used in field conditions and in other

crops to use it in practical agriculture in future, thus it is still interesting to continue use all of the three elicitors.

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Abbreviations

AMO	Ammonia monooxygenase
AOA	Ammonia-oxidizing Archaea
AOB	Ammonia-oxidizing Bacteria
BNI	Biological nitrification inhibitor
cJ	Cis-jasmone
comammox	Complete ammonia oxidizers
HAO	Hydroxylamine oxidoreductase
MJ	Methyl jasmonate
MS	Methyl salicylate
PCR	Polymerase chain reaction
qPCR	Quantitative real-time polymerase chain reaction

1. Introduction

One of the main challenges for the next decades is to feed an increasing human population without over consuming the global resources. To this end, the United Nations have established a list of 17 Sustainable Development Goals (The Global Goals 2021). One of them is "Zero Hunger". To reach this goal there is a need to increase the global food production. The harvest of high yielding crops requires sun radiation, space, water, and nutrients. Nitrogen (N) is one of the essential nutrients to grow a healthy crop, and an important component in the chlorophyll molecule for photosynthesis. This is one reason why it is important to ensure that crops have access to N to achieve high yields.

Plants obtain nutrients from the soil and N is typically absorbed as an ion, in the form of ammonium or nitrate. Contrary to ammonium (NH_4^+), nitrate (NO_3^-) is negatively charged and does not bound to negatively charged surfaces in the soil. If not assimilated by plants or microbes, nitrate can thus leach out from the soil and cause eutrophication in aquatic systems. "Life below water" is one global goal identified by the UN (The Global Goals 2021) which is affected by eutrophication and there is thus an urgent need to minimize NO_3^- pollution in aquatic ecosystems. Nitrate can also be lost by a microbial process called denitrification in which NO_3^- is sequentially reduced to nitrous oxide (N_2O) under anoxic conditions (Schlesinger 2020). This gas contributes to global warming and to the depletion of the ozone layer in the atmosphere (Hu et al. 2017; Subbarao et al. 2015). Globally, ca. 50 % of the added fertilizer is lost by leaching or denitrification (Norton et al. 2019).

Besides fertilizer input, the other major source of nitrate in soil is nitrification. In this microbially-mediated process, ammonia is first oxidized to nitrite, which is in turn oxidized to nitrate. The first and rate-limiting step is catalysed by diverse microbes found within Bacteria and Archaea. Reducing nitrification rates by targeting soil ammonia oxidizers thus represent a promising way to increase the sustainability of agriculture.

Researchers have in recent years found that substances called elicitors can be used in pest management against herbivory and pathogens (Maffei et al. 2012; Kraus et al. 2019). They can also affect plant-associated root and rhizosphere microbial communities (Huang et al. 2014). Indeed, elicitors induce the production of secondary metabolites in plants, some of which can inhibit ammonia oxidizers (Neal et al. 2012; Schreiner et al. 2011; Zakir et al. 2008). It is thus interesting to

investigate the potential of elicitors in economically important crops and determine whether they could be used to control the ammonia-oxidizing communities. Reduced nitrate production would lead to lower N losses through leaching or denitrification and could thus significantly improve the nitrogen use efficiency (i.e. the harvested product per unit of N fertilizer applied) of agricultural systems (Subbarao et al. 2015).

The hypothesis of the thesis is that plant-elicitors will alter the ammonia-oxidizing microbial communities associated to winter wheat without negatively affecting plant performance.

The first aim of the thesis was to determine the abundance, composition and activity of the ammonia oxidizers in root and rhizosphere communities of plants treated with different elicitors. We define the root communities as the microbes physically attached in and on the roots. The rhizosphere is defined as the zone of soil under the influence of the roots (Philippot et al. 2013). The second aim was to measure plant traits, defined as morphological, physiological or phenological features in one individual plant (Violle et al. 2007). The plant traits will be measure both above- and below-ground to evaluate how changes in the microbial communities affected plant performance. The third aim was to assess the effect of ammonium availability, since earlier work has shown that the release of nitrification inhibitors is regulated by root exposition to ammonium (Subbarao et al. 2013; Zakir et al. 2008).

2. Background

2.1. Ammonia oxidation in arable soils

Ammonia oxidation is the first step of nitrification, an important process in the global nitrogen cycle (Kuypers et al. 2018). Ammonia is oxidized to nitrite by ammonia oxidizing archaea (AOA) and bacteria (AOB) (Schleper et al. 2010). Microbes belonging to the bacterial genus *Nitrosospira* oxidize ammonia as well but are also able to perform the second step of nitrification, the oxidation of nitrite to nitrate. They are called complete ammonia oxidizers (Comammox) (Hu et al. 2017).

The supply of ammonium is the main factor limiting the ammonia oxidation step (Paul 2015), which in turn limits the rate of nitrification (Schleper et al. 2010; Subbarao et al. 2015). The equilibrium between ammonia and ammonium is depending on pH. Under acidic conditions the equilibrium is driven towards ammonium whereas under alkaline conditions there is more ammonia. Soil pH, and thereby the affinity to ammonia, is a major selecting factor for which group of ammonia oxidizers will predominate (Lehtovirta-Morley 2018).

Comammox, AOA and AOB are all chemolithotrophs; they fix inorganic carbon into biomass and gain energy through the oxidation of ammonia to nitrite under oxic conditions (Nardi et al. 2020; Paul 2015).

2.1.1. Ammonia-oxidizing archaea (AOA)

All known AOA belong to the phylum Thaumarchaeota within the class Nitrososphaeria (Schleper et al. 2010; Paul 2015). They use the enzyme ammonia monooxygenase (AMO) to catalyse the oxidation of ammonia to hydroxylamine (figure 1). The three subunits of the AMO are encoded by the genes a*moA*, *amoB* and *amoC* (Martens et al. 2011). The enzyme responsible for the oxidation of hydroxylamine (NH_2OH) to nitrite (NO_2^-) is not known in archaea. AOA produce N_2O as a by-product of ammonia oxidation (figure 1).

AOA are found in soils, marine waters and sediments (Schleper et al. 2010). Approximately 1-3% of all microbes in soils are AOA (Martens-Habbena et al. 2011) and their abundance is affected by environmental conditions (Coskun et al. 2017). AOA prefer soils which are rich in organic matter and have a low pH (He et al. 2012). The oxygen concentration can be low and AOA tend to prefer oligotrophic environments, which are poor in nutrients (Schleper et al. 2010) with a low amount of ammonia (Lehtovirta-Morley, 2018). Studies have shown that AOA are found in the whole soil horizon compared to AOB which generally prefer the top of the soil horizon (Schleper et al. 2010).

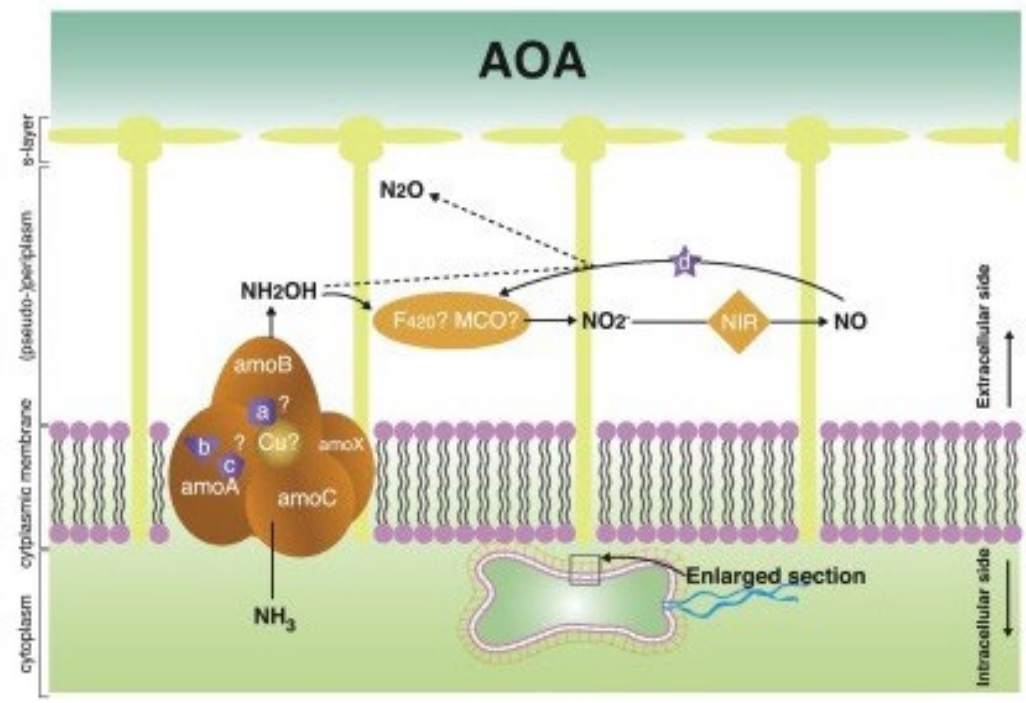

Figure 1: Ammonia oxidation pathway in ammonia-oxidizing archaea. Ammonia is first oxidized to hydroxylamine by the enzyme AMO, nitrous oxide is produced. Hydroxylamine is then oxidized to nitrite by an unknown enzyme. Nitrite is oxidized to nitric oxide by the enzyme NIR (nitrite reductase) (Beeckman et al. 2018).

2.1.2. Ammonia-oxidizing bacteria (AOB)

AOB are found in the subclasses Beta- and Gamma-proteobacteria (Coskun et al. 2017), including the beta-proteobacterial genera *Nitrosomonas* and *Nitrosospira,* the gamma-proteobacterial genus *Nitrosococcus* (Prosser et al. 2020; Soliman et al. 2018).

AOB carry the genes *amoA, amoB* and *amoC* which code for the different subunits of the enzyme AMO. Hydroxylamine is then oxidized to nitrite by the enzyme hydroxylamine oxidoreductase – HAO (Koops et al. 2005) (figure 2). As for AOA, N_2O is also produced during bacterial ammonia oxidation (Hink et al. 2018)

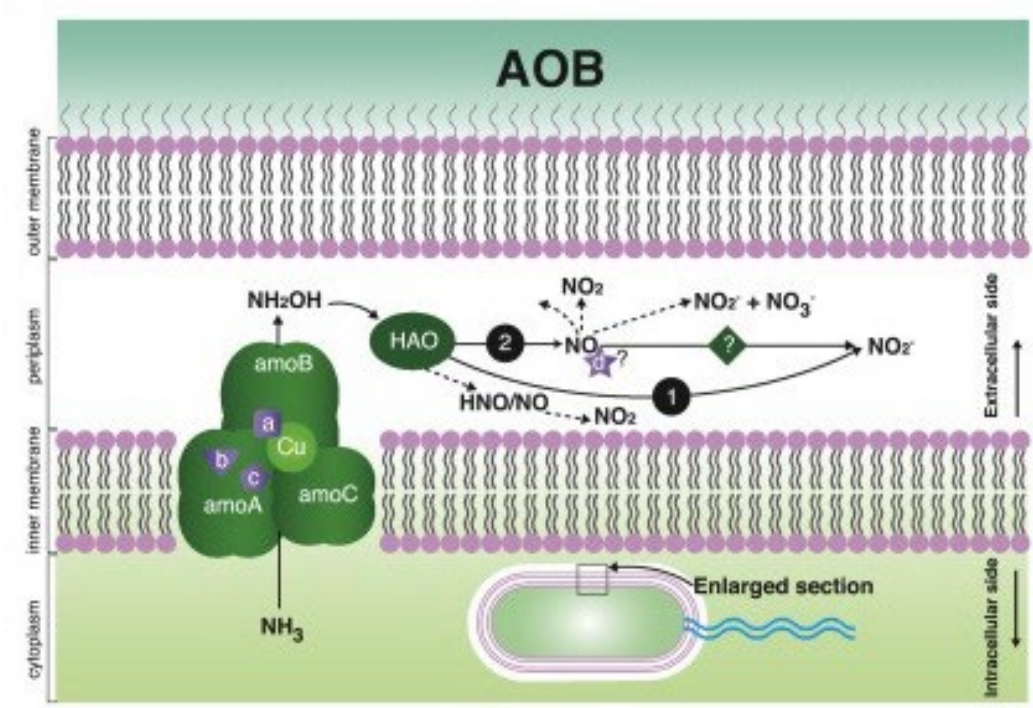

Figure 2: Ammonia oxidation pathway of ammonia-oxidizing bacteria. Ammonia is first oxidized to hydroxylamine by the enzyme AMO. Hydroxylamine is then oxidized to nitrite by the enzyme HAO. Nitrogen dioxide is produced (Beeckman et al. 2018). Nitrous oxide is produced during the ammonia oxidation but not showed in the figure (Hink et al. 2018).

AOB are found in various environments including soil, marine and freshwater systems. The Beta- and Gamma-proteobacteria have different environmental preferences. *Nitrosospira* typically dominate the AOB communities in soils (Prosser et al. 2008). AOB generally prefer soils rich in ammonia and are mostly found in alkaline environments. However, there are also exceptions and some AOB have recently been found in acid soils (Lehtovirta-Morley 2018). AOB are usually found in the top of the soil horizon (Schleper et al. 2010).

2.1.3. Complete ammonia oxidizers (Comammox)

Bacteria belonging to the genus *Nitrospira* are able to oxidize ammonia to nitrate in a single cell (Daims et al. 2015). *Nitrospira* encode *amoA*, *amoB*, *amoC* genes to synthesize the enzyme AMO which catalyse the oxidation of ammonia to hydroxylamine. The enzyme HAO is synthesised as for AOB to catalyse the oxidation of hydroxylamine to nitrite (figure 3). It is not yet determined whether comammox produce N_2O.

Comammox are common in agricultural soils, freshwater systems, wastewater treatment plants and drinking water systems (Hu et al. 2017). So far, Comammox have been mainly detected in low N soils (Lehtovirta-Morley 2018).

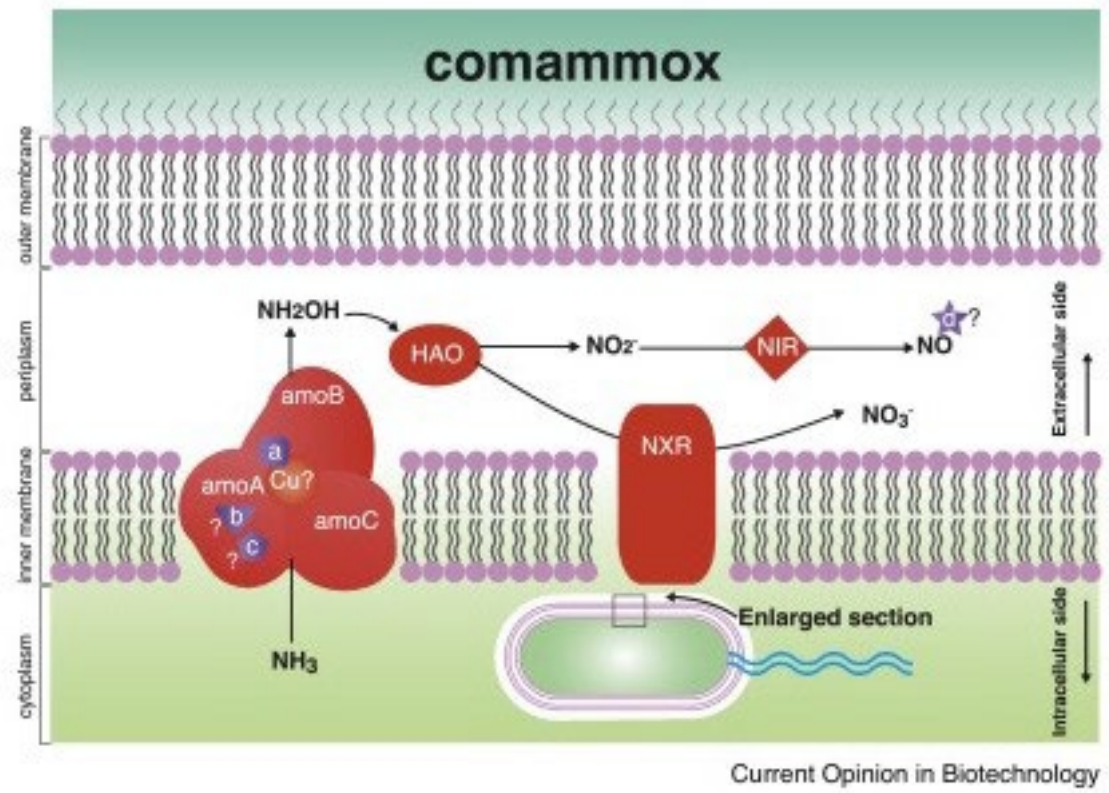

Figure 3: Ammonia oxidation pathway of Comammox Nitrospira. Ammonia is oxidized to hydroxylamine by the enzyme AMO. Hydroxylamine is oxidized by the enzyme HAO and NXR (nitrite oxidoreductase) to nitrate. Hydroxylamine can be oxidized to nitrite by the enzyme HAO (Beeckman et al. 2018)

2.2. Biological inhibition of ammonia oxidation

Some plants have the natural ability to produce and exude secondary compounds that can affect plant-associated nitrifiers. These are called biological nitrification inhibitors (BNI) and are found mostly in tropical forage grasses and crops

(Subbarao et al. 2013). The exudation of BNI is stimulated by the presence of ammonium in the root zone. Ammonium acidifies the root zone, which affect the permeability of roots. A higher root permeability stimulate the exudation of BNI (Zakir et al. 2008).

There is also the possibility to manipulate plants that do not produce BNI naturally by treating them with elicitors. The term elicitors cover a diverse group of compounds which act as signal molecules in plants. They are typically used to induce the production of secondary metabolites as a defence mechanism against pathogens and herbivores (Maffei et al. 2012; Kraus et al. 2019). Elicitors can be applied directly on the seeds or on the leaves (foliar treatment). It is known that secondary metabolites induced by elicitors are released in root exudates (Bi et al. 2007). These secondary metabolites are also known to affect the root and rhizosphere microbial communities (Huang et al. 2014). However, there are few studies connecting these two pieces together – the elicitors treatments and how they affect soil microbial communities. There is far less research on the effects of elicitors on plant-associated microbial communities compared to their benefits for plant protection against pests, and therefore it is important to understand how elicitors can affect microbial communities. In this thesis, we used the three following elicitors.

Methyl Salicylate

Methyl salicylate (MS) is a methyl ester of salicylic acid. The MS is volatile (Kalaivani et al. 2016). Phenyl propanoids compounds have been found to be naturally exudated from roots of *Sorghum bicolor* (tropical cereal crop) and *Brachiaria humidicola* (tropical forage grass) (Subbarao et al. 2013). Studies on *Sorghum* have further shown that these phenyl propanoids have an inhibitory effect on *Nitrosomonas* (Zakir et al. 2008).

Methyl Jasmonate

Methyl jasmonate (MJ) is derived from jasmone. It regulates the synthesis of glucosinolates, which are naturally found in *Brassicales*. Glucosinolates can be found in root exudates. They are involved in plant defence against pathogens and herbivores (Schreiner et al. 2011) and can inhibit nitrifying microbes in soils (Subbarao et al. 2013). For example, a study from Bending et. al (2000) showed that degrading *Brassicales* tissues release glucosinolates, which can inhibit ammonia oxidizers in soil. MJ has the ability to regulate the synthesis of sorgoleone in root exudates from *Sorghum* roots (Uddin et al. 2013). This compound has strong effects on *Nitrosomonas* (Subbarao et al. 2013).

Cis Jasmone

Cis-jasmone (cJ) has been used to confer resistance against sucking insect pests, particularly aphids. It regulates the benzoxazinoid production pathway (Moraes et al. 2008). Cereals can release benzoxazinoids from roots, where they act against microbes and insects (Neal et al. 2012). Benzoxazinoids degrade fast in aquatic environments and the degraded compounds have antimicrobial effects as well (Neal et al. 2012). A study of Moraes et al. (2008) showed results of an increased amount of benzoxazinoids in roots of plants treated with cJ. They suggest that the production or the storage of benzoxazinoids could be in the roots.

3. Material and methods

3.1. Experimental design and measurement of plant traits

Parts of the experiment were performed before the start of this master thesis.
The soil for the experiment was collected in August 2020 at SLU Lövsta research station. The soil type was high clay with a 3.3% content of organic carbon. The soil was sieved at 4 mm and then stored in a freezer at -20 ℃.

The elicitors were chosen based on earlier published knowledge of their potential to modify plant root exudate, non-toxicity and reasonable cost. Winter wheat seeds (*Triticum aestivum* cv. Julius) were used in this experiment. The seeds were first surface sterilized by soaking in ethanol (70%, 3 minutes) and sodium hydroxide (1%, 3 minutes). The seeds were rinsed with sterilized water afterwards. Elicitor solutions were prepared by mixing Tween20 10% (1% v/v) (Bio-Rad, Hercules, CA, USA) and each elicitor at different concentrations following the procedure described in (Kraus & Stout 2019a). For each elicitor treatment, 100 seeds were placed in gas tight glass flasks and incubated in aqueous solution of either cJ (1 and 5 mM; Tokyo Chemical Industry, Tokyo, Japan), MJ (1 and 5 mM; Merck KGaA, Darmstadt, Germany) and MS (1 and 10 mM; Merck KGaA) for 24 h at 25℃ on a shaker (125 rotations per minute).

After the 24-hour incubation period 10 seeds were sown in each pot. Each pot contained 1.6 kg soil and 2 cm of autoclaved clay pellets, in total 64 pots. A maximum of 4 plants in each pot were allowed to grow. There were two control treatments used in the experiment. The control treatments contained seeds incubated in either water or Tween20 10% (1% v/v) to see if the Tween20 (10%) had any effect on the seeds. Half of all the pots was fertilized with an equivalent of 100 kg ha^{-1} of ammonium chloride adjusted to the pH of the soil (pH = 6.8) to determine the effect of ammonium availability on plants treated with elicitors. Every treatment had four replicates and was randomized in 4 blocks placed in a climate chamber. The moisture was maintained at 60 % water holding capacity by weighing the pots and adding sterile water when necessary.

The winter wheat was harvested 23 days after germination when the plants were at tillering stage. Root and rhizosphere samples were collected. The roots from two individuals in each pot were collected in a falcon tube that contained phosphate buffer saline (137 mM NaCl, 2,7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4). The rhizosphere soil was separated from the roots by vortexing at maximum speed until the roots became white. Then the samples were centrifuged at 5000 g for 5 min. Pellets, corresponding to the rhizosphere samples, and cleaned roots were stored at -20 °C.

The shoots of the corresponding roots were used to measure plant traits to evaluate the effect of the elicitor treatments (Pérez-Harguindeguy et al. 2016). The following traits are related to the N status or N uptake of the plants. Leaf greenness for each plant were obtained by averaging two measurements on four different leaves (i.e. n = 8 per plant) using a SPAD-502 meter (Konica Minolta Sensing, Tokyo, Japan). Greenness is an indirect measurement of the chlorophyll content in leaves. Leaf area was estimated by analyzing leaf pictures (ImageJ; (Schneider et al. 2012) taken with a Canon EOS 100D camera (Canon Inc., Tokyo, Japan). Shoot dry weight was obtained after oven-drying at 65 °C for 4 days. N content was measured by dried plant material, cut to small pieces determined by 2520 Digestor, Kjeltec 8400 Analyzer unit and 8460 sampler unit (FOSS Analytical A/S Hilleröd, Denmark) (Nordic committee on Food Analysis, 1976). Specific leaf area (SLA) was calculated for each plant as the ratio of total leaf area to total leaf dry weight. The SLA represent the N concentration in leaves (Pérez-Harguindeguy et al. 2016).

3.2. DNA extraction and quantitative measurement

DNA from the rhizosphere and root samples were extracted using the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) with 0.24-0.30 g and 0.35-0.40 g, respectively, according to the manufacturer's instructions. Each sample had two independent extractions which were pooled before further processing.

Next step was to measure the abundance of root and rhizosphere communities of AOA, AOB and Comammox. The abundance of root communities was determined before. The thesis started by measuring the abundance of ammonia-oxidizers in rhizosphere communities. Inhibition tests of the polymerase chain reaction (PCR) reactions were done to test if the amplification of a known amount of plasmid is comparable between qPCR reactions done with and without DNA template. A known amount of the plasmid pGEM-T (Promega, Madison, WI, USA) were amplified. The specific primer M13F/M13R was used and added to both DNA extracts or non-template controls. No inhibition was detected with the used amount of DNA.

The abundance of the root and rhizosphere communities of AOA, AOB and Comammox were determined by quantitative real time polymerase chain reaction

(qPCR) in two independent runs. 16S rRNA gene was used as a measurement of the total bacterial and archaeal community in the root communities. See table 1 for reagents used in the qPCR reactions. The same reagents were used for both root and rhizosphere samples. The qPCR machine CFX Connect Real-Time System (Bio-Rad) was used using SYBR Green as the fluorescence detector. The fragments were amplified using thermal protocol (see table 2). Fluorescence was collected at 78 ◦C. For the melting curves, the following conditions were used: 95◦C for 10s followed by 65 ◦C – 95◦C 5s increment 0.5◦C. The melting curve was used as a quality control to verify that only fragments of the correct size were amplified. When DNA denatures (melts) the fluorescence signal decreases because SYBR Green only binds to double stranded DNA. The temperature is depending on the length and nucleotide composition of the fragments. If there is any non-specific amplification, a signal will be shown with peaks of another temperature than that of the target amplicon (Bio-Rad 2006). Standard curves were obtained by serial dilutions of linearized plasmids with cloned fragments of the specific genes. The amplifications were validated by melting curve analyses and agarose gel electrophoreses.

During a qPCR, the amplified product is detected and measured during the amplification. The SYBR Green dye which is used in the PCR reaction is fluorescent. The dye is binding to the double stranded DNA and the machine is measuring the fluorescence. The measured fluorescence is a measure of the amount of DNA in the amplicon in each thermal cycle. The more DNA in the sample the stronger the fluorescent signal (Bio-Rad 2006).

Table 1: Reagents used to make the qPCR reactions for both root and rhizosphere communities. Information on primer pairs is indicated in Table 2.

No. reagents (Inhibition test)	No Template Volume 1X (µl)	Inhibition test 1X (µl)	No. reagents (*16S rRNA gene)* (qPCR reaction)	Volume 1X (*16S rRNA* gene)(µl)	No. reagents (*amoA gene)* (qPCR reaction)	Volume 1X (*amoA* gene)(µl)
ddWater	6.25	2.05	ddWater	1.75	ddWater	2.25
Mastermix (2x)	7.5	7.5	Mastermix (2x)	7.5	iQ™ SYBR Green Super mix	7.5
M13 forward 15 (µM)	0.25	0.25	515 forward (15mM)	0.5	Primer forward (30 µM)	0.25
M13 r reverse 15 (µM)	0.25	0.25	926 reverse (15mM)	0.5	Primer reverse (30 µM)	0.25
Bovine Serum Albumin (20µg/ml)	0.75	0.75	Bovine Serum Albumin (20µg/ml)	0.75	Bovine Serum Albumin (20µg/ml)	0.75
Circ. Plasmid (10e 6 copies)	-	0.2	DNA extract (4 ng/rx)	4	DNA extract (4 ng/rx)	4
Total volume	15	15	Total volume	15	Total volume	15

Table 2:Primer names, sequence, target gene, expected fragment size and cycling conditions for the qPCRs in root and rhizosphere samples.

Primer names	Sequence	Gene	Fragment size	Thermal cycler protocol
CrenamoA23F CrenamoaA616R (Tourna et al. 2008)	ATG GTC TGG CTW AGA CG, GCC ATC CAT CTG TAT GTC CA	*amoA*	628	95°C 5 min)x1 (95°C 15s, 55°C 30s, 72°C 40s); 77°C 5s x40
AmoA1F AmoA2R (Rotthauwe et al. 1997)	GGG GTT TCT ACT GGT GGT, CCC CTC KGS AAA GCC TTC TTC	*amoA*	491	(95°C 5 min) x1; (95°C 15s; 55°C 30s; 72°C 40s; 80°C 5s) x40.
Ntsp- amoA 162F Ntsp-amoA 359R (Fowler et al. 2018)	GGATTTCTGGNTSGATTGGA, WAGTTNGACCACCASTACCA	*amoA*	198	(95°C 10 min)x1 (95°C 45s, 48°C 30s, 72°C 45s)x25; 72°C 7 min
515 F 926 R (Parade et al. 2016; Quince et al. 2011)	GTGYCAGCMGCCGCGGTAA CCGYCAATTYMTTTRAGTTT	*16S rRNA*	450	(95°C 5 min)x1 (95°C 15s, 50°C 30s, 72°C 30s, 78°C 5s)x35;

3.3. Amplification and sequencing of root-associated communities

Sequencing libraries were prepared for both archaeal and bacterial *amoA* genes using PCR. The amplifications were performed on a SimpliAmp Thermal Cycler (Thermo Fisher Scientific) using a two-step procedure. A duplicate run was made for the archaeal and bacterial *amoA* gene. See table 3 and 4 for the reagents, primer names, primer sequences and thermal cycler protocol. The duplicates of the PCR products were pooled and ran on an agarose gel electrophoresis to control that the fragments had the right size. The samples were purified by Sera-Mag beads (Merck KGaA, Darmstadt, Germany). In the second step, Illumina adapters with unique combinations for each sample were added for the sequencing. See table 3 for reagents and table 5 for cycling conditions. The PCR products were investigated by agarose gel electrophoresis. The second PCR products were purified by Sera-Mag bead (Merck KGaA, Darmstadt, Germany). The concentration of the samples was measured with a Qbit Fluorometer (Invitrogen, Carlsbad, CA, US). Then the samples were pooled in equimolar amounts and purified with the E.Z.N.A kit. A quality control was made using a BioAnalyzer (Agilent, Santa Clara, CA, US) to determine the DNA concentration and fragment size of the amplicons. The AOA and AOB gene *amoA* were sent to SciLife lab for sequencing. Sequencing was done on a 2 x 300 bp Illumina MiSeq platform.

Table 3: Reagents used in the PCR reactions.

Step 1	Volume 1x (µl)	Step 2	Volume 1x (µl)
Sterile water	6.75	Sterile water	3.5
2X Phusion	12.5	2X Phusion	15
Primer Forward (50 µM)	0.25	Primer tag Forward (0.2 µM)	3
Primer Reverse (50 µM)	0.25	Primer tag Reverse (0.2 µM)	3
Bovine Serum Albumin (20µg/ml)	1.25	Bovine Serum Albumin (20µg/ml)	1.5
DNA extract	4	Product from PCR 1	4
Total volume	25	Total volume	30

Table 4: Primer names, sequences, and cycling conditions for AOA and AOB.

Organism	**Primer names**	**Sequence**	**Gene**	**Fragment size**	**Thermal cycler protocol**
AOA	CrenamoA23 Front CrenamoaA616 Reverse (Tourna er al. 2008)	ATG GTC TGG CTW AGA CG, GCC ATC CAT CTG TAT GTC CA	*amoA*	629	(98°C 3 min)x1 (98°C 30s, 55°C 30s, 72°C 60s)x30; 72°C 5 min; 10°C hold.
AOB	AmoA1 Front AmoA2 Reverse (Rotthauwe et al. 1997)	GGG GTT TCT ACT GGT GGT, CCC CTC KGS AAA GCC TTC TTC	*amoA*	492	(98°C 3 min) x1; (98°C 30s; 55°C 30s; 72°C 45s) x30; 72°C 10 min;10°C hold.

Table 5: Cycling conditions used in PCR 2

Organism	Thermal cycler protocol
AOA	98°C 3 min; (98°C 30 sec, 55°C 30 sec, 72°C 40 sec) X 8; 72°C 5 min; 10°C hold.
AOB	98°C 3 min; (98°C 30 sec, 55°C 30 sec, 72°C 40 sec) X 8; 72°C 5 min; 10°C hold.

3.4. Potential Ammonia Oxidation Assay

The potential for nitrite production by ammonia oxidation in root-associated communities was measured. The ISO 15685 protocol micro-scale (see appendix) was used with some modifications because of the small amounts of roots and the small number of cells that was used in the assay compared to ordinary bulk soil.

Roots from extra plant pots were used in this test. Only roots were used because the qPCR data showed that root-associated communities were more affected than those in the rhizosphere. The roots were washed in phosphate saline buffer separating the rhizosphere soil from roots. The roots were added to 32 ml serum bottles mixed with test medium, for a total volume of 10 ml. 2.5 g soil mixed with test medium was used as a positive control. The test medium contained sodium chlorate, which inhibits the oxidation of nitrite to nitrate without negatively affecting the ammonia oxidation. The amount of nitrite will thus accumulate over time during the incubation. The samples were incubated on an orbital shaker for 10 h (175 RPM/min, 25 °C). 1.5 ml sample were sampled after 3, 5, 8 and 10 h. 1.5 ml of KCl (4mol/L) were mixed to the samples and centrifuged for 3,000 g for 2 min. The samples were filtered, then stored in a fridge until the last sampling (after 10h). Standard curves were obtained by serial dilutions of sodium nitrite.

The SEAL AutoAnalyzer AA500 was used to measure the nitrite produced by the ammonia oxidizers. Standard and reagents solutions were prepared following the AutoAnalyzer 500 Method no. A-044-19 Rev. 3 protocol from SEAL analytical. All solutions were degassed and stored in acid washed bottles (1% HCL solution for one hour) before use.

There was no consistent measurements, probably due to the low abundance and low activity of ammonia oxidizers in roots. We thus decided to use the remaining plant material to measure root traits.

3.5. Root traits

Roots from a third plant were cleaned by vortexing the root in 25 ml of phosphate saline buffer solution in a falcon tube two times, similarly to what was done with the roots used in the molecular analyses. The roots were scanned with a Espon Perfection V850 Pro. By using the software program SmartRoot (implemented in ImageJ) the length of the roots was measured. The roots were then dried in an oven at 65 °C for 3 days and their dry mass weighed. The specific root length (SRL) was measured by calculating the ratio between length and dry weight. Both these traits are used as a measurement of plant N and water uptake capacity (Abalos et al. 2019).

3.6. Data analysis

The statistical analyses were performed in R and Microsoft Excel. All the figures were created in R using a scrip made by Aurélien Saghaï.

The abundance of AOA and AOB, as well as the different plant traits were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene tests, respectively ('rstatix' package, v. 0.7.0), and log10- transformed to ensure a normal distribution when needed. There were no extreme outliers ('identify_outliers' function).

Comparing each elicitor treatment to the corresponding control the Welch's t-test was performed because the variance between the treatments was different (the student's t-test assumes homogeneity of variance). Comparisons were conducted using the Wilcoxon signed-rank test ('base' package) when the data was not normally distributed. Differences were considered significant at $p < 0.05$.

The percentage change of each elicitor treatment compared to the control was calculated as follows: (treatment value – control value)/control value x 100.

Sequence analyses of AOA and AOB communities were performed by Aurélien Saghaï. The amplicons of AOA and AOB gene were processed with the 'dada2' package (v. 1.21.0) to infer amplicon sequence variants (ASVs), which resolve differences of as little as 1 nucleotide between sample sequences. The resulting ASV tables were Hellinger transformed to limit the influence of samples with low counts and many zeros. In this transformation, the square root of the relative abundances of each ASV is computed. Differences in community composition were visualized using non-metric multidimensional scaling (NMDS) ordinations ('vegan' package v. 2.5-7). Potential differences in dispersion between treatments were explored using the betadisp function ('vegan' package) and their significance assessed using a permutation test (permutest function, $p < 0.05$). Since no significant difference in dispersion was detected, we performed a PERMANOVA (adonis2 function in 'vegan') to assess the effect of elicitor and elicitor application on the community composition of both AOA and AOB.

4. Results

4.1. Plant traits

Two controls were used in the experiment called control and T control, in which seeds were treated with water only, or water and Tween20, respectively. There was no significant difference between them in any of the statistical tests performed (figure 4). The T control was thus used when comparing each elicitors treatments to the control.

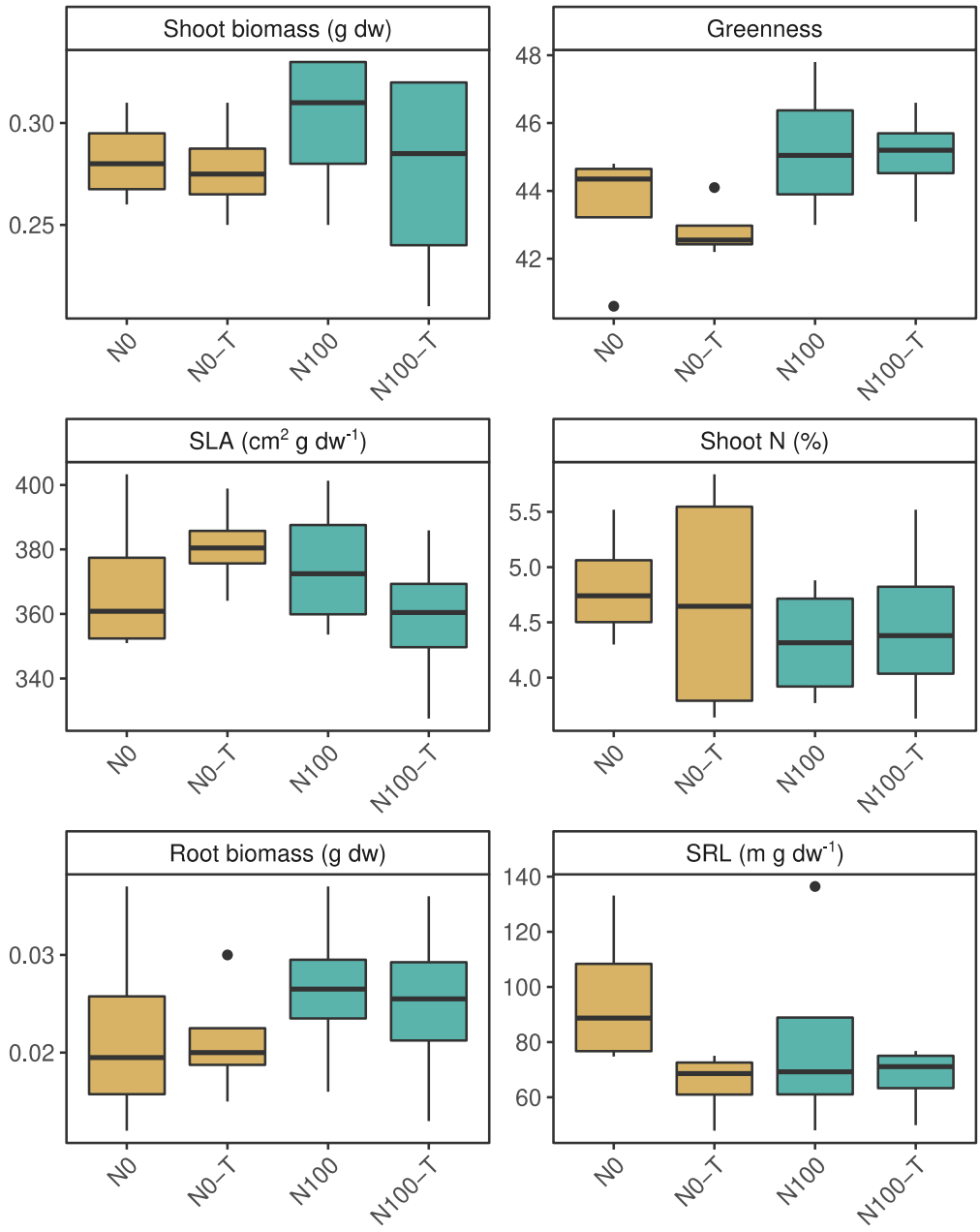

Figure 4:Plant traits in water and tween (T) controls in unfertilized (yellow left panel) and fertilized treatment (blue right panel). Shoot biomass, greenness, specific leaf area (SLA), shoot N, root biomass, specific root length (SRL). There was no significantly difference between the control and T control.

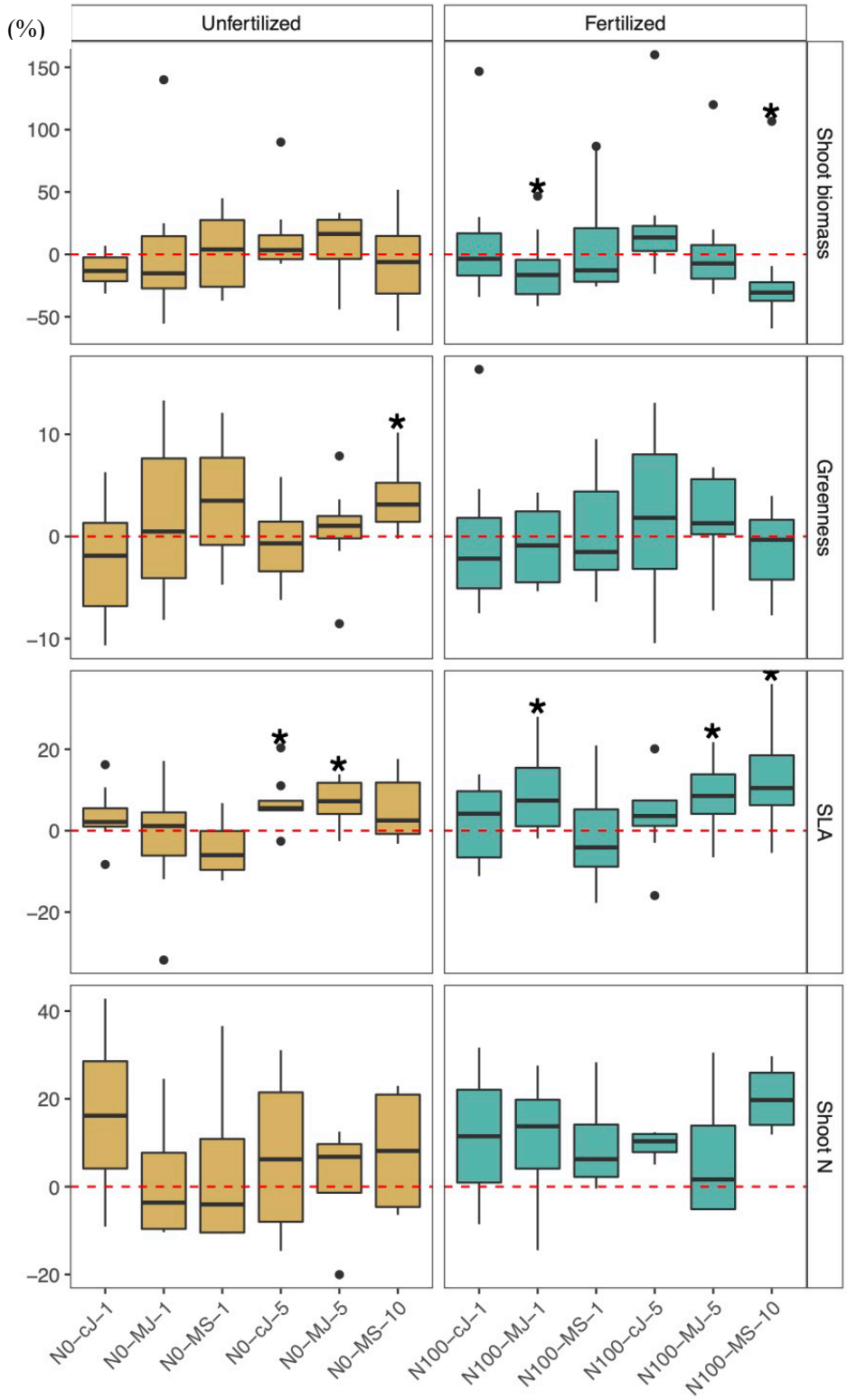

Figure 5: Comparison of the elicitor treatments to the control in unfertilized (yellow left panel) and fertilized (blue right panel) pots for the aboveground plant traits. The control is represented by the red dashed line and values above and below the line indicate positive and negative percentage changes compared to the control, respectively.

The figure 5 illustrate the plant traits measured in fertilized and unfertilized treatment above-ground. The shoot biomass in the fertilized treatments MJ-1 and MS-10 were significantly lower compared to the control. Among the non-significant results, MJ-5 tended to have a decreased shoot biomass and cJ-5 an increased biomass.

The greenness in the unfertilized treatment MS-10 was significantly higher compared to the control. Among the non-significant results fertilized cJ-5 have increased greenness.

The SLA in the fertilized treatments MJ-1, MJ-5 and MS-10 were significantly higher compared to the control. In the unfertilized treatments cJ-5 and MJ-5 were significant higher compared to the control. All treatments tended to be higher compared to the control (not all were significant), except MS-1 in both fertilized and unfertilized pots.

Regarding the shoot N content, there was no significant effect of the elicitor treatments in either fertilized as unfertilized treatment. However, the shoot N content followed the same trend as SLA in both fertilized and unfertilized treatment, except MJ-1 (unfertilized) and MS-1 (fertilized).

The root biomass (figure 6) illustrates the below-ground plant traits. The root biomass in the fertilized treatment cJ-5 was significantly higher compared to the control. We did not detect other significant effects for SRL.

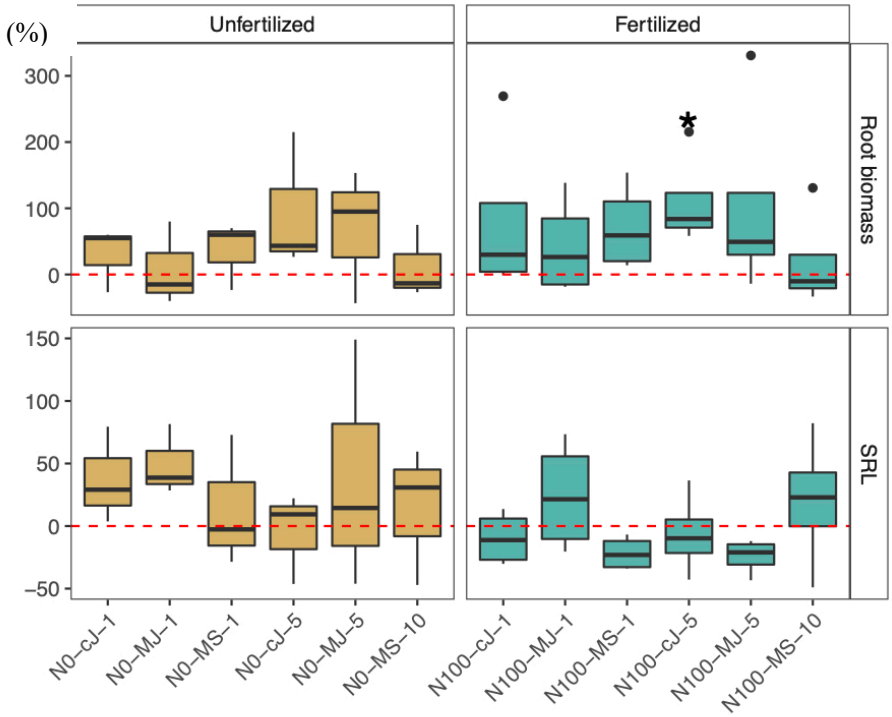

Figure 6: Comparison of the elicitor treatments to the control in unfertilized (yellow left panel) and fertilized (blue right panel) pots for the below ground plant traits. The control is represented by the red dashed line and values above and below the line indicate positive and negative percentage changes compared to the control, respectively.

4.2. Abundance of root and rhizosphere communities

There were no significant difference between the control (treated with water) and the T control (treated with Tween20) when measured genes. The T control was thus used when comparing each elicitors treatments to the control.

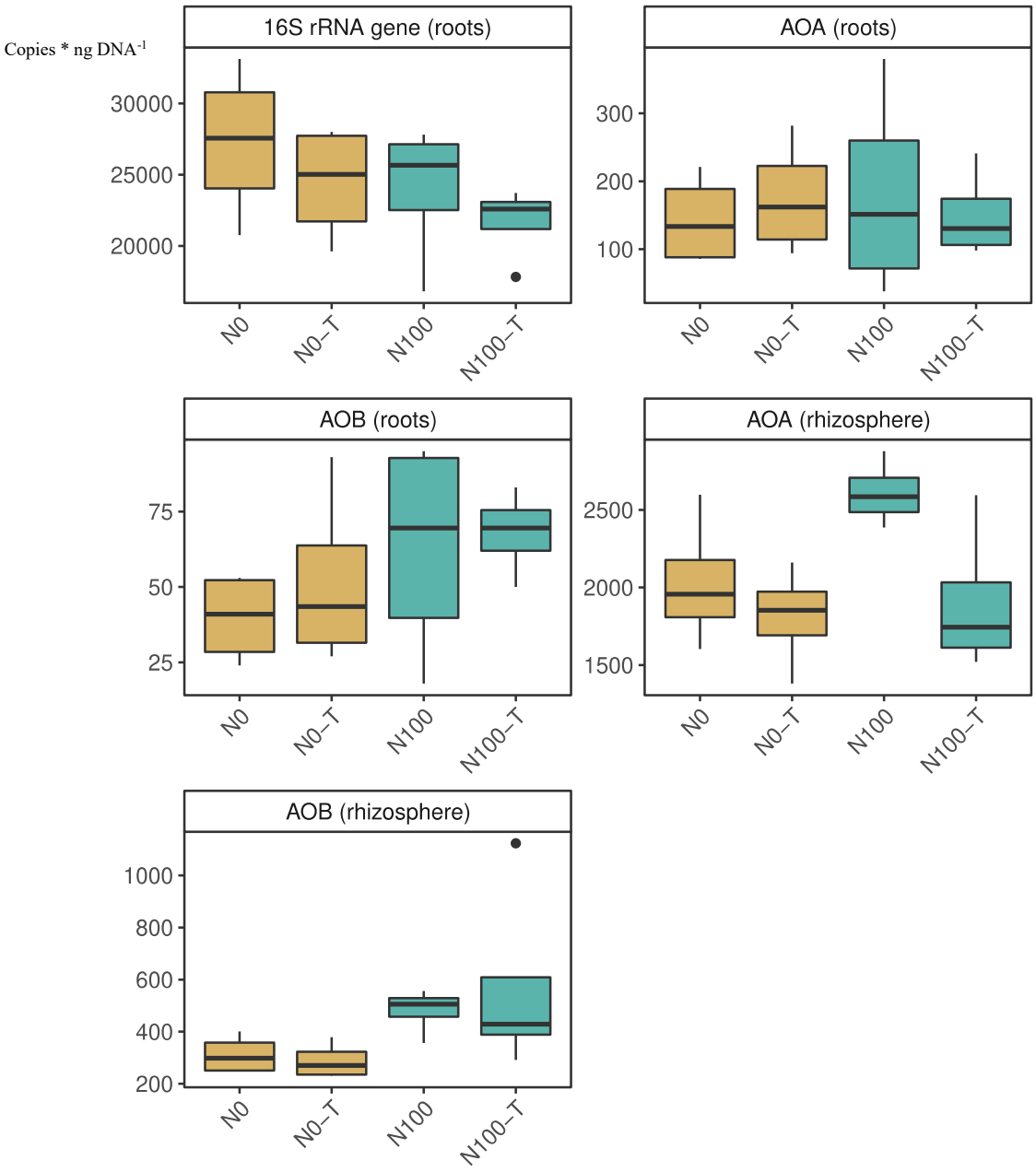

Figure 7: The determined abundance of the control and T control in unfertilized (yellow left panel) and fertilized treatment (blue right panel) of AOA and AOB root and rhizosphere communities. Determined abundance of 16S rRNA gene root community in unfertilized (yellow left panel) and fertilized (blue right panel) treatment. There were no significant differences between the control and T control.

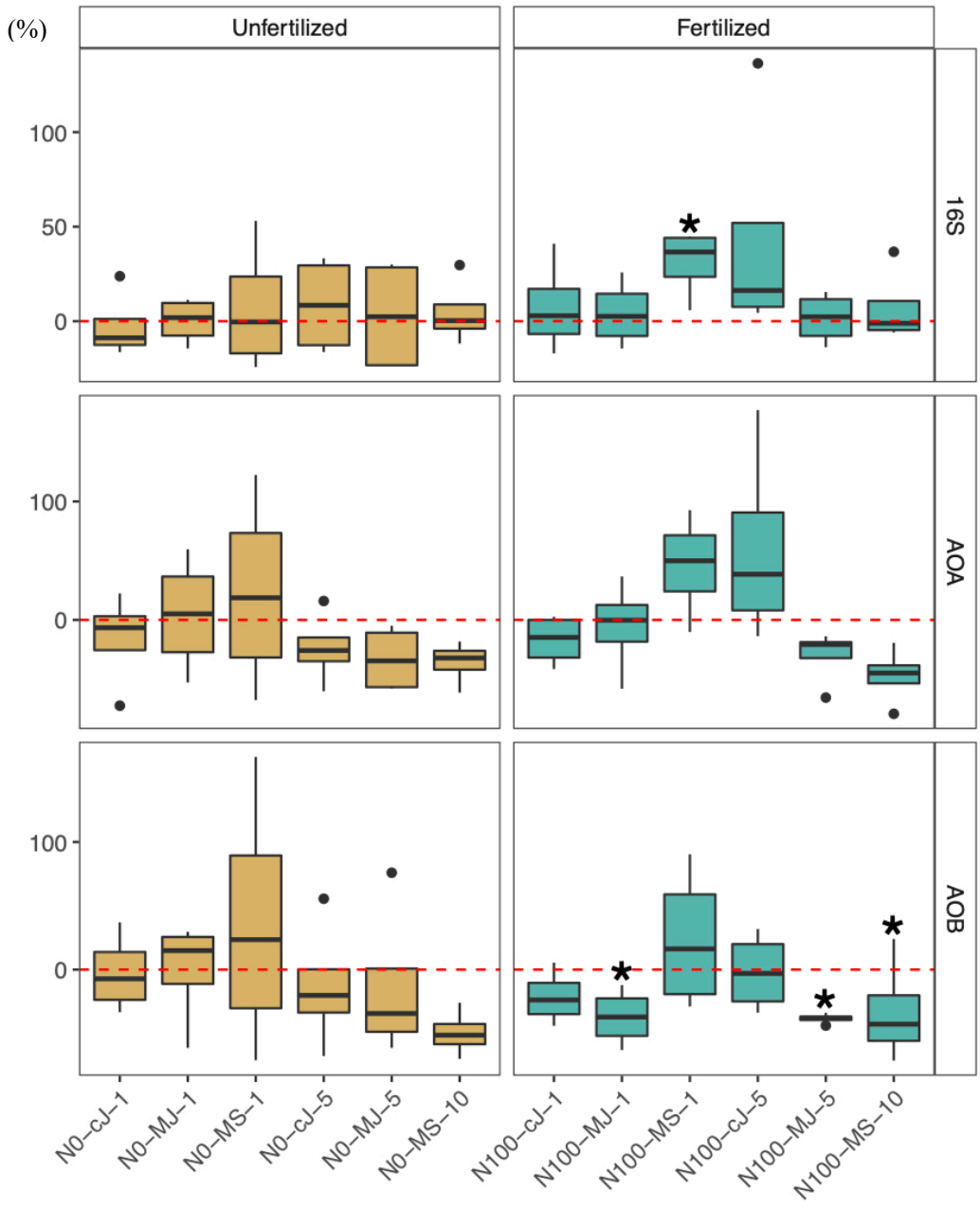

Figure 8: Comparison of the elicitor treatments to the control in unfertilized (yellow left panel) and fertilized (blue right panel) pots for the abundance of 16S rRNA gene, AOA and AOB of root communities. The control is represented by the red dashed line and values above and below the line indicate positive and negative percentage changes compared to the control, respectively.

The figure 8 illustrate the abundance of the root communities in each elicitor treatments. The 16S rRNA gene showed significantly increased abundance of the fertilized MS-1 compared to the control. This was the only treatment where the abundance of the overall archaeal and bacterial communities was significantly

higher compared to the control. Fertilized cJ-1, MJ-1, MJ-5 and MS-10 showed almost no change compared to the control.

The abundance of AOB in fertilized MJ-1, MJ-5 and MS-10 were significantly lower compared to the control. The AOA were not significantly different but showed the same trends as AOB in both fertilized and unfertilized treatments (except MJ-1).

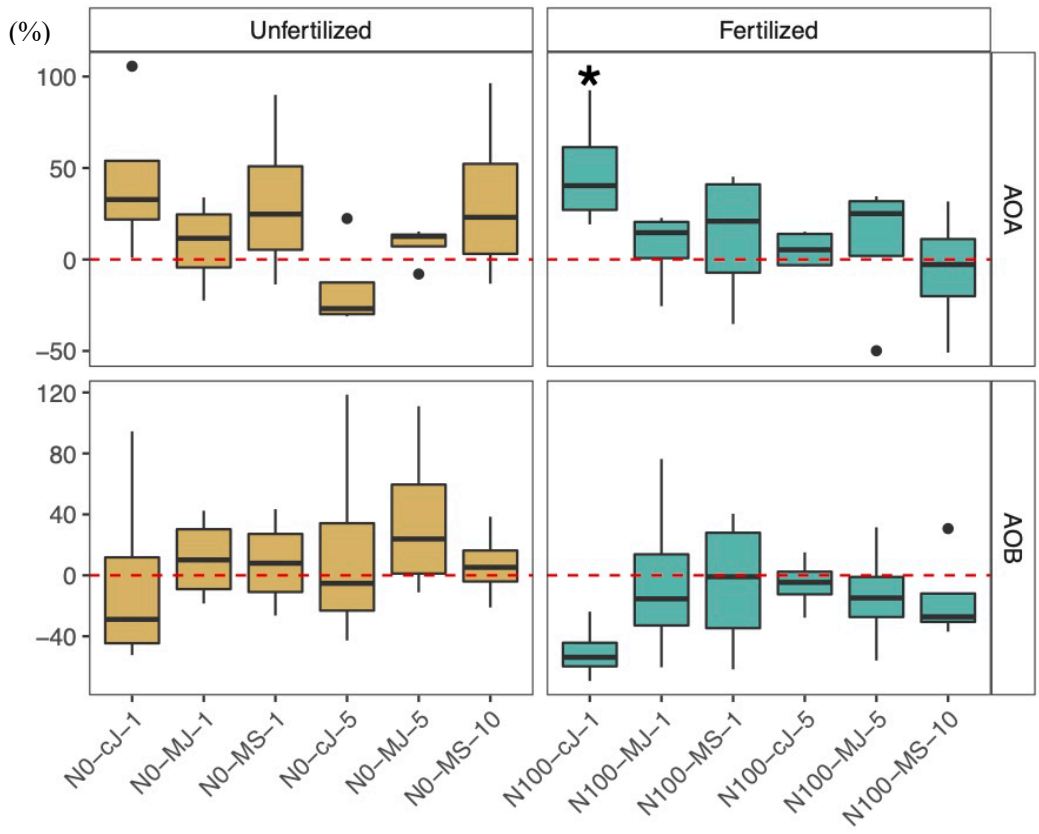

Figure 9: Comparison of the elicitor treatments to the control in unfertilized (yellow left panel) and fertilized (blue right panel) pots for the abundance of AOA and AOB of rhizosphere communities. The control is represented by the red dashed line and values above and below the line indicate positive and negative percentage changes compared to the control, respectively

For the rhizosphere community, qPCRs for the 16S rRNA gene were contaminated and were dropped. The abundance of AOA in fertilized cJ-1 were significant higher compared to the control. AOA and AOB show the opposite pattern in fertilized treatment, when the abundances of AOA were increasing the AOB were decreasing, expect the MS-10 of AOA (figure 9).

The communities of Comammox presented multiple bands on the agarose electrophoresis gel. Multiple bands indicate unspecific amplification and we could

thus not draw any conclusions on the abundance of Comammox. The Comammox were thus not investigated further in the project.

The results of the abundance of AOA and AOB in root and rhizosphere communities showed that there were more significant differences compared to the control in the root communities. For that reason, we chose to focus the community composition analysis on the roots.

4.3. AOA and AOB community composition

PERMANOVA analyses showed no effect of elicitors on the community composition of AOA ($p > 0.05$) and AOB ($p = 0.05$). Fertilization had a small but significant effect on AOB ($R^2 = 0.03$, $p < 0.001$) but not on AOA communities ($p > 0.05$).

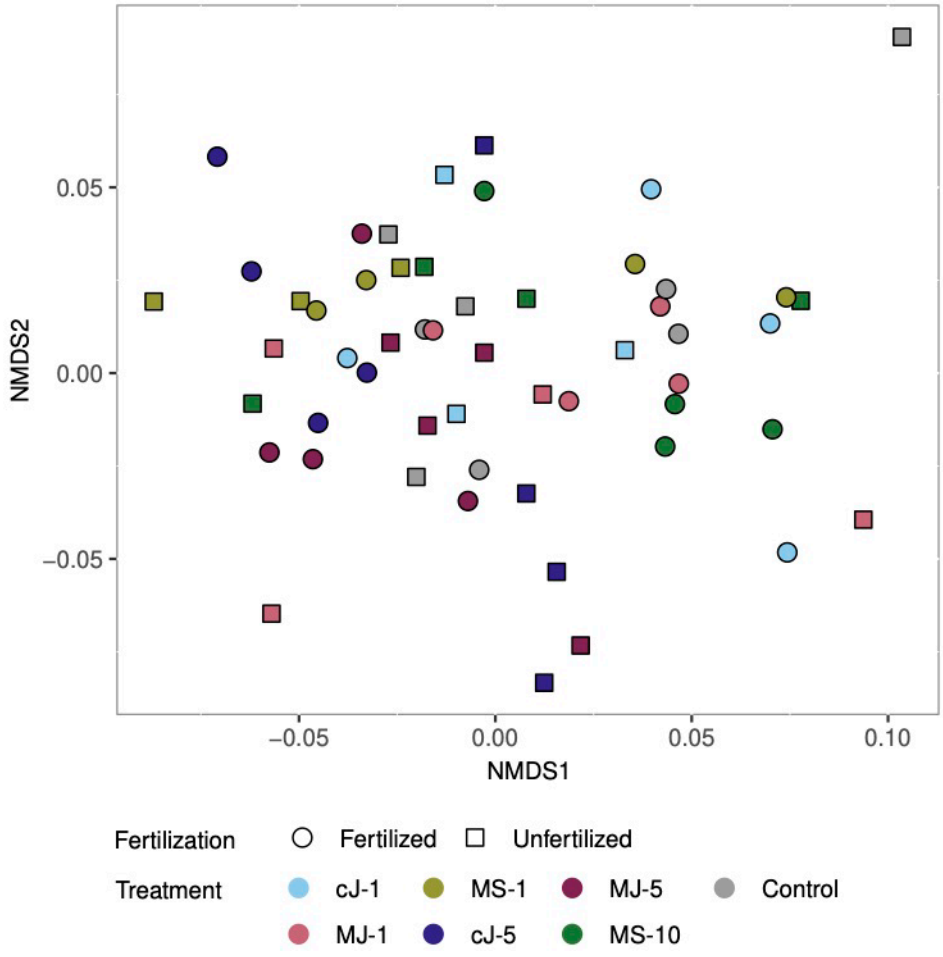

Figure 10: Non-metric Multidimensional Scaling plot of the AOA community composition. The shorter the distance between two samples the closer the community composition. The fertilized

treatment is represented by circles and the unfertilized treatment by squares. Each elicitor treatment is represented by a color.

AOA

The fertilized pots showed more similarity patterns to each other (clustered) than the unfertilized pots. In the fertilized pots, MJ-1 was largely overlapping with the control, indicating a high degree of similarity in community composition. Although not significant statistically, several treatments displayed some level of dissimilarity with the control. For example, the treatments cJ-5 and MJ-5 and MS-10 (figure 10).

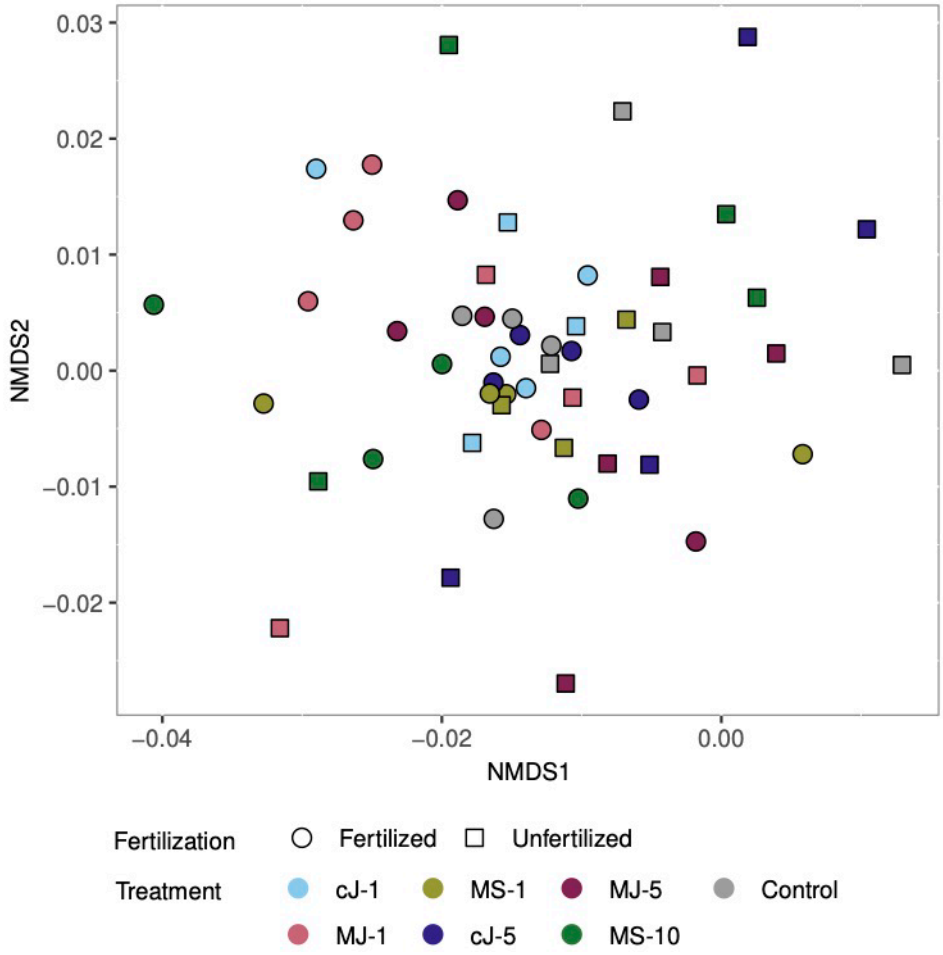

Figure 11: Non-metric Multidimensional Scaling plot of the AOA community composition. The shorter the distance between two samples the closer the community composition. The fertilized treatment is represented by circles and the unfertilized treatment by squares. Each elicitor treatment is represented by a color.

AOB

The fertilized pots showed more similarity patterns to each other (clustered) than the unfertilized pots. The fertilized MJ-1, MJ-5 and MS-10 tended to be more distant to the control, although not significant statistically (see figure 11).

5. Discussion

Stronger effects of fertilized pots in root communities

Overall, we observed more significant effects of the elicitor treatments in fertilized pots. An earlier study from (Zakir et al. 2008) showed that adding fertilizer as NH_4Cl stimulates the BNI in root exudates and inhibits ammonia oxidizers. We thus expected that the fertilization in our experiment would stimulate the exudation of potential BNI. This explains why we see more decreasing effects of the abundance and difference of the community composition compared to the control in fertilized treatments compared to the unfertilized treatment. It is the ammonium content surrounding the root which triggers the exudation of inhibitors and not the pH of the rhizosphere (Zakir et al. 2008). This could also explain why root communities (defined as microbes on and in the roots) are significantly more affected compared to the rhizosphere communities (defined microbes located outside the roots).

Methyl jasmonate

MJ seemed to be the most promising treatment because this elicitor had most significant effects at both concentrations in several plant traits and on the abundance of ammonia-oxidizing communities.

Fertilized MJ-1 and MJ-5 showed a significantly increased SLA and the shoot N displayed a consistent pattern to SLA (although not significant) with higher values compared to the control. This indicate that these elicitor treatments could have a positive effect on plant N uptake as the SLA is an indication of N uptake (Abalos et al. 2019) and the shoot N content data indicates a higher N content in the shoots.

The fertilized MJ-1 and MJ-5 displayed significantly decreased abundance of root AOB communities. The AOA communities in the fertilized MJ-5 treatment showed a trend of decreasing abundance (not significantly different), and trends of composition changes as well. The AOB in both fertilized MJ-1 and MJ-5 showed a trend of changed community composition. According to the literature, MJ induces the production of glucosinolates, which are known to have inhibitory effects on ammonia oxidizers (Bending et al. 2000). However, the study from Bending (2000) used brassicales and not cereals as for this thesis. This is further supported by work

from Subbarao et al. (2013) which have done experiment on *sorghum* (cereal) with strong effects on *Nitrosomonas.* The MJ seems to have inhibition effects of both creels and brassicales.

However, all plant traits did not significantly increase. For example, the shoot biomass in the fertilized MJ-1 significantly decreased and the fertilized MJ-5 showed a trend towards a decrease. This is important because this indicates that the elicitor treatments had a negative impact on shoot biomass and are not supporting our second hypothesis, as higher N uptake should result in increased shoot biomass. Shoot biomass correlates with increased N uptake as well (Kamiji et al. 2014). A study from Kraus et al. (2019) showed that seed treatment with MJ significantly decreased shoot biomass in the beginning of the experiment, which is in agreement with this experiment for MJ-1 treatments in the fertilized pots. Kraus et al. (2019) then showed that the plants recovered and that the shoot biomass increased after a longer plant growth. The short duration of the experiment (23 days after germination) could be an explanation of the decreased shoot biomass and maybe a longer time of plant growth could lead to different patterns. At the same time, we do not know how long the elicitors have an effect of the wheat and over time there could be a decrease of this effect. It would be interesting to include this parameter in future experiments.

Methyl salicylate

The second most effects were found in the treatment MS-10, which corresponds to the treatment with the highest elicitor concentration (seeds treated with 10 mM cJ or MJ did not germinate). The study from (Kalaivani et al. 2016) showed that using a concentration of 10 mM MS affecting the germination positively which could be an explanation as to why the seeds treated MS-10 germinated. Furthermore, the MS-1 treatment did not show any significant effects, which motivates why it is important to use higher concentrations. MS is volatile and using a low concentration could be an indication that it is less effective (Kalaivani et al. 2016). The experiment should have used 5mM of MS because it is difficult to compare different concentrations with different elicitor treatments. For future analysis all treatments should have the same elicitor concentration.

The MS-10 showed significant increases in SLA and a trend towards higher shoot N content. The abundance of the fertilized root community of AOB significantly decreased and AOA followed the same trend. By comparing the abundance of total archaeal and bacterial genes of 16S rRNA genes to the significantly decreased abundance of fertilized MS-10 of AOB and decreased abundance of AOA, we can conclude that the abundance of ammonia oxidizers is decreasing in the MS-10 treatment. In addition, the community composition also tended to differ from the control in fertilized AOA and AOB communities which indicate composition effects. These elements support the hypothesis as for the MJ

treatment, when the abundance of ammonia oxidizers decreases while both SLA and shoot N content increase. This suggests a possible inhibitory effect of this elicitor on ammonia oxidizers. Previous work has shown that the MS elicitor induces phenyl propanoids in root exudates which showed an inhibitory effect on *Nitrosomonas* (Zakir et al. 2008).

Regarding plant traits, the shoot biomass significantly decreased as for the MJ treatment. There is no study that relates to the same problem as for MJ treatment. Maybe MS have the same effect on shoot biomass as MJ and it will recover over a longer period of growth. The greenness in unfertilized MS-10 showed significantly increased effects compared to the control. Greenness is a measurement of the chlorophyll concentration and an indirect measurement of N content (Spectrum Technologies, Inc., 2021). It would then be logical if this trait would show the same pattern as SLA (Pérez-Harguindeguy et al. 2016) and shoot N content, which is a measurement of N content as well. But Xiong et al. (2015) showed that the analyze using SPAD readings is complex and not always reliable. They showed that the environmental factors and features of different crops have a strong effect on the N content per leaf. They showed that the relationship with the SPAD analyze of N content in the leaf is uncertain. This can motivate why the greenness did not show any strong pattern of increased greenness and maybe this plant trait should not be used for future analyses.

Cis jasmone

Treatment cJ-5 had distinct effects on plant traits compared to MS and MJ. For example, the root biomass in fertilized cJ-5 treatment significantly increased. No other plant traits were significantly different compared the control, including the other below ground trait SRL. A high SRL indicates that there is reduced N losses (Abou-Mansour et al. 2008) and maybe an increase in N uptake. We did not see any significant effects or trends of the SRL traits. The other above ground fertilized plant traits show an increasing trend which indicates an increased N uptake. If cJ would have stronger effects on only belowground traits it would be expected that SRL would show some increased trend as well. To see if cJ only affects belowground traits it would be interesting to measure other traits, for example root N content. This trait indicates potential N uptake (Abalos et al. 2019).

The abundance of the fertilized root community was not affected and showed no trends. The community composition of the fertilized AOA displayed some degree of dissimilarity to the control, but not for AOB. An article by (Neal et al. 2012) showed that cJ have effects on soil microbes but there was no mention of the effect on ammonia oxidizers. This thesis shows that cJ does not affect the ammonia oxidizers as the other elicitors do, but the (slight) effect on AOA community composition tells us that this is still interesting to continue investigating this elicitor.

Potential ammonia assay

The potential ammonia oxidation activity of the AOA and AOB could not be determined in this project because the activity of the ammonia oxidizers was too low. The ISO protocol for micro scale was refereeing to measurement of slurries and not roots. It is difficult to use roots because it contains a low number of ammonia oxidizers compared to the bulk soil. There is no published study measuring potential ammonia oxidation on roots. We hypothesized that if the elicitor treatment would induce inhibitory compounds this should result in a lower rates of nitrite production compared to the control. For future analyses it would thus be interesting to measure the differences in potential ammonia oxidation between the control and the fertilized elicitors treatment of root communities.

6. Conclusion

In agreement with our hypotheses we found that applying methyl jasmonate and methyl salicylate on winter wheat decreased the abundance of ammonia oxidizers in the root communities without negatively affecting plant performance. The thesis also showed that adding fertilizer as ammonium chloride increased the effect of the elicitor treatments, spatially limited to the root communities.

Methyl jasmonate was the treatment with most significant effects both for both 1 mM and 5 mM. Methyl salicylate (10mM) was the treatment with second most significant effects. In both cases the plant traits were positively affected except for the shoot biomass, but it is possible that this effect is temporary and that a longer growth period would allow an increase in the shoot biomass. The third elicitor investigated here, cis-jasmone, showed fewer effects but seem to positively affect below-ground plant performance.

Future analysis should try to include measurements of the potential ammonia oxidation rates to strengthen the hypothesis that elicitors can be used to induce inhibition of ammonia oxidizers. From a wider perspective it would be interesting to do field experiments and see the effects of elicitor treatment (5mM) on both plant defence and ammonia-oxidizing root communities. We should also continue to use cJ, MJ and MS and see if these elicitors have similar effects on different crops and how they can be used in practical agriculture.

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Appendix

In the appendix is the average number of copier per ng DNA of every ammonia oxidizing gene and 16S rRNA gene from qPCR present. The ISO 15685 protocol for micro-scale is attached below.

Table 6: Average number of copies per ng DNA of AOA, AOB, Comammox and 16S rRNA gene.

Organism	Average number of copies /ng DNA
AOA	$2.07*10^3$
AOB	$3.73*10^2$
Comammox	$2.33*10^2$
16S rRNA	$9.34*10^4$

The ISO 15685 protocol micro-scale page 1-5 and 9-11.

Soil quality — Determination of potential nitrification and inhibition of nitrification — Rapid test by ammonium oxidation

1 Scope

This International Standard specifies a rapid method for the determination of the potential rate of ammonium oxidation and inhibition of nitrification in soils. This method is suitable for all soils containing a population of nitrifying microorganisms. It can be used as a rapid screening test for monitoring soil quality and quality of wastes, and is suitable for testing the effects of cultivation methods, chemical substances [except volatiles, i.e. $H > 1$ (Henry's constant)], extracts of biosolids and pollution in soils.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO 10390, *Soil quality — Determination of pH*

ISO 10694, *Soil quality — Determination of organic and total carbon after dry combustion (elementary analysis)*

ISO 11260, *Soil quality — Determination of effective cation exchange capacity and base saturation level using barium chloride solution*

ISO 11261, *Soil quality — Determination of total nitrogen — Modified Kjeldahl method*

ISO 11277, *Soil quality — Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation*

ISO 11465, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

ISO 14238, *Soil quality — Biological methods — Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes*

ISO 14256-2, *Soil quality — Determination of nitrate, nitrite and ammonium in field-moist soils by extraction with potassium chloride solution — Part 2: Automated method with segmented flow analysis*

EN 14735, *Characterization of waste — Preparation of waste samples for ecotoxicity tests*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
inhibitory dose
ID
amount of a chemical added to soil that effectively inhibits biological activity by a stated percentage after a given time, in comparison with an untreated control

NOTE It is expressed as a percentage. For example, ID25 and ID50 indicate a 25 % and 50 % inhibition of biological activity, respectively.

3.2
biosolids
organic products used in agriculture, which include sewage sludge, compost, manure and some industrial wastes

4 Principle

Ammonium oxidation, the first step in autotrophic nitrification in soil, is used to assess the potential activity of microbial nitrifying populations. Autotrophic ammonium-oxidizing bacteria are exposed to ammonium sulfate in a soil slurry buffered at pH 7,2. Oxidation of the nitrite performed by nitrite-oxidizing bacteria in the slurry is inhibited by the addition of sodium chlorate. The subsequent accumulation of nitrite is measured over a 6 h incubation period, and is taken as an estimate of the potential activity of ammonium-oxidizing bacteria. As the generation time of ammonia-oxidizing bacteria is long (> 10 h), the method provides a measure of the potential activity of the nitrifying population at the time of sampling. It does not measure growth of the nitrifying population.

Chemical test substances and extracts of biosolids can be tested by adding them to the slurry at various concentrations. The test can be used for comparison of different soils (e.g. polluted and reference soils). Effects of polluted soils, waste samples and other solid materials on nitrification can be evaluated by measuring the method on mixtures of these samples with a reference soil.

Substances known to be active at pH < 7,2 should be added in due time before starting the test to allow substances to exhibit their effects on nitrifying bacteria.

5 Reagents

5.1 Distilled water.

5.2 Potassium dihydrogenphosphate, $c(KH_2PO_4 = 0{,}2$ mol/l).

5.3 Dipotassium hydrogenphosphate, $c(K_2HPO_4 = 0{,}2$ mol/l).

5.4 Sodium chlorate, $c(NaClO_3 = 0{,}5$ mol/l).

5.5 Diammonium sulfate, $(NH_4)_2SO_4$.

5.6 Sodium hydrogencarbonate, $c(NaHCO_3 = 5$ mmol/l).

5.7 Potassium chloride, $c(KCl = 4$ mol/l).

5.8 Stock solution A.

Prepare stock solution A as follows.

Potassium dihydrogenphosphate, KH_2PO_4 (5.2)	28 ml
Dipotassium hydrogenphosphate, K_2HPO_4 (5.3)	72 ml
Distilled water (5.1)	100 ml

5.9 Test medium.

Prepare the test medium as follows.

Stock solution A (5.8)	10 ml
Sodium chlorate, $NaClO_3$ (5.4)	10 ml to 30 ml
Diammonium sulfate, $(NH_4)_2SO_4$ (5.5)	0,198 g

Dilute to 1 000 ml with distilled water (5.1).

The test medium contains 1 mmol/l of potassium phosphate buffer, 5 mmol/l to 15 mmol/l of sodium chlorate and 1,5 mmol/l of diammonium sulfate, and has a pH of approximately 7,2.

The sodium chlorate concentration selected within the range given should be sufficient for effective inhibition of biological nitrate formation, while not having negative effects on ammonium oxidation. If necessary, test the influence of the sodium chlorate concentration.

Test chemicals to be added to the test medium shall be dissolved in the phosphate buffer described and added before diluting to 1 l. For test chemicals with low water solubility, prepare solutions by mechanical dispersion or by using vehicles such as organic solvents, emulsifiers or dispergents of low toxicity to ammonium-oxidizing bacteria. If any vehicle is used to add a test chemical to the slurry or a soil, additional control shall be performed to assess possible effects of this vehicle on ammonium oxidation.

Add extracts of biosolids, when tested, separately to the test medium before dilution to 1 l.

NOTE When a carbon source is needed, e.g. when testing biosolids with high nitrate levels, add 10 ml of $NaHCO_3$ (5.6) to the test medium before diluting to 1 l.

6 Apparatus

6.1 Ordinary laboratory equipment.

6.2 Orbital shaking incubator, thermostatically controlled.

7 Sampling, storage and characterization of samples

Guidance on sampling, sample processing and storage is given in ISO 10381-6. For the testing of chemicals or biosolids, or for mixing tests with polluted soils, experimental soil with an ammonium-oxidizing activity of between 200 ng N/g and 800 ng N/g of dry mass of soil/h, determined from a preliminary experiment, should be chosen. When testing chemicals, the soil should have a low adsorption capacity (e.g. sandy soils low in C_{org} < 1 %); on the other hand, the soil should have a pH between 5,5 and 7,5.

Soil samples can usually be stored in a refrigerator at approximately 4 °C for periods of up to three months prior to use (ISO 10381-6). Some soils can only be kept in a refrigerator for up to two weeks. Storage of soil samples in a freezer (−20 °C) is not generally recommended.

It has been shown for a number of soils from temperate climates that storage at −20 °C for up to 12 months does not inhibit ammonium oxidation activity (Reference [4]). Where such information is available for a particular soil, storage under such conditions is permissible.

Measurement of the following variables is recommended for characterization of the soil:

— particle size distribution (ISO 11277);

— water content (ISO 11465);

— water-holding capacity (ISO 14238);

— pH (ISO 10390);

— effective cation exchange capacity (ISO 11260);

— organic matter content (ISO 10694);

— total nitrogen content (ISO 11261).

8 Procedure

8.1 Testing soils

8.1.1 General

The test design should include at least three replicates of approximately 25 g of moist soil. The water content of soil for the calculation of dry mass is determined gravimetrically according to ISO 11465.

8.1.2 Initial incubation

Mix soil samples or waste materials with test medium (5.9) to form slurries. The volume of test medium should be adjusted to give a precise total liquid volume, e.g. 100 ml. Calculate the volume of medium to be added by subtracting the volume of water in the initial soil or waste sample from the desired liquid volume, e.g. 100 ml. Incubate the slurries in 250 ml flasks, placed upright on the orbital shaking incubator (6.2), thermostatically controlled at (25 ± 2) °C. Rotation should be sufficient to keep solids suspended (175 r/min).

A liquid volume greater than 100 ml is required in the slurry if the water-holding capacity of the soil is > 200 % (organic soils).

8.1.3 Sampling of soil slurry

Take samples (2 ml) of the soil slurry after 2 h and 6 h of incubation, provided that ammonium oxidation is known to be linear over this period. The soil slurry should be well shaken at sampling times to ensure that the ratio of solution to soil is constant during the test. Dispense samples into test tubes and add 2 ml of KCl (5.7) to stop the ammonium oxidation. Then centrifuge the samples at, for example, 3 000$_g$ for 2 min, or filter. Filter paper should be of high filtration speed, while its chemical purity may be less than the highest grade. Determine nitrite by a suitable method of chemical analysis (see ISO 14256-2). At this stage, the solutions can be stored in a refrigerator (4° C to 8 °C) but analysis should be carried out within 24 h.

If necessary, check the linearity of the ammonium oxidation over time by sampling soil slurry a number of times during the 6 h of incubation. This is likely to be necessary if laboratories are not familiar with the soil types being used in the test. Some cases of non-linearity can be corrected by ensuring aerobic conditions or supplying a carbon source.

8.2 Testing the effect of chemicals

Determine ammonium oxidation according to the procedure for soils by adding test chemicals to the test medium (5.9) at different concentrations. Include controls with no added test chemical in the test design as a reference. The number of replicates of the control should be twice the number of replicates chosen for each concentration of test chemical.

Conduct a preliminary range-finding test to select the concentrations of test chemical. In the main test, arrange at least five different concentrations in a geometric progression. The lowest concentration tested should have no effect on ammonium oxidation and the highest should inhibit by 50 % to 100 %.

When a vehicle for dissolution of a chemical is used, it should not exceed 100 mg/l in the test medium. Its effect on ammonium oxidation should be tested by having additional controls containing the vehicle at the highest concentration used in the test.

NOTE Volatile test substances require special arrangements which are not within the scope of this International Standard.

8.3 Testing polluted soils

The most straightforward method is to compare the polluted soil with an unpolluted reference soil with comparable soil properties, or to make gradient studies on soil samples taken at various levels of pollution. Take a sufficient quantity of soil in order to prepare at least four replicates.

In the absence of a suitable unpolluted soil for reference, or in cases of low potential nitrification activity, the following test procedure is recommended. Pre-incubate the polluted soil and an unpolluted soil of known nitrification activity at 200 ng N/g dm of soil/h to 800 ng N/g dm of soil/h at a water content of 40 % to 60 % of water-holding capacity or −0,01 MPa to −0,03 MPa suction pressure for two days at 20 °C in darkness. Mix moist amounts of the two soils comparable to 75 g of dry mass in a 1:1 ratio on a dry-mass basis. Store the mixture, the polluted soil and the control soil for 24 h at 20 °C in darkness. Measure the potential ammonium oxidation as described in 8.1 using four replicates of the mixture and of each soil.

8.4 Testing water extracts of biosolids

The effect on soil nitrification by extracts of biosolids, e.g. sewage sludge, can be tested. Prepare water extracts by 24 h extraction with demineralized water at a ratio of 1:10 [g of biosolid (dry mass):ml of water] and pH 7,5 (see EN 14735). Separate the aqueous phase from the particles by centrifugation, e.g. at 1 300$_g$ for 1 h. Test the effect of the extract on soil in a concentration series, including controls with no extract. Follow the test procedure given in 8.1, adding the extract of biosolid just before the addition of test medium. Include extract samples without soil in the test design for subtraction of ammonium oxidation by nitrifying bacteria in the extracts.

9 Calculations

Calculate the rate of ammonium oxidation (ng NO_2-N/g of dry mass of soil/h) from the difference between NO_2-N concentrations at different measuring times.

Calculate the inhibition of ammonium oxidation activity by the test chemical or extract of biosolid as a change in the activity in reference soil, expressed as a percentage.

In mixtures of polluted and unpolluted soils, the polluted soil is considered toxic if the mixture shows ammonium oxidation significantly lower than 90 % of the mean activity of the two soils kept separate. Using four replicates, this is calculated using Equation (1):

$$\bar{A}_m + s_m < 0{,}9 \times \frac{\left(\bar{A}_c + \bar{A}_p\right)}{2} \quad (1)$$

where

$\bar{A}_m$ is the mean ammonium oxidation activity in the soil mixture;

s_m is the standard deviation of ammonium oxidation activity in the soil mixture;

$\bar{A}_c$ is the mean ammonium oxidation activity in the control soil;

$\bar{A}_p$ is the mean ammonium oxidation activity in the polluted soil.

10 Test report

The test report shall contain the following information:

a) a reference to this International Standard;

b) soil characteristics;

c) rate of ammonium oxidation (ng NO_2-N/g of dry mass of soil/h), individual values, mean values and standard deviations for each treatment and soil;

d) test of reaction linearity:

— if reaction linearity was tested at three sampling times or more, a plot of nitrite-N concentration versus time is reported;

Annex B
(informative)

Micro-scale measurement of potential nitrification rates

B.1 General

The following procedure, proposed in Reference [3], is suitable for the determination of potential nitrification in small soil samples. All the quantities of solids and liquid substances were scaled down tenfold compared to the original method. Nitrite concentrations are determined spectrophotometrically using a microplate reader.

B.2 Reagents

Prepare reagents as described in 5.1 to 5.8.

B.2.1 Test medium

Prepare the test medium as described in 5.9. A final sodium chlorate concentration of 5,625 mmol/l is recommended.

B.2.2 Reagents for nitrite determination using a microplate reader

B.2.2.1 Buffer, ammonium chloride (NH_4Cl): dissolve 5,08 g in 400 ml of distilled water, adjust to pH 8,5 using ammonium hydroxide (25 % NH_4OH) and fill up to 500 ml (0,19 mol/l).

B.2.2.2 Colour reagent (freshly prepared), sulfanilamide (CAS 63-74-1): dissolve 0,5 g in 25 ml of distilled water in a 50 ml volumetric flask, add 5 ml of concentrated phosphoric acid (H_3PO_4) (cool) and 0,025 g of N-(-1-naphthyl)-ethylenediaminedihydrochloride (CAS 1465-25-4) and fill up to 50 ml.

B.2.2.3 Sodium nitrite stock solution (1 000 mg NO_2-N l^{-1}): dilute 4,925 7 g of $NaNO_2$ in 1 l of distilled water.

B.2.2.4 Sodium nitrite standard solution (10 µg ml^1): add 0,5 ml of stock solution to a 50 ml volumetric flask and fill up with distilled water.

Sodium nitrite standards [(0; 0,01; 0,02; 0,1; 0,2; 0,4; 0,8) µg NO_2-N ml^{-1}], add 0 µl; 50 µl; 100 µl; 500 µl; 1 000 µl; 2 000 µl; and 4 000 µl of standard solution (10 µg ml^{-1}) and 25 ml of 4 mol/l potassium chloride solution (5.7) to 50 ml volumetric flasks and fill up with distilled water.

B.3 Apparatus

B.3.1 Incubation horizontal shaker [approximately 175 min^{-1}, (25 ± 1) °C].

B.3.2 Centrifuge, for reaction vessels (2,0 ml).

B.3.3 Microplate reader.

B.4 Procedure

B.4.1 General

The test design should include four replicates of approximately 2,5 g of moist soil. Determine the water content for calculation of dry mass gravimetrically, according to ISO 11465.

B.4.2 Initial incubation

Mix soil samples with 10 ml of the test medium in 50 ml polyethylene flasks to form slurries. The volume of the test medium should be adjusted to give a precise volume of 10 ml. Incubate the slurries on an orbital shaker (175 min^{-1}, 25 °C).

B.4.3 Sampling of soil slurry

Take samples (1 ml) after 2 h and 6 h of incubation. Dispense the samples into test tubes (2,0 ml) containing 1 ml of 4 mol/l potassium chloride solution (5.7) to stop the ammonium oxidation. Keep the slurries for 20 min and occasionally shake by hand. Thereafter, centrifuge the samples at 3 000$_g$ for 2 min. Transfer 1,5 ml of the supernatant into closable test tubes which can be stored in a refrigerator prior to NO_2 analysis for 24 h.

B.4.4 Measurement of nitrite production

Transfer 150 µl of samples and nitrite standards into microplate wells in three replicates for the measurement of nitrite production. Add 90 µl of buffer (ammonium chloride 0,19 mol/l, pH 8,5) and 60 µl of colour reagent to each well. Measure the absorbance in a microplate reader spectrophotometrically at a wavelength λ of (540 ± 10) nm after a 15 min colour reaction time. The absorbance values for the nitrite standards compute the nitrite concentration for each sample. At the end, calculate the potential nitrification rates as ng NO_2-N/g of dry mass of soil/h as follows:

$$c_{s,2h}\ [\text{ng } NO_2\text{-N/g}] = \frac{c_{l,2h} \cdot 2 \cdot (10 + m) \cdot 1\,000}{m_{dm}} \quad \text{(B.1)}$$

$$c_{s,6h}\ [\text{ng } NO_2\text{-N/g}] = \frac{c_{l,6h} \cdot 2 \cdot (10 + m) \cdot 1\,000}{m_{dm}} \quad \text{(B.2)}$$

$$\text{Potential nitrification } [\text{ng } NO_2\text{-N / g of dry mass of soil / h}] = \frac{c_{s,6h} - c_{s,2h}}{4} \quad \text{(B.3)}$$

where

$c_{s,2h}$ is the nitrite concentration in the soil sample after 2 h incubation;

$c_{s,6h}$ is the nitrite concentration in the soil sample after 6 h incubation;

$c_{l,2h}$ is the nitrite concentration in µg NO_2-N/ml in the liquid samples after 2 h incubation;

$c_{l,6h}$ is the nitrite concentration in µg NO_2-N/ml in the liquid samples after 6 h incubation;

2 is the dilution factor resulting from the addition of KCl (5.7);

10 is the volume of test medium [ml] (5.9);

m is the mass of water in the moist soil sample;

1 000 is the conversion factor [ng/µg];

m_{dm} is the dry mass of sample [g];

4 is the time difference (6 h – 2 h = 4 h).

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