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Nitrite Production by Controlled Nitrification of Liquid Pig Manure

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Abstract

Intensive livestock production leads to a regional surplus of nitrogen in production areas. To avoid environmental damages by over-fertilization, volatilization or leaching the manure could be transformed to a more valuable product.

We produced a nitrite-containing foliar spray from liquid pig manure in a simple batch process which could be applied in crop production as a foliar fertilizer with additional antifungal effects. For this purpose, liquid manure was diluted and inoculated with agricultural soil to establish nitrification in batch shaking cultures. Addition of high substrate levels ($NH_4^+ \geq 20$ mM) and pH values around pH 8 led to selective inhibition of nitrite oxidizing bacteria (NOB) by free ammonia (FA) and subsequently to an accumulation of NO_2^- in the batches up to concentrations of 30 mM. In the accumulation batches a NO_2^- building rate of 71 μM h^{-1} could be achieved starting from fresh inoculum.

Nitrite produced in this one-step batch process was tested on it's ability to inhibit fungal growth at pH values below 5. Different plant pathogens were tested in a newly developed filter paper test allowing quick and easy testing of antifungal components under the same culturing conditions. After addition of acidic amino acids (L-glutamic acid (Glu) and L-aspartic acid (Asp)) the nitrified liquid manure (NLM) showed an inhibitory effect of fungal growth on *Fusarium graminearum*, *Phoma eupyrena*, *Aspergillus nidulans*, *Ramularia punctiformis*, *Sclerotinia sclerotiorum* and *Ustilago maydis*.

In summary the developed foliar spray showed potential as a fungicide in lab experiments demonstrating the possibility to convert an agricultural waste product into an economical interesting resource that may be even applicable in organic farming.

Zusammenfassung

Intensive Viehwirtschaft führt in den Produktionsregionen zu einem Überschuss an Stickstoff. Um dadurch möglicherweise auftretende Umweltschäden durch Überdüngung, Ausgasung oder Auswaschung zu vermeiden, könnten die anfallenden Güllen zu einem wertvolleren Produkt umgewandelt werden.

Wir haben ein nitrithältiges Blattspray aus Schweinegülle in einem einfachen Batchprozess hergestellt, welches als Blattdünger mit zusätzlicher fungizider Wirkung in der Pflanzenproduktion zum Einsatz kommen könnte. Dazu wurde Schweinegülle verdünnt und mit Erde von landwirtschaftlich genützten Boden inokuliert um eine Nitrifizierung in den Batch-Kulturen zu erreichen. Die Zugabe von hohen Substratmengen ($NH_4^+ \geq$ 20 mM), bei pH Werten um 8, hat zu einer selektiven Inhibierung von Nitritoxidierern (NOBs) durch freien Ammoniak geführt, was die Akkumulation von Nitrit in der Kulturbrühe zur Folge hatte. Dabei konnten Konzentrationen von bis zu 30 mM erreicht werden bei einer Nitrit-Bildungsrate von 71 μM h^{-1}, ausgehend von frischem Inokulum. Das hergestellte Nitrit wurde auf die Fähigkeit zur Inhibierung von Pilzwachstum bei niedrigen pH Werten (< 5) getestet. Dazu wurde eine Feuchtekammer entwickelt, welche den schnellen und einfachen Vergleich von Pilzwachstum unter Zugabe von verschieden Komponenten bei gleichen Kulturbedingungen erlaubt. Nach der Absenkung des pH durch die Zugabe von sauren Aminosäuren (L-Glutaminsäure (Glu) bzw. L- Asparaginsäure (Asp)) zeigte die nitrifizierte Schweinegülle einen wachstumshemmenden Effekt gegen *Fusarium graminearum*, *Phoma eupyrena*, *Aspergillus nidulans*, *Ramularia punctiformis*, *Sclerotinia sclerotiorum* und *Ustilago maydis*.

Das entwickelte Blattspray bewies Potential als Blattdünger mit fungizider Wirkung in Laborexperimenten und zeigt die Möglichkeit auf, ein landwirtschaftliches Abfallprodukt in eine wirtschaftlich interessante Ressource zu verwandeln, die auch im biologischen Landbau eingesetzt werden könnte.

Contents

1 Introduction

1.1 Nitrogen Cycle

Nitrogen underlies a constant conversion in it's different forms by physical and biological processes. Galloway et al. [1] describe the role of anthropogenic and natural creation of reactive forms of nitrogen out of N_2 by fossil fuel combustion and inorganic fertilizer on a global scale which clearly shows the impact of human action on the transformation of the nitrogen cycle. Inefficient use and consequently nitrogen loss to air, water and land lead to severe environmental and human health problems [2]. To reduce loss of nitrogen in agricultural industry and increase efficiency in use of nitrogen in cropping systems, improvement in livestock management is required. In areas with intensive animal livestock production a nitrogen surplus occurs because nitrogen content in animal wastes exceed the crop needs in a given region. Restitution of animal wastes to regions where they would be needed as nitrogen source is expensive and energy intensive due to the high water content of animal excreta [3]. The reduction in mass and volume of these wastes are one option to face this problem, this thesis follows the idea of using animal wastes as a substrate to produce useful products in agricultural crop production. Liquid pig manure was used as a resource in an nitrification process to produce a nitrite-containing foliar fertilizer with an additional antifungal effect.

Figure 1.1 shows a simplified overview of the microbial processes participating in the nitrogen cycle. In this thesis we will focus on the processes of nitrification and denitrification (Fig. 1.1 2,3) since we want to use that ability of the microbes in a simple batch process. Resent studies show that a wide range of microorganisms contribute to the conversion processes in the nitrogen cycle [4]. The following introduction will focus on autotrophic organisms capable to carry out nitrification [5] as well as microbes performing denitrification, which is a more widespread metabolic activity within the microbial community [4].

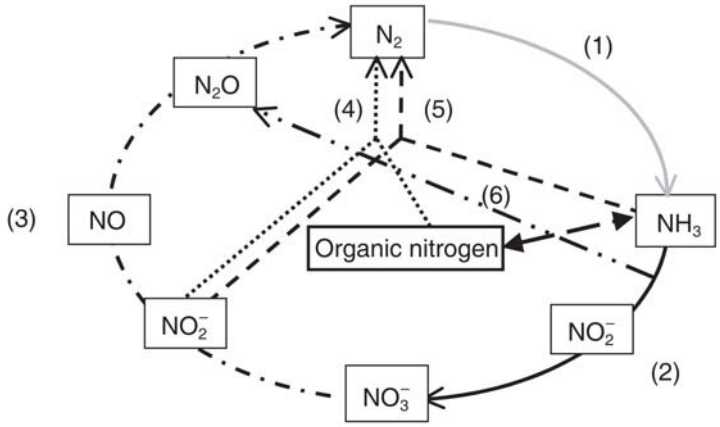

Figure 1.1 Microbial processes in the N-Cycle. Hayatsu et al. 2007 [4] (1) Nitrogen fixation, (2) nitrification, (3) aerobic and anaerobic denitrification,(4)(5) co-denitrification (fungi), (6) N_2O production during ammonia oxidation.

1.1.1 Nitrification

The conversion of ammonium (NH_4^+) over nitrite (NO_2^-) to nitrate (NO_3^-) by microbial activity is know as nitrification. This process is carried out by two different classes of proteobacteria. The oxidation of ammonium to nitrite is carried out by ammonia oxidizing bacteria (AOB) whereas the further oxidation to nitrate is performed by so called nitrite oxidizing bacteria (NOB). AOBs are classified in the genera *Nitrosmonas*, *Nitrosococcus* and *Nitrosospira* [6], whereas NOBs are distinguished between the genera *Nitrobacter*, *Nitrospina*, *Nitrococcus* and *Nitrospira* [7]. Natural habitats of these bacteria are soil, freshwater and marine environments but they were even found in extreme environments. The first step of nitrification (Fig. 1.2), also called ammonia oxidation, happens in two steps in AOBs [8]. Initially, ammonia is oxidized by the membrane bound enzyme ammonia monooxygenase (AMO) to hydroxylamine:

$$NH_3 + O_2 + 2\,H^+ + 2\,e^- \longrightarrow NH_2OH + H_2O \tag{1.1}$$

In a subsequent step hydroxylamine is further oxidized to nitrite by hydroxylamine oxidoreductase (HAO), located in the periplasmic space, anchored in the cytoplasmic membrane:

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5\,H^+ + 4\,e^- \tag{1.2}$$

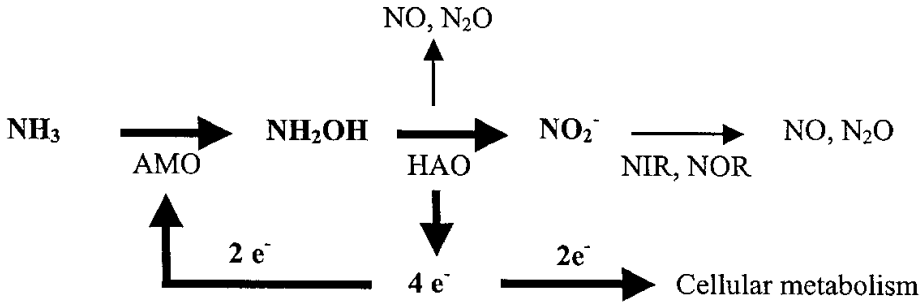

Figure 1.2 Ammonia oxidation in AOB. Arp et al. 2003 [9] AMO = ammonia monooxygenase, HAO = hydroxylamine oxidoreductase, NIR = nitrite reductase, NOR = nitric oxide reductase. Bold arrows show the major fluxes in the process.

The two enzymes function in a co-depended way serving electrons (for the AMO) and substrate (for the HAO) for each other. Two of the four electrons generated by the HAO reaction flow into the electron transport chain, finally generating energy in form of ATP. Combining the two reactions leads to the well known reaction for the first step in nitrification:

$$NH_4^+ + 1.5O_2 \longrightarrow NO_2^- + H_2O + 2\,H^+ \tag{1.3}$$

Arp et al. [9] stated a model for the electron transport chain for energy generation in *Nitrosomonas europaea* (Fig. 1.3). Two important nodes could be indentifyed so far. On the one hand the oxidation of hydroxylamine generating 4 electrons on the other hand the ubiquinone pool which seems to act as one universal electron transport system back to the AMO reaction as well as to the final electron acceptor O_2 (catalized by the terminal cytochrome oxigenase). By this reaction a proton motive force is build up across the cytoplasmic membrane which is used to generate energy. It is not clear until now if cytochrome complex III contributes in building up a proton gradient although electrons pass the complex.

The second step in nitrification carried out by NOBs is also called nitrite oxidation:

$$NO_2^- + 0.5O_2 \longrightarrow NO_3^- + H_2O \tag{1.4}$$

Key enzyme in this reaction is the nitrite oxidoreductase (NXR). Two electrons can be shuttled in the electron transport chain per oxidized NO_2^-, but not all mechanisms in this energy generating step of the NOBs are clarified until now.

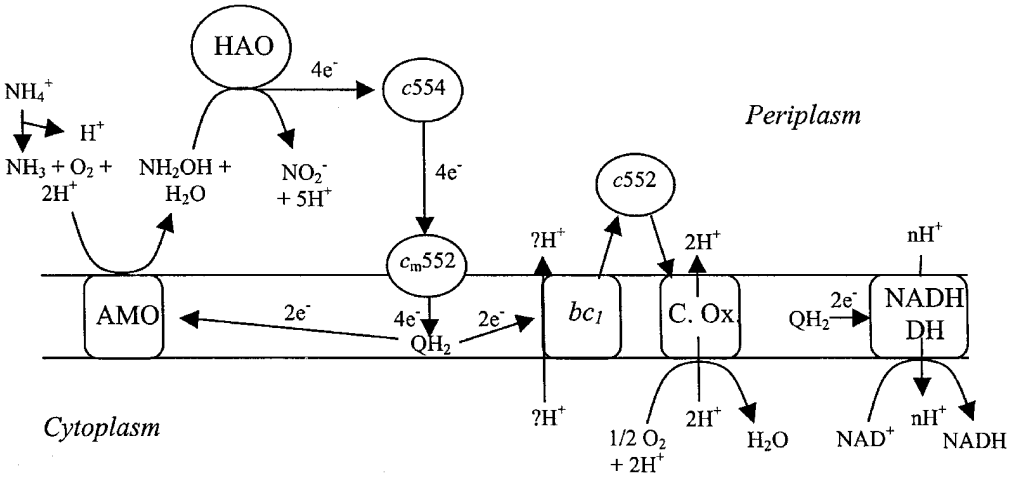

Figure 1.3 Schematic electron flow in the energy generating process of *N. europaea*. Arp et al. 2003 [9]. AMO = ammonia monooxygenase, HAO = hydroxylamine oxidoreductase, bc_1 = cytochrome complex III, C.Ox. = cytochrome oxidase, NADH DH = NADH dehydrogenase, c = cytochrome, QH_2 = quinol. Electrons generated in the HAO reaction are distributed by the ubiquinole pool to AMO and into the electron transport chain. Development of a proton gradient across the cytoplasmic membrane is used to generate energy.

Nitrobacter species were thought to be the main NOB organisms carrying out the majority of the metabolic conversion from nitrite to nitrate in microbial nitrification communities. Recent studies on the molecular level show that in many cases *Nitrospira* species have that role and thus became an interesting subject in research [10], [11]. Lücker et al. [12] proposed a genome based model of the energy metabolism of *Candidatus Nitrospira defluvii* (Fig. 1.4). Differences in the metabolic behavior of *Nitrospira* in contrast to *Nitrobacter* are quite remarkable. The CO_2 fixation process seems to be carried out in a reductive TCA cycle in *Nitrospira* whereas in *Nitrobacter* and *Nitrococcus* the Calvin-Benson-Bassham (CBB) cycle occurs [13]. NXR in *Nitrobacter* is capable to carry out a reverse reaction from NO_3^- to NO_2^- driven by electrons from organic compounds. The formation of NO_2^- out of NO_3^- has not been demonstrated experimentally in *Nitrospira*.

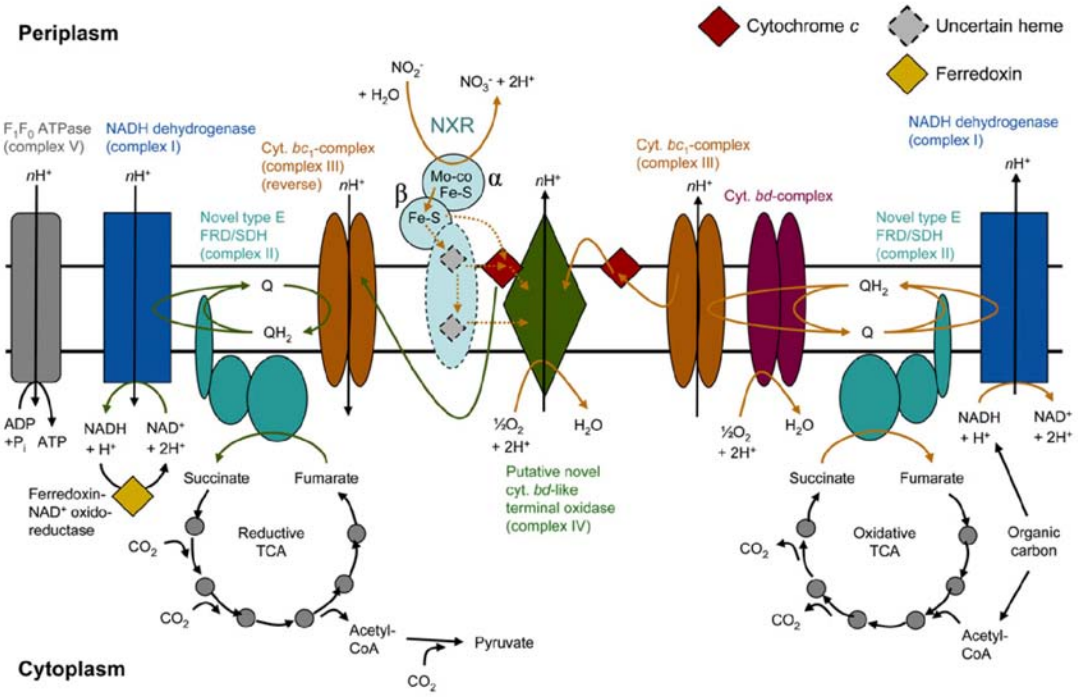

Figure 1.4 Actual model of the energy metabolism in *Ca. N. defluvii* based on genomic data. Lücker et al, 2010 [12]. Orange arrows show the proposed pathway of electrons for the oxidative part of the electron transport chain, whereas green ones indicate the flow of electrons from NO_2^- to NAD^+. Dashed lines point out that these parts of the model are uncertain. NXR = nitrite oxidoreductase; FDR = fumarat reductase; SDH = succinate dehydrogenase.

1.1.2 Denitrification

The ability to reduce NO_3^- to the end product N_2 over the intermediate steps NO_2^-, nitric oxide (NO), nitrous oxide (N_2O) is called denitrification. This reduction process is widespread over the microbial community [4], the following equations give an overview of the process in consecutive order:

$$NO_3^- + 2\,e^- + 2\,H^+ \longrightarrow NO_2^- + H_2O \tag{1.5}$$

$$NO_2^- + e^- + 2\,H^+ \longrightarrow NO + H_2O \tag{1.6}$$

$$2\,NO + 2\,e^- + 2\,H^+ \longrightarrow N_2O + H_2O \tag{1.7}$$

$$N_2O + 2\,e^- + 2\,H^+ \longrightarrow N_2 + H_2O \tag{1.8}$$

A detailed description on denitrification, the involved enzymes and genes can be found in a review of Bothe et al. [8]. In this review it's also pointed out, that some bacteria are not able to reduce nitrate fully to N_2, instead the end product is nitrite or nitrous oxide. In this thesis we wanted to carry out an incomplete denitrification by *Raoultella terrigena* (formerly *Klebsiella terrigena*), a bacterium only capable to carry out the reduction step of nitrate to nitrite [14].

1.1.3 Nitrite Accumulation

The selective inhibition of NOBs in a nitrification process by free ammonia (FA) and free nitrous acid (FNA) was first described by Anthonisen et al. in 1976 [15]. In recent years strategies were developed to implement this inhibition in waste water treatment processes to shorten the denitrification process and thus reduce process costs of treatment plants [16]. In this so called SHARON process (Single reactor system for High activity Ammonia Removal Over Nitrite) nitrification is stopped at the stage of nitrite to subsequently reduce the nitrite to N_2. The major advantages are a reduction in aeration costs for nitrification and lower carbon demand for denitrification.
We saw a possibility to use this effect in our small batch reactors to achieve high nitrite concentrations in one single step and keep the production of nitrate at a minimum at the same time.

1.2 Antimicrobial Effect of Acidified Nitrite

Nitrite has been known to have a antimicrobial effect since ancient times and was used e.g as curing salt in food preservation. Castellani et al. [17] described factors influencing the bacteriostatic effect of sodium nitrite and pointed out that the pH strongly determines the antimicrobial action of nitrite. Since he investigated a stronger inhibition of bacterial growth at low pH values he also stated, that the active form causing the inhibition is free nitrous acid (FNA). Further experimental data showed that FNA has a inhibitory effect on various microorganisms [18], [19], [20], [21]. Zhou et al. [22] investigated the role of FNA in waste water treatment plants and described three possible inhibition mechanisms which will be explained in more detail in the following paragraphs.

Uncoupling energy generation from growth It was shown that FNA acts as a protonophore and as such increases the permeability of the cell membrane for protons [23]. In this model (Fig. 1.5) FNA diffuses in the cell where it deprotonates, thus lowering the pH. To counteract that the cell is forced to pump protons out of the cell using ATP which is no longer available for energy generation. Mortensen et al. [24] found that pH is decreasing inside of yeast cells during exposure to FNA supporting this theory. Additionally activity of the ATPases in the cells was much lower when nitrite was present outside of the cell at low pH. This could also be explained by the higher proton concentration inside of the cell.

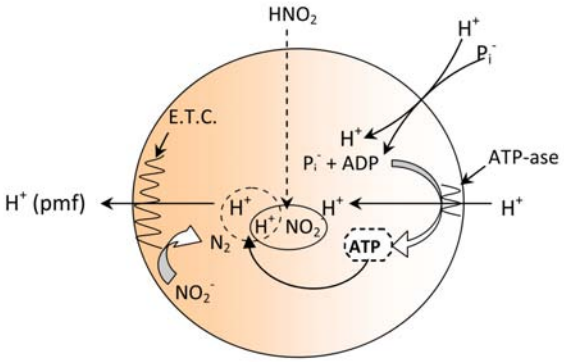

Figure 1.5 Uncoupling of ATP production and energy generation in a denitrifying cell. Zhou et al, 2011 [22] E.T.C. = electron transport chain; pmf = proton motiv force. FNA acts as protonophore increasing the permeability of the cell membrane for protons. To counteract, H^+ has to be actively pumped out of the cell by use of ATP. Thus ATP is no longer available in the cell to generate energy.

Enzymes affected by FNA Zhou et al. [22] reviewed data showing different interactions of FNA with enzymes. FNA was found to be the main factor regulating the expression of nitrite reductase NirK in *Nitrosomonas europaea* [25]. In addition investigation showed that FNA may interfere with the enzyme assemblage, resulting in inactive enzymes. It was also found that FNA could bind to copper containing enzymes like N_2O reductase, which would lead to an accumulation of toxic N_2O in the cell caused by inhibition of the enzyme. Another interaction of FNA with enzymes could happen in the TCA cycle where sulfhydryl containing enzymes are key regulators. These inhibitions would lead to a severe disturbance of the energy generation in the cell.

Nitric oxide (NO) inihibition Studies also concluded that the antimicrobial effect of acidified nitrite is related to the release of NO or other toxic reactive nitrogen intermediates [26].
However the process of inhibition on microbial growth observed in the presence of nitrite and low pH is not well understood yet. It is suggested that in many cases the effect is a combination of different inhibitory effects on cell viability.
NO also is known to nitrosylate amino acids and thus change their functional properties. Our group showed that flavohemoglobin proteins, which convert NO to NO_3^- in a dioxygenase reaction, are necessary to protect fungal cells against the deleterious effect of NO_2^- at low pH [27]. As such, flavohemoglobins may act to reduce the effective level of NO and thus prevent nitrosylation of structural and regulatory proteins.

1.3 Fungal Plant Pathogens

Fungal diseases on plants cause serious problems in crop production due to toxin production affecting human and livestock nutrition as well as a general reduction in quality of crops leading to economical damage. It was estimated that plant diseases contribute to an average loss in food production of 5 - 10% in developing countries [28].
The following section should give a short overview about the fungi used as test organisms in this thesis and the diseases they cause on plants. General information on fungi were collected from reference books [29], [30], [31].

Alternaria alternata Fungi of the genus *Alternaria* are found in atmosphere as well as soil and seeds. *A. alternata* is known as a plant pathogen infecting many different plant species, but it also occurs as storage pest causing spoilage of crops. The fungus occurs on leafs, stems, flowers and fruits of annual plants, mainly vegetables. Mycotoxins produces by *A. alternata* are separated mainly in three different classes described in detail by Logrieco et al. [32].
Diseases caused by *Alternaria* appear as spots on leafs (development of concentric rings) and blights but also stem, tuber and fruit rots are common. *A. alternata* is known to trigger the tomato fruit rot, black point disease on cereals or the blackmould on different crops among other diseases.

Alternaria solani another pathogen of the genus *Alternaria* shows similar symptoms as described above, but mainly causes diseases on tomato and potato (early blights, stem-, root-, fruit rots).

Aspergillus nidulans Among the *Aspergilli* we find many fungi which play an important role in industry. *A. nidulans* developed to act as a non-pathogenic model organism for filamentous fungi and thus is now one of the best investigated fungi. Since first test on growth inhibition by acidified nitrite where carried out on *A. nidulans* by Thorsten Schinko [27] it was also chosen to serve as a positive control in the growth tests of this thesis.

Aspergillus brasiliensis This fungus is a member of the black aspergilli and was described to be a own species by Varga et al. in 2007 [33]. Species of this genus are well known to cause food spoilage but are also used in industry as a production organism for enzymes and organic acids. Even though *A. brasiliensis* is no plant pathogen it is known to cause major damages by infecting agricultural products on a world-wide scale.

Botryotinia fuckeliana The anamorph form of the fungus is *Botrytis cinerea* and well known to cause the so called noble rot on grapes which lead to the production of sweet wines. Under humid and wet condition the pathogen leads to major damage in grape production triggering the grey mold disease on grapes [34]. Furthermore the pathogen infects vegetables, ornamentals, fruits and some field crops, the diseases appear as blossom blights, fruits rots, stem cankers but also tuber rots and leaf spots were observed.

Cochliobulus sativus is the teleomorph stage of the disease causing fungus *Bipolaris sorokiniana*. It is common on gasses, reduces leaf area by building of brownish spots, but also attacks stems, roots and kernels. *C. sativus* is known for the crown rot and common root rot, which is responsible for variable yield losses in crop production every year.

Fusarium graminearum The genus of *Fusarium* is a widespread important plant pathogen causing severe damage in cereal production due to mycotoxin production of the fungus. The main groups of mycotoxins synthesized by *Fusarium* are trichotechenes, zearalenones

and fumonisins. The building of deoxynivalenol (DON) by *F. graminearum* is a major threat in livestock feeding causing digestive disorders among other symptoms already at low concentrations. The corresponding disease on small-gain cereals is called fusarium head blight (FHB).

Phoma eupyrena The genus *Phoma* cause various diseases on vegetables and annual plants and are often present with other weak pathogens. *P. eupyrena* is known to be pathogen of the Phoma blight disease on fir and douglas-fir [35] as well as causing tuber rots on potatoes [36].

Ramularia punctiformis This pathogen is the anamorph of the fungus *Mycosphaerella punctiformis* and is also closely related to the well known plant pathogen *R. collo-cygni* causing the ramularia leaf spot disease (RLS) on barley [37], [38]. Species of this genus are responsible for fungal leaf spot diseases on many crops world-wide including cereals, banana, citrus and strawberries.

Sclerotinia sclerotiorum Fungi from the genus Sclerotinia belong to the ascomycota and cause destructive diseases on succulent crops like vegetables but also on grasses. They can affect the plant in all stages of growth and also after harvest. *S. sclerotiorum* is also called a soil borne pathogen because it overwinters as sclerotia on the field where it can infect plants again in the next cropping period.

Trichoderma longibrachiatum The genus *Trichoderma* is found in many habitats, mainly in the soil. Certain species where found to act parasitic or antagonistic against plant pathogenic fungi. Therefore they were studied as a bio control organism in soils [39], [40]. In this thesis *T. longibrachiatum* is tested to estimate the potential of a combined use with the produced fungicidal foliar spray.

Ustilago maydis The *Ustilanginales* belong to the basidiomycota and include the so called smut fungi which are important plant pathogens causing crop losses world-wide every year [41]. *U. maydis* infects maize and is dependent on living host tissue (like other smut fungi) for proliferation and development and was therefore well studied in recent

years to better understand plant pathogen interactions [42]. The fungus reduces yield by forming galls on the aboveground parts of the plant, teliospores which mature inside of the galls overwinter in the crop debris or in the soil and can stay infective over several years.

Aim of Work

Although 79% of the atmosphere consists of dinitrogen N_2, nitrogen is a limiting resource in agriculture which requires foresighted management. Intensive livestock units accumulate nitrogen through protein rich fodder which leads to a regional surplus of nitrogen, whereas shortages of nitrogen occur in other regions at the same time. A simple exchange of animal manures containing the surplus of nitrogen to this regions fails predominantly because of expensive transport, making the animal manures to a waste product requiring treatment to prevent environmental damages.

In this thesis we pursue the idea to convert a agricultural waste product into a resource for organic farming by implementing a well studied microbial process of soil environments into a batch process. In soils microbial communities convert ammonia over nitrite to nitrate to generate energy. Consequently the idea was to take the ammonium in liquid pig manure as a substrate in this process and convert it to nitrite as a end product. Since nitrite is known to have an antimicrobial effect under certain conditions we wanted to use the obtained product as a foliar spray against fungal pathogens on plants. Thus we would create the opportunity to reuse the surplus nitrogen in animal manures again for plant protection in organic crop production.

2 Material and Methods

2.1 Nitrogen Quantification

2.1.1 Sampling and Preparation of Batch Samples

To perform nitrogen analysis, 500 μL samples were taken in regular time steps from the different nitrification and denitrification experiments. Samples were stored in 1.5 mL micro tubes at -20°C until preparation and subsequent nitrogen quantification.
To prolong the correct function of the selective membranes of the ion selective electrode (ISE) used in potentiometric NO_2^- and NO_{3^-} measurement (see 2.1.3) , samples had to be treated and cleaned up before measurement. The micro tubes with the sample were centrifuged (Eppendorf Centrifuge 5424) at 14680 rpm for 5 min. Supernatant was pipetted in 250 μL portions into a 96-well filter plate (Millipore MAHVN45) with a cut-off of 0.45 μm. The liquid was pushed through the filter by applying pressure with an 30 mL syringe (Braun Omnifix © 30 mL) put on the top of the micro wells for removal of particles. Samples were collected in an 96 deep-well plate put underneath the filter plate.
Subsequently the filtrate was pipetted into a mini-spin column with a molecular weight cut-off of 10 kD (Roti©-Spin MINI-10), put into a new 1.5 mL micro tube to collect the filtrate. The mini-spin column was centrifuged at 14680 rpm for 10 min to remove macromolecules from samples and filtrate was stored at -20°C until measurement.
A control clean-up was carried out to test if analyte is lost during the sample preparation procedure. For that purpose a raw liquid manure sample was diluted to a concentration of 10 mM NH_4^+ and treated as described above together with a successful nitrified liquid manure (NLM) batch (NO_2^- = 23 mM). After each preparation step samples were taken and subsequently tested for NH_4^+ (colourmetric approach), NO_2^- and NO_3^- (via ISEs). As a last step the used mini-spin columns were washed with 200 μl deionised water (H_2O DI) which was also tested to detect possible restrained ions in the mini-spin membrane.

Table 2.1 Solutions for colourmetric NO_2^-, NO_3^- and NH_4^+ quantification. All solutions were prepared fresh on a daily basis, H_2O DI was used to dilute reagents and stock solutions.

NO_2^- / NO_3^-		
Griess 1	N-Naphthylethylendiamin Dihydrochlorid	0.77 mM
Griess 2	Sulfanilic Acid	58 mM
	HCl	3 M
VCl_3	Vanadiumchloride (III)	50.9 mM
	HCl	1 M
NH_4^+		
Colour Reagent	Na-Salicylat	1.06 M
	Na-Pentacyanonitrosylferrat(II) Dihydrat	4.29 mM
	NaOH	0.3 M
Oxidation Reagent	Na-Dichloroisocyanurat	3.9 mM

2.1.2 Colourmetric Nitrite, Nitrate and Ammonium Quantification

To monitor the different nitrogen-converting processes in the batches the determination of NO_2^-, NO_3^- and NH_4^+ concentrations were crucial. For this purpose a photometric method for measuring nitrogen species in soil solutions described by Hood-Nowotny [43] was used. The detection of nitrate in the samples with this method led to negative results, which was found to be depended on the nitrite concentration in the sample. An ion selective electrode measurement system provided by Lazar Research Laboratories (ArrowSTRAIGHT^{TM} Nitric Oxide Measurement System) was set up to achieve an independent quantification of the two ion species.

2.1.2.1 Colourmetric Ammonium Quantification

The assay for NH_4^+ quantification was based on a modified indophenol method derived from the berthelot reaction [44], shown in Fig. 2.1. For colour reaction 50 μL of standard or sample in duplicates, 25 μL colour reagent and 10 μL oxidation reagent (Table 2.1) were pipetted in described order into a microtiter plate, using multichannel pipettes to guarantee a quick addition of the reagents. The plates were incubated at room temperature for 30 min and absorbance at 660 nm was measured in an microtiter plate reader

(Enspire 2300 Multilabel Reader). A 1000 mM NH_4Cl stock solution was used to prepare standards by serial dilution (1.25 - 0.019 mM), samples were diluted 100-fold before measurement.

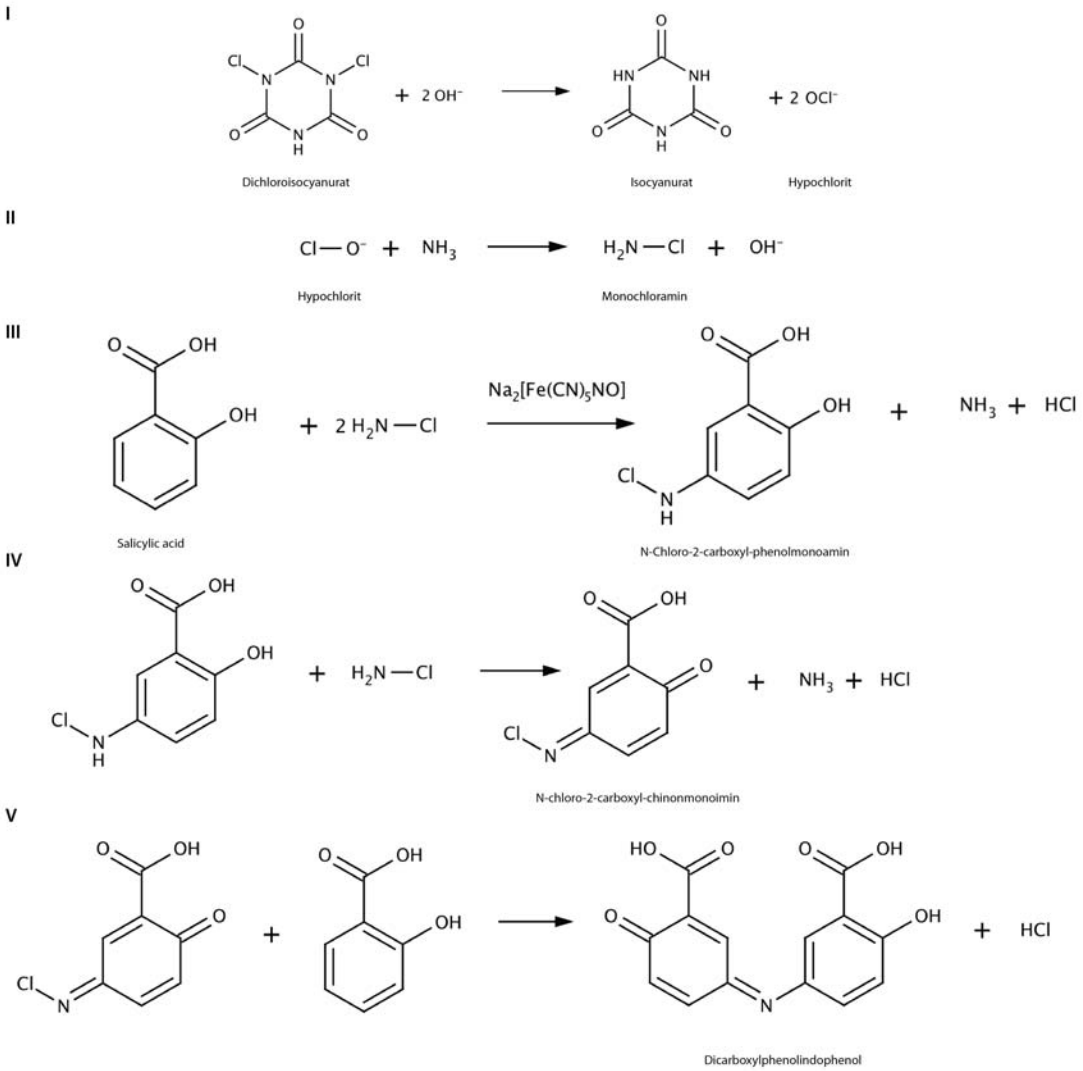

Figure 2.1 Modified indophenol reaction based on the berthelot reaction. Reaction takes place in alkaline environment where almost all of the NH_4^+ is NH_3 due to the principle of Le Chatelier-Braun. The NH_3 reacts with hypochlorit (derived from the dechlorination of dichloroisocyanurat (I)) to monochloramin (II). In subsequent steps (III + IV) monochloramin reacts with salicylic acid to N-Chloro-2-carboxyl-phenolmonoamin and further to N-Chloro-2-carboxyl-quinonmonoimin. In this steps Na-Pentacyanonitrosylferrat(II) Dihydrat serves as a catalyst. Finally the N-Chloro-2-carboxyl-quinonmonoimin reacts with another salicylic acid molecule to the blue coloured indophenol which can be detected at 660 nm.

2.1.2.2 Colourmetric Nitrite Quantification

A colourmetric assay based on the Griess reaction [45] (Fig. 2.2) was used. For colour reaction, 50 μL standard or sample was pipetted in duplicates into a microtiter plate adding 50 μL of a 1:1 mixture of Griess 1 and 2 (Table 2.1 - mixed immediately before use) using a multichannel pipette to guarantee quick addition of the reagents. Plates were incubated at 37°C for 60 min and subsequently the absorbance at 540 nm was measured in a microtiter plate reader (Enspire 2300 Multilabel Reader). A 1000 mM $NaNO_2^-$ stock solution was used to prepare standards by serial dilution (0.625 - 0.010 mM), samples were diluted 100-fold before measurement.

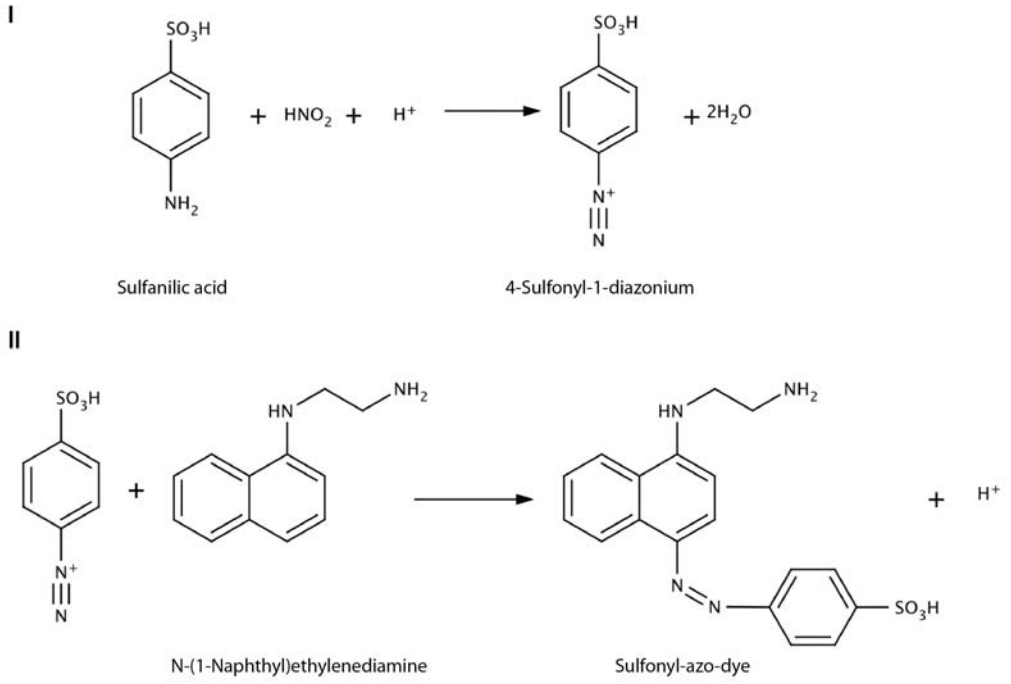

Figure 2.2 Colourmetric assay to determine NO_2^- and NO_3^- concentrations in samples. Sulfanilic acid reacts in an acidified environment with nitrous acid to form a diazonium-salt of the aromatic acid (I). In a second step this diazonium-salt reacts with N-(1-Naphthyl)ethylenediamine to the pink-colored sulfonyl-azo-dye which can be detected at 540nm (II).

2.1.2.3 Colourmetric Nitrate Quantification

For determination of nitrate in the samples, the same colourmetric reaction as for nitrite measurement was used, but with previous reduction of NO_3^- to NO_2^- through the addition of 50 μL VCl_3-solution (Table 2.1).

$$NO_3^- + 2\,V^{3+} + 2\,H^+ \longrightarrow NO_2^- + 2\,V^{4+} + H_2O \tag{2.1}$$

The final concentration of NO_3^- was obtained by subtraction of the values gained in NO_2^- measurement. A 1000 mM $NaNO_3^-$ stock solution was used to prepare standards by serial dilution (0.625 - 0.010 mM), samples were diluted 100-fold before measurement.

2.1.3 Potentiometric Nitrite and Nitrate Quantification via ion selective electrode (ISE)

2.1.3.1 General Principals of ISEs

ISEs detecting a wide range of different ions were developed to quantify concentrations in solutions. The key part plays an ionophore impregnated membrane that makes the electrode selective for a certain type of ion. When the electrode is placed in a solution an electrical potential develops across the membrane, which is compared to a reference potential from a reference electrode. The difference between these two potentials can be measured by a volt meter and further related to the activity of the ion in solution. The relation between the activity of the ion in solution and the measured potential is described by the Nernst equation:

$$E = E_0 + S \cdot \log \cdot a_{ci} \tag{2.2}$$

E = measured electrode potential
E_0 = reference potential
$a_{c,i}$ = activity of the ion in solution
S = electrode slope

The activity of the ion in solution is proportional to it's concentration and depends on the ionic strength of the solution:

$$\mathrm{a_{ci}}=\gamma_{\mathrm{ci}} * \frac{c_i}{c_0} \tag{2.3}$$

$a_{c/i}$ = activity of the ion in solution
$\gamma_{c,i}$ = activity coefficient
c_i = concentration of ion in real solution
c_0 = concentration of ion in standard state

To ensure accurate results an ionic strength adjustment buffer (ISA) is used for standards as well as samples to set the ionic strength to an equal value and bring the sample to the wanted pH where the electrode can work precisely.
Calibration of the electrodes is done by measuring two standards of defined concentrations of the ion of interest. The mV values are plotted versus the logarithm of the related concentrations of the standards to gain a response curve of the ISE. Working in the linear range of the electrode gives you values for slope and reference potential. By measuring the sample potential the concentration of the ions in the sample can be calculated by rearranging the Nernst equation:

$$\mathrm{E}=\mathrm{E_0} + \mathrm{S} \cdot \log \cdot [\mathrm{Ion}] \tag{2.4}$$

$$[\mathrm{Ion}]=10^{\frac{E-E_0}{S}}$$

[Ion] = concentration of ion in sample

2.1.3.2 Construction and Principles of ISEs in Use

NO_2^- For nitrite quantification a nitrogen oxide gas sensing electrode (Van London pHoenix.CO; Model# NOX1501) was used. The electrode consists of an inner glass pH electrode placed in the outer body of the electrode, which is filled with a 0.1 M $NaNO_2$ reference solution. A hydrophobic gas permeable membrane separates the internal filling solution from the sample solution outside. Placed in a nitrite containing sample (acidified by addition of sample buffer), gaseous anhydrides of the nitrous acid diffuse through the membrane until the partial pressure of the different nitrogen oxides is equal on both

sides. Since the internal concentration of nitrous acid [HNO_2] is affected by the diffused nitrogen oxides

$$2\,H^+ + 2\,NO_2^- \longleftrightarrow 2\,HNO_2 \longleftrightarrow NO + NO_2 + H_2O \longleftrightarrow N_2O_3 + H_2O \qquad (2.5)$$

and [NO_2^-] can be assumed as fixed due to the high concentrated internal solution, the electrode potential varies in a Nernstian manner with changes in [H^+]. Since [H^+] is proportional to [HNO_2] which is again dependent on [NO_2^-] in the sample outside of the electrode, the measured potential difference of the inner pH electrode can be used to determine [NO_2^-] in the sample.

NO_3^- For nitrate quantification a ISE with a polymer membrane was used (ASI Analytical Sensors & Instruments, Ltd - Model NO43). In this type of ISE an ion exchange material is incorporated into a PVC matrix. The electrode potential which arises from the membrane surface is related to the concentration of the ion species of interest. In our case the reference electrode consisted of a gel-filled chamber inside of the electrode so no reference filling solution was needed. The measured potential difference was used to determine [NO_3^-] in the sample as described above.

2.1.3.3 Assembly of the ISE Measurement System

A simple three way valve usually used to connect HPLC tubings purchased by GE Healthcare was used as an injection valve. The valve was used in two positions, one for rinsing the measuring cell, where a syringe (Braun Omnifix© 30 mL) with H_2O DI was placed and position two for sample injection with a disposable syringe (ONCE 1mL syringe with B.Braun Sterican needle Ø= 0.45 mm). A valve at the injection port was necessary to stop back flow after sample injection due to difference in altitude of measuring cell and waste container. This back flow resulted in unstable mV readings on the volt meter and had to be avoided.
The measuring cell was an acrylic flow cell where the electrode was placed approximately 1 mm above the bottom of the cell. This was crucial to reduce the sample volume needed to guarantee sufficient contact between sample and the sensing element of the electrode in the flow cell. Tests were carried out to determine the minimum sample volume needed to achieve repeatable results. For this purpose a defined standard solution was injected

repeatedly in the flow cell using different sample volumes. A volume of 300 μL was found to provide repeatable results, after repositioning of the electrodes and reducing the length of the tubings a volume of 250 μL was sufficient, which was also used throughout the experiments.
Sample was discarded by rinsing with H_2O DI in a 1000 mL beaker. The electrodes where connected to a portable voltmeter (JENCO Electronics, Model 6230N), where mV values were read. Values were saved in a table calculating program (LibreOffice Calc 3.5.3.2) where also further calculations were done.

2.1.3.4 Quantification of Nitrite and Nitrate via ISEs

Before first measurements were done the slope of the electrode response was tested. For nitrite detection it was stated to be about 56 mV according to the manual and 56±4 mV for the nitrate sensing electrode at 25°C and a 10-fold change in concentration. For both electrodes slope values where found to be in the stated range.
All standards and samples were tested in duplicates, all solutions where used at room temperature. Standards (10, 1 and 0.1 mM) were prepared freshly on a daily basis from a 100 mM stock solution (Table 2.2) by serial dilution in a microtiter plate with H_2O DI. Samples were prepared as described in section 2.1.1 and diluted 10-fold with H_2O DI in microtiter plates before measurement resulting in an end volume of 250 μL. Also from standard dilutions 250 μL were pipetted in a new microtiter plate. Immediately before measurement 25 μL of the sample buffer (Table 2.2) were added to samples and standards. A syringe (ONCE 1 mL) was used to inject the buffered sample in the injection valve. Using the meter in the autolock mode the display showed mV values when a stable end point was achieved (no change in mV value for 10 seconds). If no stable mV reading could be achieved the mV value after 45 seconds counting from injection was used, due to the stated response time in the electrode manuals.
For calibration three standards where measured (10, 1 and 0.1 mM) and plotted like described in section 2.1.3.1 (Graphpad Prism Version 5.0a). Subsequently samples were measured in random order and values were recorded. Between each sample injection the flow cell was rinsed with approximately 10 mL H_2O DI to be sure that all of the sample was washed out. Recalibration of the electrodes was done every 3-4 hours according to the manuals. For electrode storage over night or over weekend 5 mL of the appropriate storage solutions was injected in the flow cell (Table 2.2).

Table 2.2 Solutions for potentiometric NO_2^- and NO_3^- quantification via ISE. Solutions were prepared once and stored at 4°C, H_2O DI was used and concentrated H_2SO_4 to set the pH of the buffer solutions.

NO_2^-			
Standard Stock Solution	$NaNO_2$		0.1 M
Sample Buffer	Na_2SO_4	pH 1.3	1 M
Storage Solution	$NaNO_2$ Sample Buffer 10 % (v/v)		0.001 M
NO_3^-			
Standard Stock Solution	$NaNO_3$		0.1 M
Sample Buffer	Na_2SO_4	pH 2.5	1 M
Storage Solution	$NaNO_3$		0.01 M

2.2 Nitrification of Liquid Pig Manure

The available ammonium in liquid pig manure should be oxidized to nitrite and further to nitrate by microbial activity as it occurs in nature. A simple batch culture approach was chosen to establish nitrification in the liquid samples.

Preparation of Inoculum Conical tubes (50 mL) were prepared similar as described by Inselsbacher [46]. The tips of the tubes were cut of and a sieve was placed at the bottom of the tube to allow packing of the column. To achieve better aeration in the column agricultural soil (Tullnerfeld) was mixed with 50 % quartz sand (w/w) and subsequently 30 g were packed loosely in the tube. Soil was fed with 0.5 mL 10-fold diluted liquid manure per day and incubated at room temperature (RT) over 2 weeks. Every inoculum used in experiments was ground in a mortar to get a homogeneous mixture of the used matrix.

Nitrification in Batch Cultures In 100 mL Erlenmeyer flasks 20 mL of solutions containing NH_4^+ as a substrate for nitrification were placed. H_2O DI served as negative, NH_4Cl solutions as positive controls. Different dilutions of liquid pig manure were used

in the first experiments, a 10-fold dilution turned out to be the most suitable to start nitrification thus it was used in all following approaches. The batches were inoculated with 1 or 2 g of the used inoculum, covered with a piece of aluminum foil allowing aeration, and incubated on a rotary shaker (170 rpm) at 20°C. Samples of 500 μL were taken over the cultivation time in regular time steps and stored at -20°C until nitrogen quantification. pH was controlled using a indicator paper (Macherey-Nagel pH-Fix 2.0-9.0), for accurate results a pH meter was used (SCHOTT© Instruments Lab 850; pH electrode Blueline 14 pH).

Nitrification in Soil Columns In another approach a lab-scale soil system should serve as a continuous fixed-bed reactor to convert ammonium to nitrite and nitrate. For this purpose 50 mL conical tubes were prepared as described above. Underneath the tubes another conical tube was placed to catch up the liquid manure passed through the column. A peristaltic lab pump (GE-Healthcare Peristaltic Pump P-1) which was set on the lowest possible flow rate (1.65 mL h^{-1}) was used to pump a 10-fold dilution of the liquid manure on top of the soil column. Samples were taken before and after passing the soil column and tested on NH_4^+,NO_2^- and NO_3^-.

2.3 Denitrification of Nitrified Liquid Manure

Nitrification batches of liquid pig manure were stopped at different time points, resulting in nitrification broths with different concentrations of NH_4^+, NO_2^- and NO_3^-. Since the goal was to achieve a highest possible NO_2^- concentration, the following denitrification approach was used to reduce build NO_3^- to NO_2^-.
Raoultella terrigena TFI-08 was plated on TSA-agar (Table 2.5) to obtain single colonies. Plates were incubated at RT for 2 days and subsequently a single colony was suspended in 2 mL PBS-buffer (Table 2.5) using a sterile pipette tip. From the tested solutions, 4 mL were placed in small glass test tubes in triplets. H_2O DI served as a negative, nitrate broth (Table 2.5) as a positive control. Triplets were covered with an aluminum cap and autoclaved (121°C, 20 min). After cooling down to RT each test tube was inoculated with 10 μL of the bacteria suspension and subsequently incubated at 20°C.

2.4 Fungal Growth Inhibition of Nitrified Liquid Manure (NLM)

NO_2^- in combination with a pH below 5 showed an inhibitory effect on the growth of *A.nidulans* as recently shown in our lab [27]. Nitrification batches, where accumulation of NO_2^- could be achieved, were used in the further described experiments. To achieve a pH below 5 L-glutamic acid (Glu) and L-aspartic acid (Asp) respectively were used as acidifying agents. For positive controls NLM was replaced by $NaNO_2$ (10 mM final concentration), in negative controls NLM was omitted and replaced by H_2O DI.

2.4.1 Preparation of Spore Suspensions

The tested fungi were grown on malt-extract agar (MEA) plates until spores could be earned. Spores were transferred with a sterile toothpick in 2 mL PBS/0.1% Tween buffer (Table 2.5) and mixed well. From this stock suspension dilution series in 10^{-1} steps were prepared in PBS/0.1% Tween buffer. Spores from an adequate dilution were counted in a Fuchs-Rosenthal chamber and the number of spores per mL was calculated. At least 4 big squares of the chamber were counted, calculations were done with the following formula:

$$S_s = \frac{S_c}{A_c} * t * D_s \tag{2.6}$$

S_s = spore per mL suspension
S_c = spores counted
A_c = counted area in $mm^2 \hat{=}$ counted big squares
t = depth of the chamber in mm
D_s = dilution factor of the suspension

Suspensions were stored on 4°C until experiment but not longer than 5 days.

2.4.2 Growth Test on Agar Plates

Preparation of Solutions Each solution used in fungal growth tests was filtered through a disposable syringe filter with a cut-off of 0.22 μm, to get a sterile solution. H_2O DI served as negative control, a 100 mM $NaNO_2$ stock solution was prepared to serve as a positive control. All solutions were tested on pH by using a pH paper before use in the experiment.

Table 2.3 Fungal growth test on agar plates with plated solutions. Stock solutions: NO_2^- = 100 mM; L-glutamic acid = 100 mM; L-aspartic acid = 100 mM; nitrified liquid manure (NLM) (NO_2^- = 21 mM). Concentrations in the table are the end concentrations of each component after combination of stocks. 200 μL of each combination were plated on MEA plates, pH was measured with an pH glass electrode before use.

Component 1	**Component 2**	**pH**
$NaNO_2$ 10 mM	L-glutamic acid 10 mM	3.9
$NaNO_2$ 10 mM	L-aspartic acid 10 mM	3.5
H_2O DI	L-glutamic acid 10 mM	3.3
H_2O DI	L-aspartic acid 10 mM	3.1
$NaNO_2$ 10 mM	H_2O DI	10.8
NLM $[NO_2^-]$= 19 mM	L-glutamic acid 10 mM	5.0
NLM $[NO_2^-]$= 9.5 mM	L-glutamic acid 10 mM	3.9

Growth Test on Agar Plates In a first approach 200 μL of the combinations of NO_2^--source and the acidifying agent (Table 2.3) were plated on a MEA plate and subsequently 4.8 μL of 4 dilutions of the before counted *N.nidulans* suspension was pipetted in triplets on the plate resulting in 4 triplets with 10000, 1000, 100 and 10 spores per spot. After drying of the spots the plates were incubated bottom-up at 37°C and subsequently documented by taking pictures.

In a second approach the same amount of spores of *A.nidulans* were pipetted in spot triplets on a MEA-plate. After drying 8 μL of the nitrite - acid combinations (Table 2.4) were pipetted directly on the spots with the intention to achieve higher concentrations of the tested agents around the spores. Plates were also incubated at 37°C and documented by taking pictures.

Table 2.4 Fungal growth test on agar plates with spotted solutions. Stock solutions: NO_2^- = 100 mM; L-glutamic acid = 100 mM; L-aspartic acid = 100 mM; nitrified liquid manure (NLM) (NO_2^- = 21 mM). Concentrations in the table are the end concentrations of each component after combination of stocks. 8 μL of each combination was pipetted on *A.nidulans* spots on MEA plates, pH was checked with an pH paper before use.

Component 1	**Component 2**	**pH**
$NaNO_2$ 10 mM	L-glutamic acid 10 mM	4.0
$NaNO_2$ 50 mM	L-glutamic acid 50 mM	4.0
H_2O DI	L-glutamic acid 10 mM	3.0
$NaNO_2$ 10 mM	H_2O DI	10
$NaNO_2$ 10 mM	HCl	4.5
NLM $[NO_2^-]$= 19 mM	L-glutamic acid 10 mM	5.0
NLM $[NO_2^-]$= 9.5 mM	L-glutamic acid 10 mM	4.0
NLM $[NO_2^-]$= 9.5 mM	H_2O DI	6.0

2.4.3 Fungal Growth Test on Filter Paper in a Artificial Moist Chamber

Since no inhibitory effects could be detected with the above described tests on agar plates, another experimental setup had to be developed to achieve higher concentrations of the tested agents and also to simulate more natural growth conditions for the fungi. First experiments were carried out with *A.nidulans* grown at 37°C to get quicker results to adjust the set-up. Later on, experiments were done on different fungal plant pathogens grown at 20°C.

The goal was to grow the fungi on a cellulose filter paper in an small artificial moist-chamber (Fig. 2.3). For that purpose filter paper (Whatman 3mm Chromatography Paper) was cut into two square sizes (10x10 cm and 2x2 cm), autoclaved (121°C for 20 min) and dried in an compartment dryer. The bigger piece of filter paper was placed on the bottom of a Greiner square petri dish (120x120 mm). The smaller pieces were placed in 9 mini petri dishes (Ø= 35 mm), which were put in the big square dish without lid.

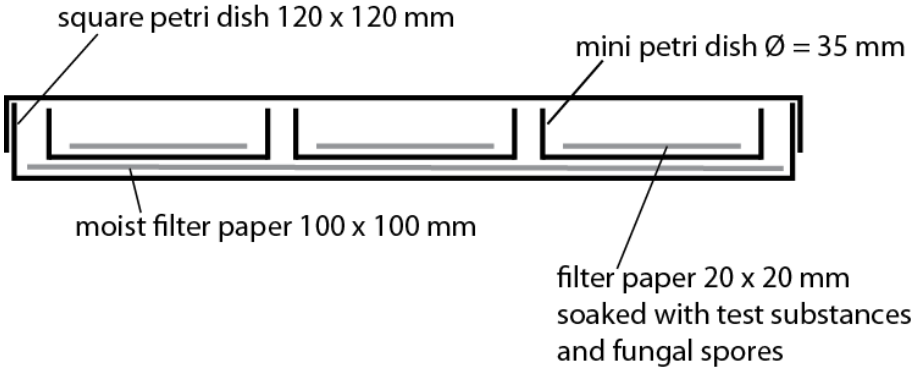

Figure 2.3 Scheme of the artificial moist chamber used in fungal growth tests. Filter paper was autoclaved before use and the chamber was sealed with a vinyl tape to guarantee a moist environment.

Spore solutions were diluted in a 2% malt extract broth (Table 2.5) to a concentration of 10^6 spores mL^{-1}. 60 μL of the spore solutions was pipetted on each small filter paper resulting in $6*10^4$ spores placed on each paper. Afterwards 30 μL of the nitrite source (H_2O, NO_2^-, NLM) as well as 30 μL of the acidifying agent (H_2O, Glu, Asp) were pipetted on top of the spore solution resulting in a combination scheme as follows:

Spores H_2O H_2O	Spores NO_2^- H_2O	Spores NLM H_2O
Spores H_2O Glu	Spores NO_2^- Glu	Spores NLM Glu
Spores H_2O Asp	Spores NO_2^- Asp	Spores NLM Asp

NO_2^- = from $NaNO_2$ solution (2.5 and 5 mM)
NLM = nitrified liquid manure with accumulated NO_2^- (2.5 and 5 mM)
Glu = L-glutamic acid (2.5 and 5 mM)
Asp = L-aspartic acid (2.5 and 5 mM)

Subsequently 8 mL of H_2O DI was pipetted on the big filter paper, the lid of the big square dish was closed and sealed with vinyl tape to guarantee a moist environment. The moist chambers were incubated either at 37°C in the first experiments with *A.nidulans* to get quicker results, later at 20°C in the tests on the different plant pathogens. Results were documented by taking pictures of the small filter papers in a 10-fold enlargement with a digital camera positioned over a stereo microscope (Olympus SZ61).
This set up provided an efficient method to check inhibitory effects on fungal growth under the same environmental conditions but with different combinations of possible influential agents.

2.5 Buffers, Media & Solutions

Trypticase Soy Agar (TSA)	17 g Bacto^{TM} Tryptone 3 g soy peptone 2,5 g glucose 5 g NaCl 2,5 g K_2HPO_4 15 g agar fill up to 1000 mL with H_2O DI autoclave (121°C; 20 min)
Malt Extract Agar (MEA)	48 g malt extract agar fill up to 1000 mL with H_2O DI autoclave (121°C; 10 min)
Nitrate Broth (NB)	3 g meat extract 4 g soy peptone 1 g Protease Peptone No. 3 1.516 g KNO_3 (for 15 mM NO_3^-) 3.03 g KNO_3 (for 30 mM NO_3^-) fill up to 1000 mL with H_2O DI autoclave (121°C; 20 min)
Malt Extract Broth 2%	20 g malt extract fill up to 1000 mL with H_2O DI autoclave (121°C; 20 min)
10x Phosphate buffered saline (PBS)	15.6 g NaH_2PO_4 x $2H_2O$ 87.66 g NaCl fill up to 900 mL with H_2O DI adjust pH with NaOH fill up to 1000 mL with H_2O DI autoclave (121°C; 20 min)
PBS/0.1% Tween	dilute 10x PBS 10-fold add 0.1% (v/v) Tween© 20

2.6 Reagents & Equipment

	Distributor
Agar	Carl Roth
L-aspartic acid	Fluka
Bacto^{TM} Tryptone	BD Biosciences
Bacto^{TM} Protease Peptone No.3	BD Biosciences
Conical Tubes 15; 50 mL	Greiner Bio One
Filter Paper	Whatman
Glucose	Carl Roth
L-glutamic acid	Sigma-Aldrich
HCl 12 M	Carl Roth
H_2SO_4 12 M	Carl Roth
K_2HPO_4	Carl Roth
KNO_3	Carl Roth
Malt Extract	Merck
Malt Extract Agar	Merck
Meat Extract	Carl Roth
Microtiter Plate 96-well	Greiner Bio One
Micro Tubes 1.5; 2 mL	Starlab
Mini Spin Columns	Carl Roth
NaCl	Carl Roth
Na-Dichloroisocyanorate	Alfa Aesar
NaH_2PO_4 x $2H_2O$	Carl Roth
$NaNO_2$	Sigma-Aldrich
$NaNO_3$	Carl Roth

Na-Pentacyanonitrosylferrat	Alfa Aesar
Na-Salicylate	Alfa Aesar
Na_2SO_4	Carl Roth
NaOH	Carl Roth
NH_4Cl	Carl Roth
N-naphtylethylendiamin	Fluka
Petri Dishes	Greiner Bio One
Soy Peptone	Carl Roth
Sulfanilic Acid	Fluka
Syring 1 mL	ONCE
Syringes 2; 5; 10; 20 mL	B.Braun
Syringe Filters	Carl Roth
Tween© 20	Carl Roth
VCl_3	Sigma-Aldrich

3 Results

3.1 Nitrogen Quantification

3.1.1 Calibration Curves

From values of all calibrations done throughout the measurements, means were calculated and plotted in figure 3.1. For the NO_3^--ISE (Fig. 3.1 a) average slope and reference potential were -52.5 and -65.27 respectively, R^2 was 0.9837. For the nitrogen gas sensing electrode measuring NO_2^- two mean response curves were calculated due to a reassembly of the electrode in between the measurements (Fig. 3.1 b). Slopes were similar (46.41 before, 45.31 after reassembly), reference potentials were 250.6 before and 273.0 after the reassembly. R^2 values were 0.9426 before and 0.9926 after reassembling of the electrode. For the colourmetric measurement of NH_4^+ the average values for slope and y-intersept were 0.7038 and 0.0237 respectively and a r^2 of 0.9657 was calculated (Fig. 3.1 c).

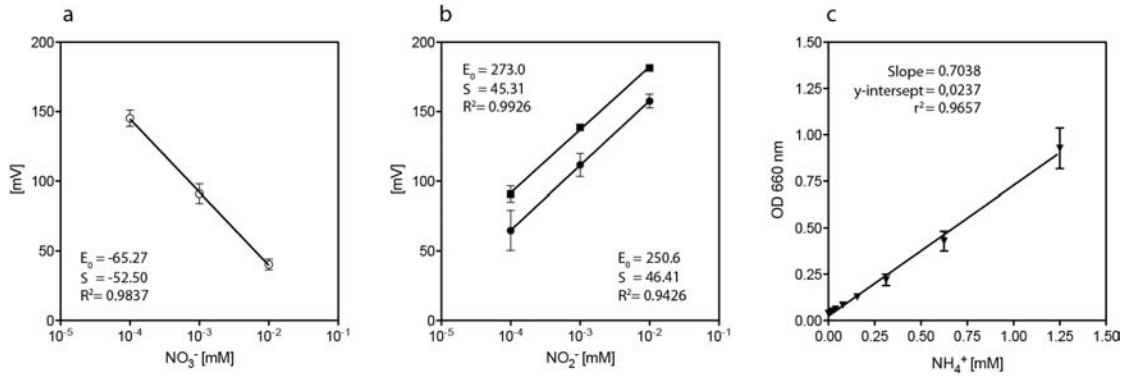

Figure 3.1 Calibration curves of the used nitrogen quantification methods. 10, 1 and 0.1 mM of $NaNO_3$ **(a)** and $NaNO_2$-standard **(b)** was plotted on a log-scale axis against the mV output of the ISE on a linear axis, subsequently linear regression was performed. Mean of all calibration values used during the experiments shown as data points ($\circ = NO_3^-$; $\bullet$ show the mean before, $\blacksquare$ after a reassembly of the nitric oxide electrode during NO_2^- measurements.) **(c)** Dilution series of a 1.25 mM NH_4Cl standard was plotted against the OD-values at 660 nm and linear regression was performed. Mean of all calibration values used during the experiments shown as ▼.Error bars show the standard deviation.

3.1.2 Interaction of NO_2^- on Colourimetric NO_3^- Quantification

Since first colourmetric measurements of NO_3^- led to negative results in the nitrification batches, the assays for NO_2^- and NO_3^- quantification were tested for interactions. For that purpose a defined concentration of the analyte (10 mM NO_2^- or rather NO_3^-) was added to a dilution series of the possibly interfering ion. Samples prepared from the same standards were measured via the colourmetric assay and via the ISE and plotted in figure 3.2.

In colourmetric assays measured NO_2^- (Fig. 3.2 a) was 11.13 ± 0.152 mM over all tested concentrations of NO_3^-. Nitrate was 10.87 ± 0.632 mM for added nitrite ranging from 0 to 2.8 mM. If NO_2^- is exceeding 2.8 mM, lower values for NO_3^- were measured (Fig. 3.2 b). Nitrite concentrations measured via ISE was 9.59 ± 1.109 mM over all tested concentrations of NO_3^- (Fig. 3.2 c). Nitrate concentration was found to be 10.64 ± 1.196 mM over all tested concentrations of NO_2^- (Fig. 3.2 d). Based on these results further quantification of NO_2^- and NO_3^- were done with the ISE. In conclusion the colourmetric assay for NO_3^- quantification was not suitable for our purposes since we expected NO_2^- concentrations in nitrification batches to exceed 2.8 mM.

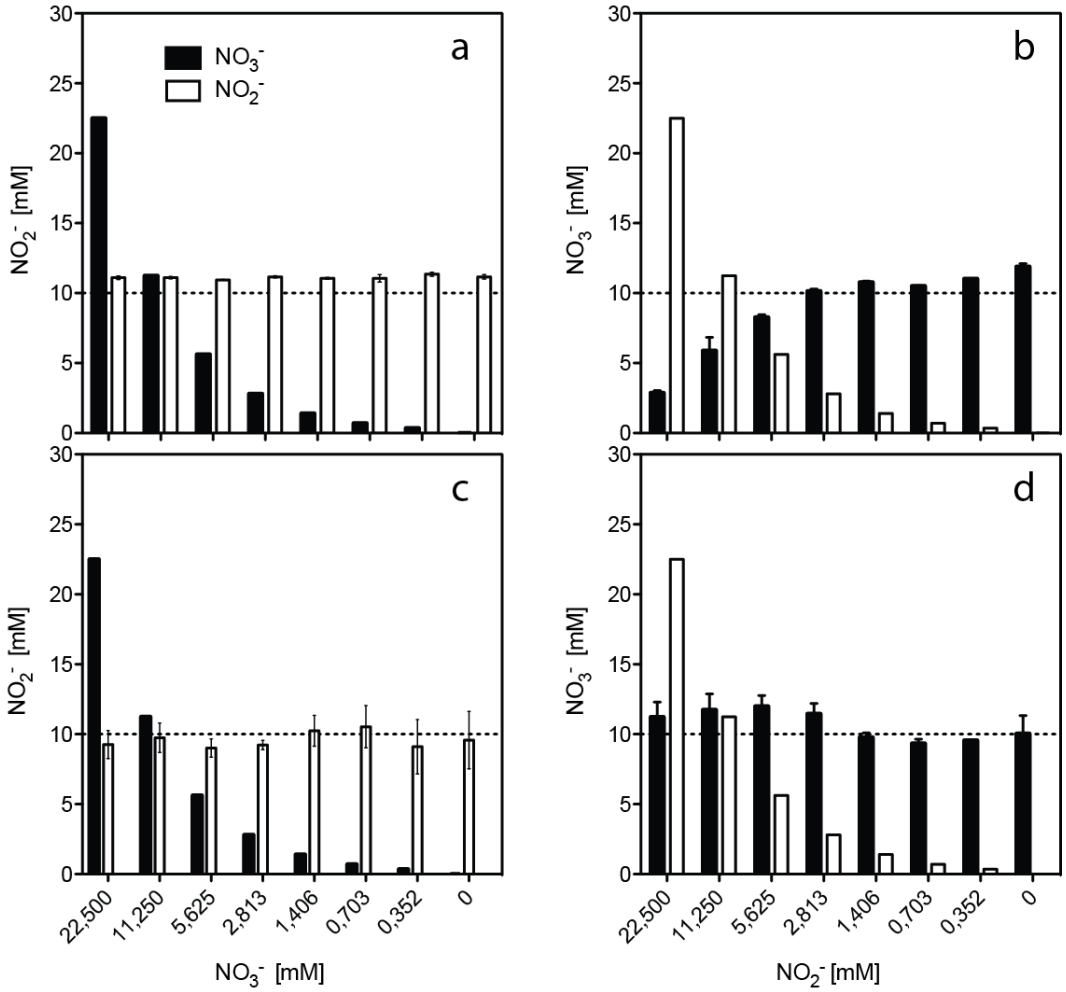

Figure 3.2 Interaction of NO_2^- and NO_3^- comparing the colourmetric with the potentiometric quantification method via ISEs. 10 mM of one ion (dotted line) was added to a dilution series of the contrary ion which influence should be tested.**(a)** colourmetric quantification of 10 mM NO_2^- in the presence of varying amounts of NO_3^-; **(b)** colourmetric quantification of 10 mM NO_3^- in the presence of varying amounts of NO_2^-; **(c)** potentiometric quantification of 10 mM NO_2^- in the presence of varying amounts of NO_3^-; **(d)** potentiometric quantification of 10 mM NO_3^- in the presence of varying amounts of NO_2^-; Error bars show the standard deviation.

3.1.3 Sample Treatment for NO_2^- & NO_3^- Quantification via ISE

Samples taken from the nitrification batches had to be treated before potentiometric quantification could be done (section 2.1.1). Measured concentrations of NH_4^+ (colourmetric approach), NO_2^- and NO_3^- (via ISEs) are shown in figure 3.3. Raw 10-fold diluted liquid manure was treated (Fig: 3.3 a) as well as a sample of a successful nitrified batch (Fig: 3.3 b). In the unfiltered raw liquid manure (1) a NH_4^+ concentration of 9.26 mM $\pm$ 0.406 was detected. After the first and second filtration step (2) (3), NH_4^+ was found to be 9.09 $\pm$0.353 and 9.09 $\pm$ 0.122 mM respectively. Finally 0.11 $\pm$ 0.130 mM NH_4^+ was found in the sample after the washing step of the sample preparation. All ion concentrations measured in this experiment are summarized in table 3.1.

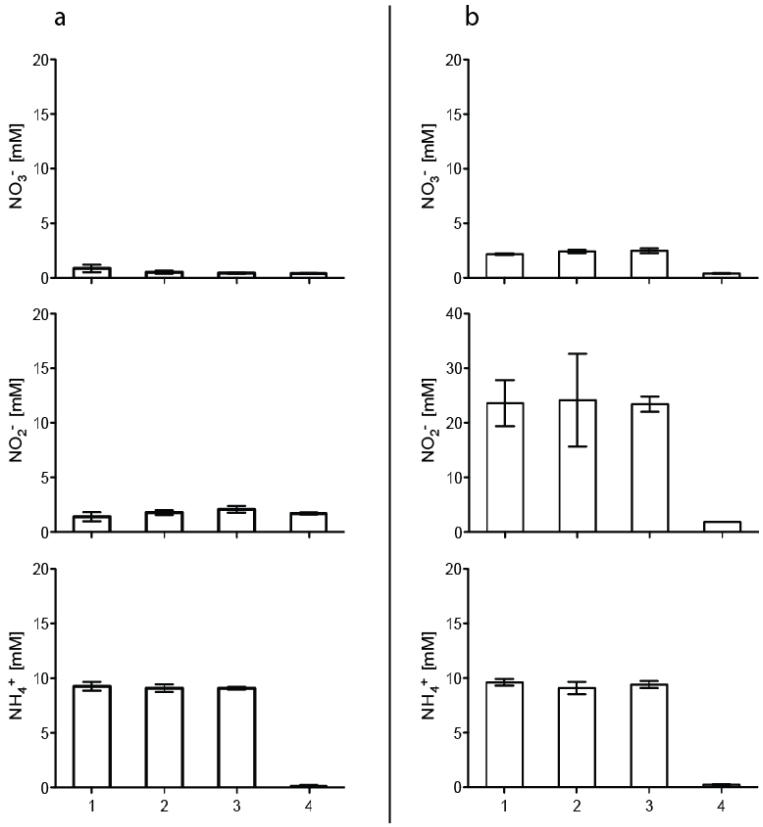

Figure 3.3 Sample treatment before quantification with ISE Raw 10-fold diluted liquid manure **(a)** and NLM **(b)**. **1:** untreated sample; **2:** after first filtration (cut-off = 0.45 μm); **3:** after filtration with Roti©-Spin MINI-10 (cut-off = 10 kD); **4:** H_2O DI used to wash mini spin columns after sample preparation. Error bars show the standard deviation.

Table 3.1 Ion concentrations of NH_4^+, NO_2^- and NO_3^- throughout sample treatment. Treatment was performed as described in section 2.1.1. **(a)** = 10-fold diluted raw liquid manure; **(b)** successful nitrified liquid manure.

	Ion concentrations in mM (mean±SD)			
Treatment[1]:	(1)	(2)	(3)	(4)
(a)				
NH_4^+	9.26 ± 0.41	9.09 ± 0.35	9.09 ± 0.12	0.11 ± 0.13
NO_2^-	1.41 ± 0.42	1.79 ± 0.21	2.08 ± 0.31	1.71 ± 0.10
NO_3^-	0.87 ± 0.34	0.53 ± 0.14	0.44 ± 0.04	0.41 ± 0.03
(b)				
NH_4^+	9.61 ± 0.31	9.09 ± 0.57	9.41 ± 0.33	0.24 ± 0.04
NO_2^-	23.60 ± 4.20	24.16 ± 8.47	23.43 ± 1.40	1.86 ± 0.00
NO_3^-	2.18 ± 0.07	2.43 ± 0.15	2.49 ± 0.23	0.41 ± 0.03

[1] (1) untreated sample; (2) after filtration (cut-off = 0.45 μm); (3) after mini-spin column (molecular weight cut-off = 10 kDa); (4) washing filtrate of final washing step

3.2 Nitrification of Liquid Pig Manure

3.2.1 Characterization of Raw Liquid Pig Manure

Raw liquid pig manure was tested on the ions of interest as well as urea to define the initial concentrations before nitrification experiments were carried out. Obtained values from measurements throughout the experimental work were collected to calculate averages and standard deviations. Ammonium was found to be 136 ± 4.6 mM, nitrite and nitrate were 0.2 ± 0.06 and 0.5 ± 0.2 mM respectively. Urea could not be detected in the liquid manure. In raw liquid pig manure a pH of 8.50 was measured, in the 10-fold diluted liquid manure it was 8.38.

3.2.2 Nitrification in Batch Cultures with Biovin as Inoculum

A nitrification experiment in 100 mL Erlenmeyer flasks was inoculated with Biovin and incubated for 14 days (336 h) on a rotary shaker at 20°C. Biovin is an organic fertilizer produced from humified grapevine pomace [47]. As a standardized product with a high biological activity and buffer capacity it was our first choice to start a nitrification process in the liquid pig manure. As shown in figure 3.4 (a,b) the concentrations of the measured

ions didn't change over the incubation time either in the negative (a) nor in the positive (b) control of the assay. NO_3^- and NO_2^- values were constant at 24 mM and 0.1 mM respectively in both controls. NH_4^+ was detected at a concentration of 8.5 mM in the negative, and 10.5 mM in the positive control, this concentrations were also detected throughout the hole incubation time.
In the three assays with raw (Fig. 3.4 c) and diluted liquid manure (d = 5-fold, e = 10-fold diluted), NH_4^+ concentrations decreased whereas NO_2^- and NO_3^- concentrations were stable over the incubation time. In raw liquid manure a NH_4^+ concentration decrease of 65% from initially 168 to 60 mM was measured. In the 5-fold diluted liquid manure a decrease of 43% from 33.6 to 19 mM and in the 10-fold diluted assay a decrease of only 7 % from 17.9 to 16.6 mM was observed. pH values in raw undiluted liquid manure increased from 8.5 to 9.5 within 3 days. In the 10-fold diluted assay pH increased from initial 8.38 to 8.8. In both assays pH remained constant over the experiment after the initial increase. Further experiments were carried out with 10-fold diluted liquid manure because little loss in substrate could be expected. By inoculating the liquid manure with Biovin no nitrification could be established in the batches.

3.2.3 Nitrification in Soil Columns

Two experiments were carried out using small soil columns as fixed-bed reactor for the nitrification of liquid manure (see section 2.2). In a first approach (Fig: 3.5 a), 16 mL of 10-fold diluted liquid manure was pumped at a flow rate of 1.7 mL h^{-1} on the top of the soil/sand packed column and collected after passing the column. NH_4^+ concentration dropped from 16 mM in the loaded liquid manure to 0.5 mM in the passed liquid. NO_2^- concentration stayed unchanged at 0.1 mM before and after the column and NO_3^- slightly increases from 0.6 to 1.6 mM.
In a second approach (Fig: 3.5 b) two fractions of the liquid manure passed through the column were collected. NH_4^+ concentration in the diluted liquid manure decreased from 19 to 5.8 mM in the first fraction and increased again to 9.3 mM in the second one. NO_2^- could not be detected in neither of the three fractions, whereas NO_3^- increased from 0.4 before column to 1.0 mM in the first fraction, but afterwards decreased again to 0.4 mM in the second fraction.

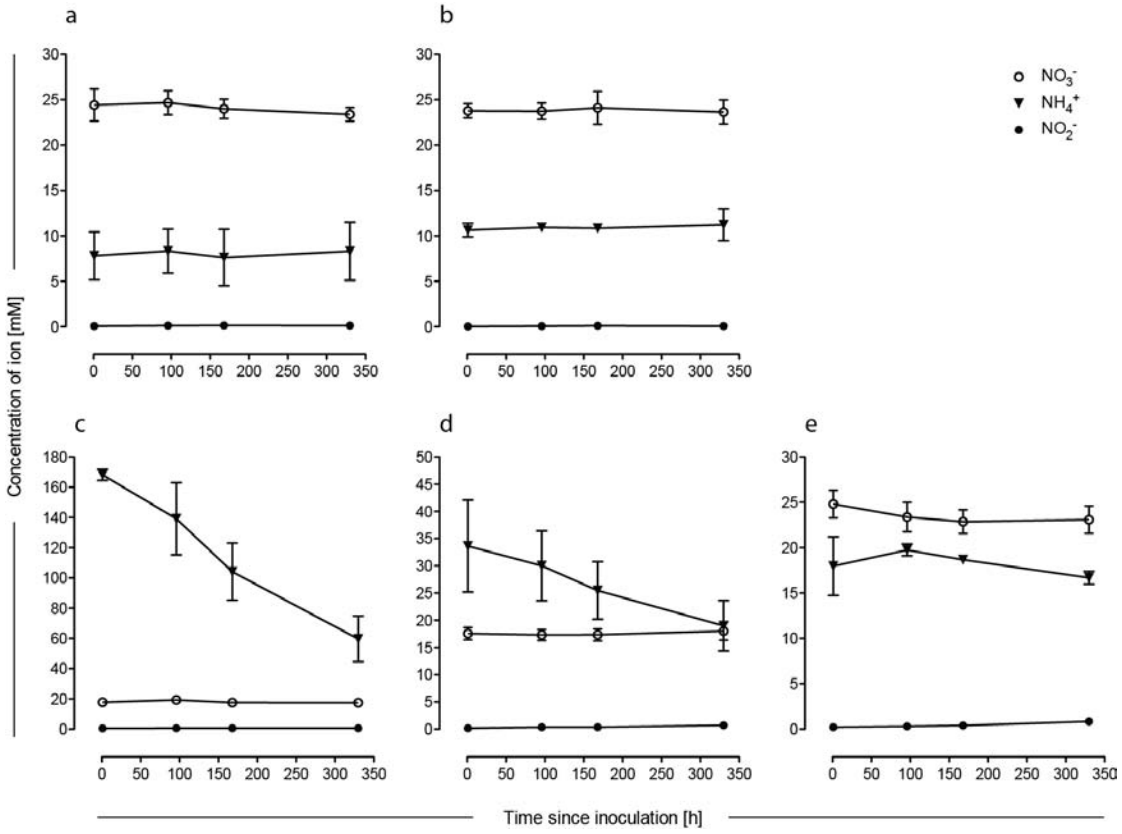

Figure 3.4 Nitrification in batch cultures with Biovin as inoculum. 20 mL of liquid in triplets were inoculated with 1 g of Biovin and put on a rotary shaker at 170 rpm for 14 days (336 h) at 20°C.**(a)** negative control: H_2O DI; **(b)** positive control: NH_4Cl solution 1 mM; **(c)** liquid manure undiluted; **(d)** liquid manure 5-fold diluted with H_2O DI; **(e)** liquid manure 10-fold diluted with H_2O DI; Error bars show the standard deviation.

3.2.4 Nitrification in Batch Cultures with Soil as Inoculum

First successful nitrification was observed in a batch culture experiment using soil from the column experiment described above as inoculum. An increase of NO_2^- concentration in the approach with 10-fold diluted liquid manure was observed after 11 days of inoculation (data not shown). It was assumed that the NH_4^+ put on the soil columns increased the number of AOBs in the soil [48], [5]. All further experiments were incubated with fed-soil (described in section 2.2) resulting in successful nitrification in the batches.
Figure 3.6 shows a successful nitrification approach over 52 days on 10-fold diluted liquid

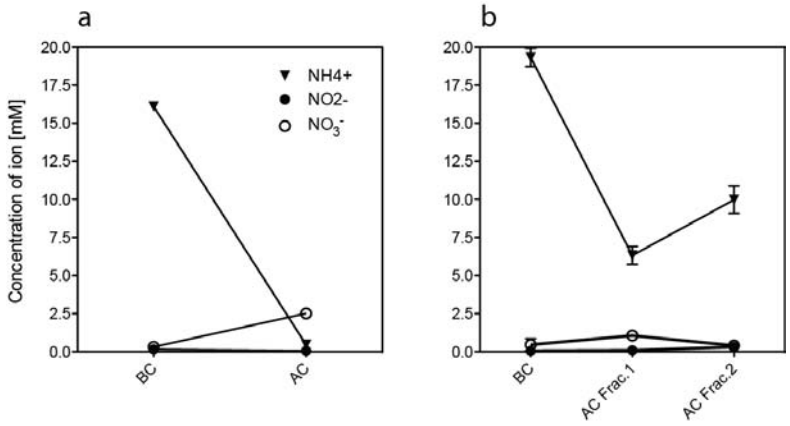

Figure 3.5 Nitrification approach in small soil columns and constant addition of diluted liquid manure. **(a)** 30 g of 50% soil / 50% sand (w/w) placed in 50 mL Greiner tube on a sieve. Addition of 16 mL diluted liquid manure via a tube pump at a flow rate of 1.7 mL h^{-1}. **(b)** 50 mL Greiner tube with three sieves kept in distance, 10 g soil-sand mixture placed on each sieve. Diluted liquid manure addition via tube pump at a flow rate of 1.3 mL h^{-1}, liquid manure passed through the column collected in 2 fractions. 1^{st} fraction 0-94 h pumping time, 120 mL loaded on column; 2^{nd} fraction 94 - 162 h pumping time, 88 mL diluted liquid manure loaded on column. BC = before column; AC = after column; AC Frac.1 = after column first fraction; AC Frac.2 = after column second fraction. Error bars show the standard deviation.

manure using fed-soil as an inoculum. Complete oxidation of NH_4^+ to NO_3^- could be achieved in this approach. In the negative control (Fig: 3.6 a) no NH_4^+ and NO_2^- was detected over the hole experiment, for NO_3^- a small increase up to 1.5 mM in the last taken sample could be observed. In the positive control (Fig: 3.6 b) the NH_4^+ concentration was decreasing from 10.7 to 1.0 mM 23 days (552 h) after inoculation where fresh substrate (NH_4Cl solution 15 mM) was added resulting in 2.5 mM NH_4^+ in the batch. Another decrease to 0.5 mM 38 days (912 h) after inoculation took place followed by new addition of fresh substrate ((NH_4Cl solution 50 mM) resulting in 10.1 mM NH_4^+ in the batch decreasing again to 1.5 mM at 52 days (1248 h) when experiment was ended. NO_2^- and NO_3^- concentrations showed a simultaneous increase until the first addition of new substrate to 4.4 and 3.7 mM respectively. After that NO_2^- values decrease to 0 mM whereas NO_3^- constantly increases to 29.9 mM in the batch at the end of the experiment, what is consistent with a complete conversion of NH_4^+ to NO_3^-. The approach with 10-fold diluted liquid manure (Fig: 3.6 c) showed a more rapid decrease of NH_4^+ from 9.4 to 0.4 mM 18 days (432 h) after inoculation. At 23 days (552 h) fresh substrate was added (5-fold diluted liquid manure) resulting in 4.5 mM NH_4^+ in the batch. A quick decrease to 0.2 mM at 30 days (720 h) followed until fresh substrate after 38 days (912 h) was

added (undiluted liquid manure) resulting in 14.3 mM NH_4^+ decreasing to 0.5 at the end of the experiment. NO_2^- and NO_3^- concentrations behaved similarly like in the positive control, although NO_2^- increases faster than NO_3^- until the first addition of substrate. Highest level of NO_2^- was detected 28 days (672 h) after inoculation (7.2 mM), highest level of NO_3^- was measured at the end of experiment (40 mM).

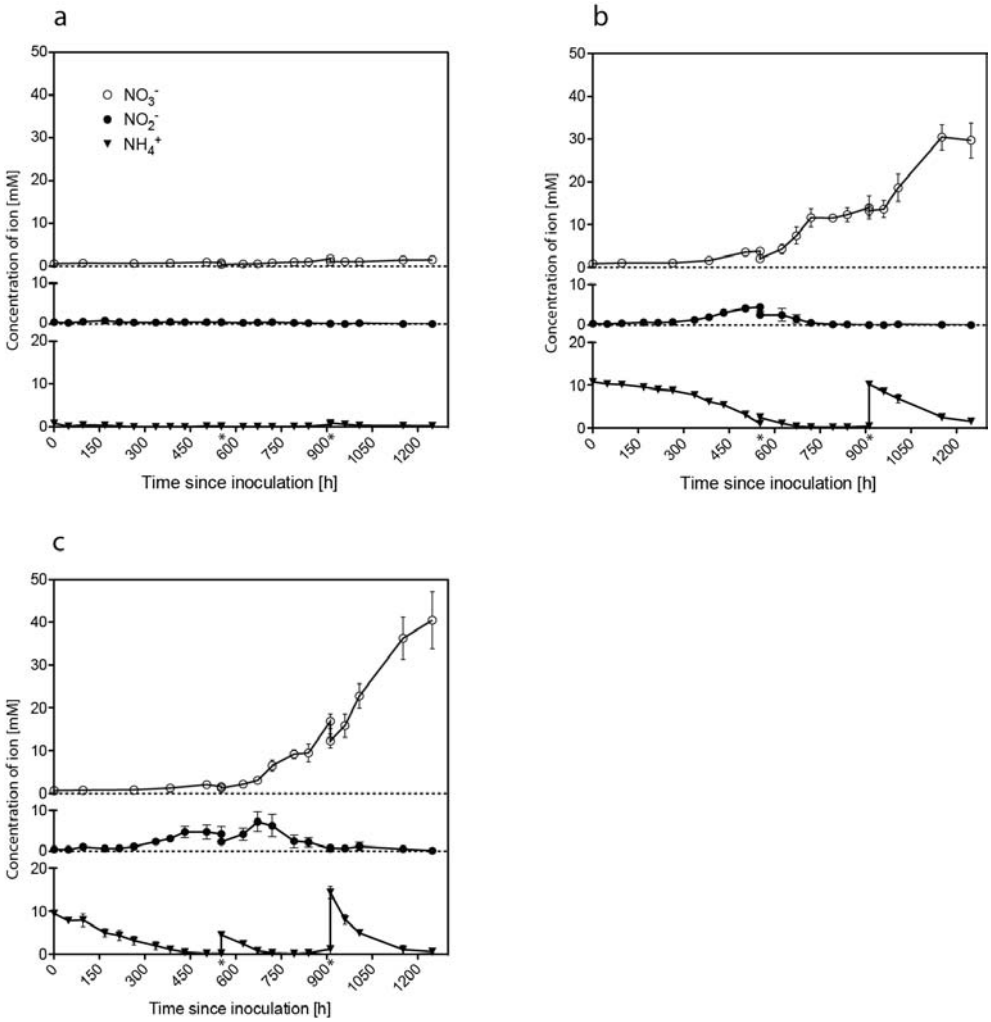

Figure 3.6 Nitrification in batch cultures with fed soil as inoculum. 20 mL of liquid placed in 100 mL Erlenmeyer flask in triplets and inoculated with 2g fed soil as described in material and methods. Incubation at 20°C on a rotary shaker at 170 rpm for 52 days. Fresh substrate added 23 days (552 h) and 38 days (912 h) after inoculation (∗). **(a)** negative control: 20 mL of H_2O DI; **(b)** positive control: 10 mM NH_4Cl solution; **(c)** 10-fold diluted liquid manure; ▼ = NH_4^+; • = NO_2^-; ○ = NO_3^-. Error bars show the standard deviation.

3.2.5 Nitrification with Accumulation of Nitrite in Batch Cultures

Since it was clear, that nitrification of liquid pig manure works in batch cultures with soil as inoculum, batches were set up to produce larger amounts of NLM for the subsequent reduction of the produced NO_3^- to NO_2^- by partial denitrification with *Raoultella terrigena* TFI-08. Within a scale-up attempt batches were fed with high amounts of NH_4^+ added in irregular time steps. At the end of incubation they showed a high concentration of NO_2^- (21 mM), whereas NO_3^- was low (5 mM). FA and FNA were shown to be capable to selectively inhibit the oxidation step from NO_2^- to NO_3^- carried out by NOBs [15], [49]. This selective inhibition allows an accumulation of NO_2^- in a nitrification processes.
A new process design was set up to check if the accumulation of NO_2^- can be repeated in the liquid pig manure batches by addition of high amounts NH_4^+ and consequently exceed the threshold concentration of FA for inhibition of the ammonia oxidizing community. For this purpose addition of raw undiluted liquid manure (16 days after inoculation) to reach a concentration over 20 mM of ammonium after the initial amount of ammonium in the batches was almost used up (Fig.3.7) was carried out. After 28 days NO_2^- reached a concentration of 25 mM whereas NO_3^- was found to be 4 mM, indicating an accumulation of NO_2^- in the batch.

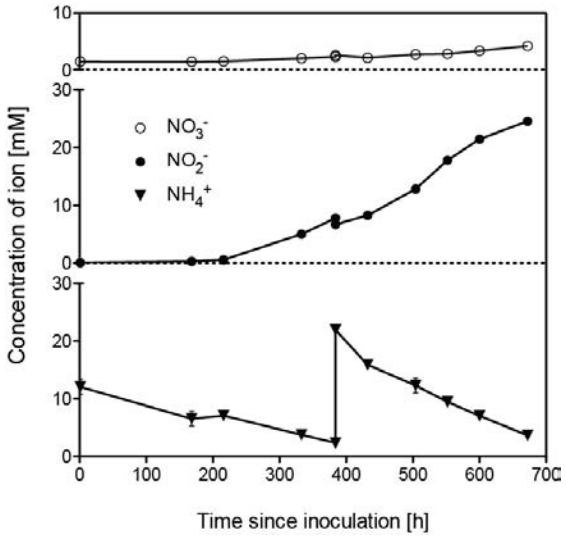

Figure 3.7 Nitrification in batch cultures with accumulation of nitrite. 20 mL of 10-fold diluted liquid manure placed in 100 mL Erlenmeyer flask in triplets and inoculated with 2 g fed soil. Incubation at 20°C on a rotary shaker at 170 rpm for 28 days. ▼ = NH_4^+; • = NO_2^-;○ = NO_3^-; error bars show the standard deviation.

3.2.6 Substrate and Product Building Rates

To know how fast NH_4^+ decreases and oxidized nitrogen (nitrite and nitrate) increases in the batches, linear regression was performed through data points which showed a linear de- or increase in ion concentration. Slope values gained from this procedure show the change in ion concentration over time (mM h^{-1}). Data was analyzed from the nitrification shown in figure 3.6 and from nitrification with accumulation of NO_2^- shown in figure 3.7. Results are summarized in table 3.2.

Table 3.2 Change of substrate and products concentrations in nitrification batches over time Linear regression through data points showing a linear de- or increase of ion concentration in the batches. Slope value gives the change of ion concentration in mM h^{-1}.

	NH_4^+		NO_2^-		NO_3^-	
	Slope	r^2	Slope	r^2	Slope	r^2
NLM[1] (a,b,c)						
1	-0.018	0.9901	0.016	0.9453	0.005	0.9692
2	-0.031	0.9990	0.040	0.9378	0.015	0.9847
3	0.000	0.0000	-0.036	0.8807	0.026	0.9061
4	-0.098	0.9641	0.004	0.4800	0.109	0.9684
Positive control[2] (d,e,f)						
1	-0.027	0.9717	0.014	0.9760	0.010	0.9475
2	-0.014	0.9395	-0.012	0.8254	0.056	0.9451
3	-0.032	0.9992	0.000	0.2337	0.076	0.9696
NLM[3] (g,h,i)						
1	-0.028	1.000	0.042	0.9920	0.005	0.9978
2	-0.066	0.9650	0.071	0.9757	0.004	0.6711

[1] 10-fold diluted liquid manure, nitrified for 52 days - no accumulation of NO_2^-; Figure 3.6 (c)
[2] NH_4Cl solution 10 mM, nitrified for 52 days - no accumulation of NO_2^-; Figure 3.6 (b)
[3] 10-fold diluted liquid manure, nitrified for 28 days - accumulation of NO_2^-; Figure 3.7

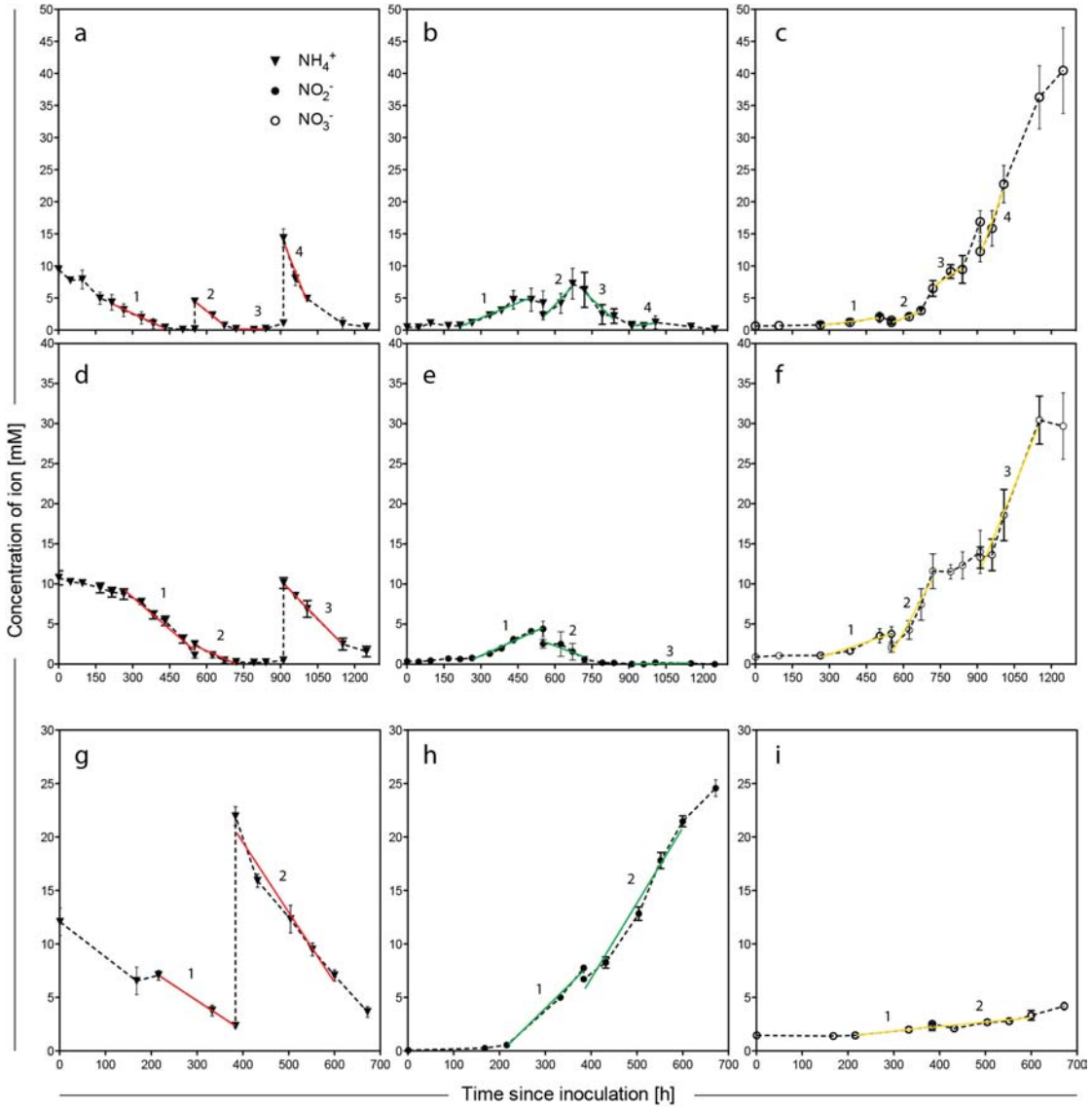

Figure 3.8 Change in ion concentrations in nitrification batches. Linear regressions were performed through data points where de- or increase in ion concentration was linear, linear phases indicated by numbers in the graphs, data shown in Table 3.2. **(a,b,c)** diluted liquid manure (Figure 3.6 c) no accumulation of NO_2^-, substrate completely oxidized to NO_3^-; **(d,e,f)** NH_4Cl solution 10 mM (Figure 3.6 b); **(g,h,i)** 10-fold diluted liquid manure (Figure 3.7) accumulation of NO_2^-;error bars show the standard deviation; ▼ = NH_4^+; ● = NO_2^-;○ = NO_3^-

3.3 Denitrification of Nitrified Liquid Manure

Reduction of NO_3^- to NO_2^- was carried out in denitrification experiments by adding *R.terrigena* to previously sterilized samples containing NO_3^-. Figure 3.9 shows results of the denitrification approach with a 15 mM nitrate broth serving as a positive control. NO_3^- concentration decreased in this control from 13.3 to 1.2 mM 72 hours after inoculation with *R.terrigena*. Simultaneously NO_2^- increased from 0.3 to 7.8 mM and NH_4^+ from 1.4 to 2.4 mM. The negative control showed no change in NO_2^- and NO_3^- but an decrease in NH_4^+ from 3.5 to 2.5 mM. The two samples with successful nitrified liquid manure showed no changes in any of the measured ions.

Results using a slightly different approach are shown in figure 3.10. In the positive control (nitrate broth 30 mM) NO_3^- decreased from 35.7 to 1.5 mM in 120 hours after addition of *R.terrigena*. Simultaneously NO_2^- increased from 0.7 to 31.2 mM and NH_4^+ from 2.0 to 6.9 mM. Again no changes in concentration of NO_2^- and NO_3^- could have been observed in the negative control, whereas NH_4^+ slightly increased again from 0.2 to 1.0 mM. No significant changes in ion concentrations could be detected in the sample containing sterile NLM either.

Further optimization of partial denitrification by *R.terrigena* TFI-08 was not done, as in the meantime accumulation of NO_2^- could be achieved in liquid manure batch cultures (section 3.2.5).

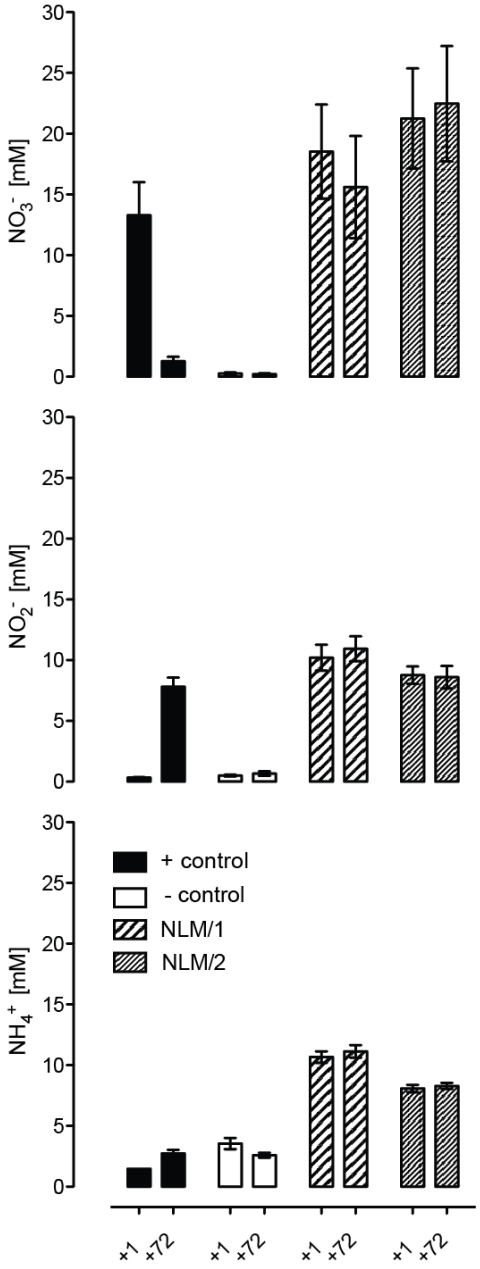

Figure 3.9 Denitrification of NLM with added glucose by *R.terrigena*. 5 mL of sample placed in small glass test tubes in triplets, autoclaved (120°C, 20 min) afterwards inoculated with *R.terrigena*. + control = nitrate broth [NO_3^-] = 15 mM; - control = H_2O DI + Glucose 1 M + raw liquid manure (1:30); NLM/1 = nitrified liquid manure + Glucose 1 M + raw liquid manure (1:30); NLM/2 = nitrified liquid manure + Glucose 1 M; error bars show the standard deviation.

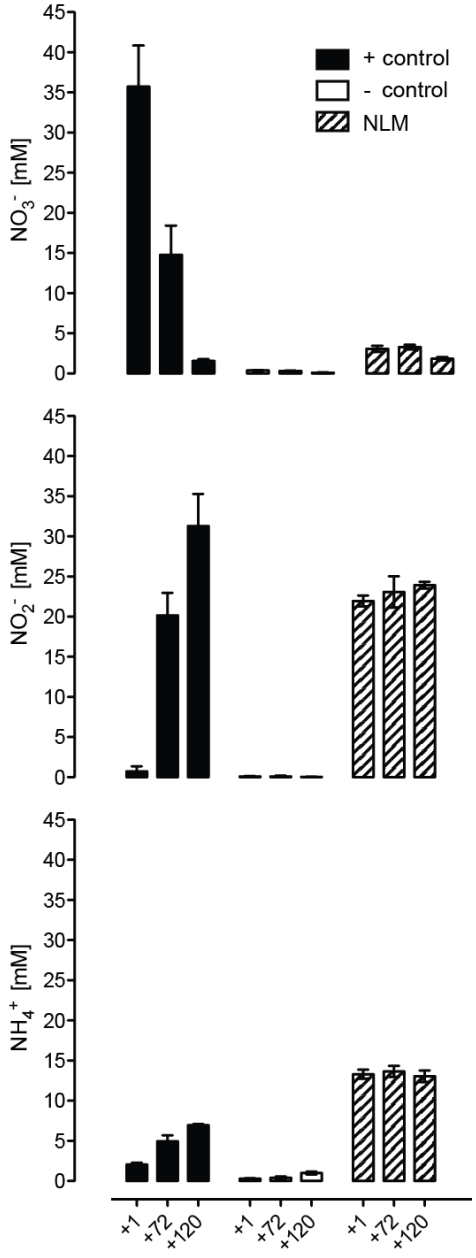

Figure 3.10 Denitrification of NLM by *R.terrigena*. 3 mL of sample placed in small glas test tubes in triplets, autoclaved (120°C, 20 min) afterwards inoculated with *R.terrigena*. + control = nitrate broth [NO_3^-] = 30 mM; - control = H_2O DI; NLM = nitrified liquid manure [NO_2^-] = 21 mM; error bars show the standard deviation.

3.4 Fungal Growth Inhibition by Acidified Nitrite

3.4.1 Growth Test on Agar Plates

Successful nitrified liquid manure containing up to 19 mM NO_2^- was tested for antifungal activity at low pH. 200 μL of tested substances (Table 2.3) were plated on MEA and spores were pipetted in spots as describe in section 2.4.2. Growth of *A.nidulans* showed no difference to the positive control (Figure 3.11 a). Plates c, d and f in figure 3.11 even showed more growth than the positive control indicating a nutritious effect on the fungus. Plates b, e, g and h in figure 3.11 showed similar growth like on the positive control but no plate could achieve an inhibition in fungal growth.

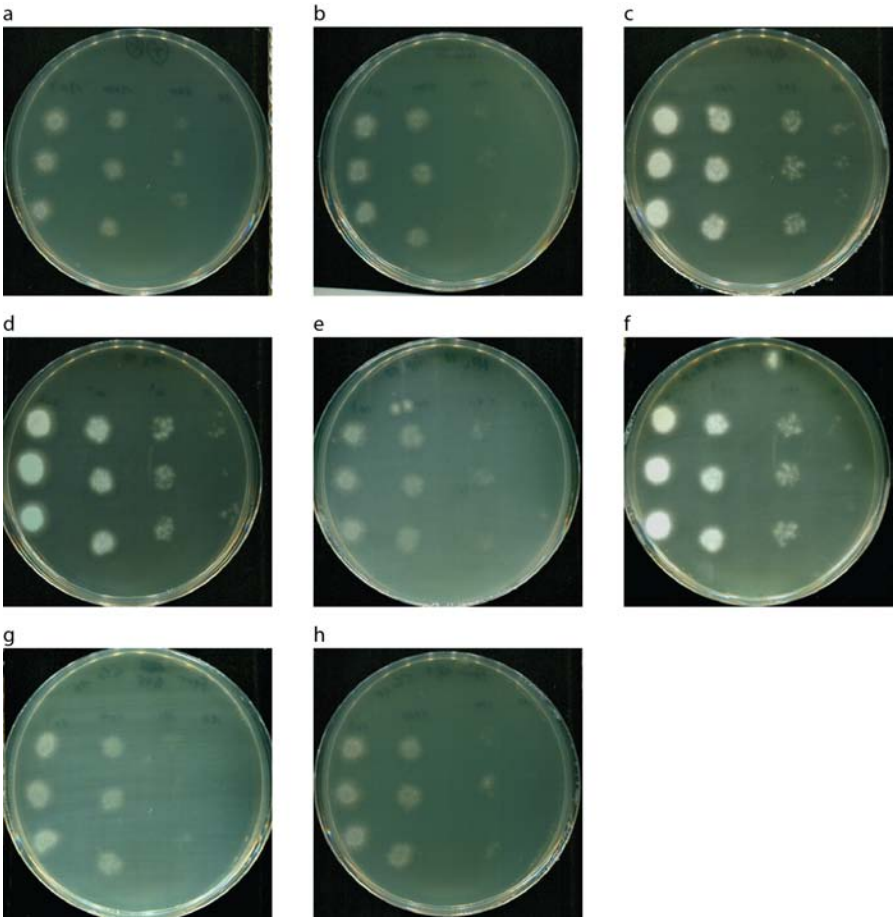

Figure 3.11 Growth test of *A.nidulans* wild type on MEA plates with plated solutions. 200 μL of solutions (Table 2.3) plated on MEA plates and spore suspensions spotted in 4 triplets of *A.nidulans* wild type counting 10^4, 10^3, 10^2 and 10 spores per spot. Incubation at 37°C for 1 day. **(a)** positive control with H_2O DI; **(b)** Glu 10 mM; **(c)** Asp 10 mM; **(d)** $NaNO_2$ 10 mM; **(e)** $NaNO_2$ 10 mM + Asp 10 mM; **(f)** $NaNO_2$ 10 mM + Glu 10 mM; **(g)** NLM $[NO_2^-]$ = 9.5 mM + Glu 10 mM; **(h)** NLM $[NO_2^-]$ = 19 mM + Glu 10 mM. Glu = L-glutamic acid, Asp = L-aspartic acid.

Also when spores of *A.nidulans* were covered with the tested solutions on agar plates (see section 2.4.2) no inhibition of fungal growth could have been shown (Figure 3.12). The plate with spores covered with H_2O DI again served as positive control (Fig.3.12 a). As clearly visible in the figure the other plates didn't show less growth as expected. Contamination in plate c and h by another fungus had no influence on the test, due to it's low amount and enough uncontaminated spots to compare to the positive control. Test substances applied to agar plates in this approach are probably rapidly diluted.

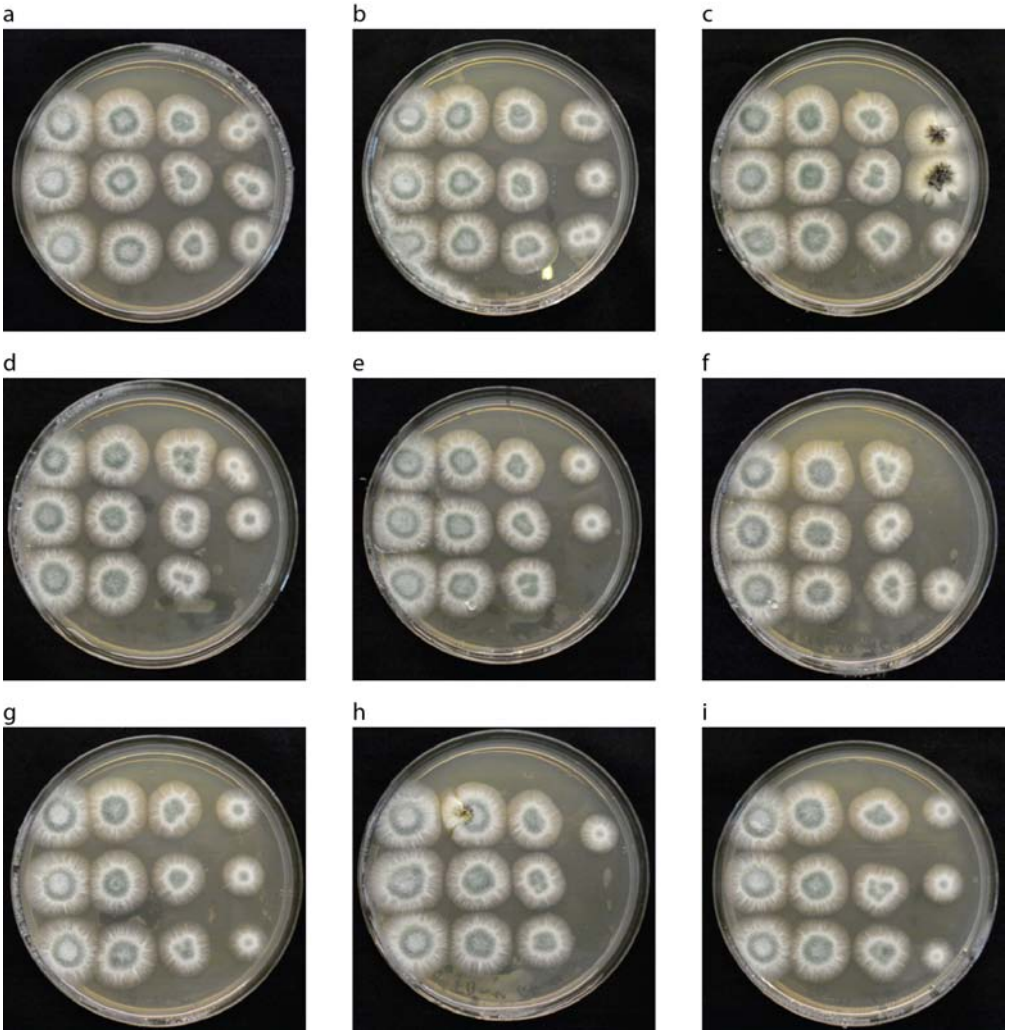

Figure 3.12 Growth test of *A.nidulans* wild type on MEA plates with spotted solutions. Spore suspensions pipetted in 4 triplets counting 10^4, 10^3, 10^2 and 10 spores per spot. After drying, the spots were covered by 8 μL of the further described solutions. **(a)** positive control with H_2O DI; **(b)** $NaNO_2$ 10 mM + HCl, pH 4; **(c)** H_2O DI + $NaNO_2$ 10 mM, pH 10; **(d)** Glu 10 mM; **(e)** $NaNO_2$ 10 mM + Glu 10 mM; **(f)** $NaNO_2$ 50 mM + Glu 50 mM; **(g)** NLM $[NO_2^-]$ = 19 mM + H_2O DI; **(h)** NLM $[NO_2^-]$ = 9.5 mM + Glu 10 mM; **(i)** NLM $[NO_2^-]$ = 19 mM + Glu 10 mM. Glu = L-glutamic acid. Incubation at 37°C for 3 days.

3.4.2 Growth-Test on Filter Paper in a Artificial Moist Chamber

Since tests on agar plates turned out to be unsuccessful, a filter paper based assay was developed to prevent dilution of NO_2^- and buffering of acidic pH. Growth inhibition on *A. nidulans* could be achieved, thus further tests on different fungi were performed using the same concentrations of NO_2^- and acidifying agent. Pictures of the fungi are shown in figure 3.13, concentrations of the tested agents are indicated in the pictures.

A.nidulans (Fig. 3.13 a) served as a positive control for growth inhibition. Combination of NO_2^- derived from the sodium salt or NLM and the amino acids (acidified nitrite) showed a inhibition in growth of the fungus. Whenever only one component is present growth resembled the positive control using malt extract broth 1%.

A. alternata (Fig. 3.13 b) showed a different growth in the acidified nitrite samples than in the control samples with one component. While the control samples showed more growth of mycelium, in samples containing acidified nitrite the fungus seemed to build more conidia. The diameter of the fungal spot on the filter paper was similar on all tested solutions.

A.brasiliensis (Fig. 3.13 c) showed no differences throughout the tested solutions. Diameters of fungal spots were also similar, no inhibition in growth could be observed.

C.sativus (Fig. 3.13 d) showed again a inhibition in growth when exposed to acidified nitrite. Growth in the control samples with only one component added led to similar growth in morphology and diameter as in the negative control. Building of more conidia in this samples was also observed than in the samples with acidified nitrite. In these samples the outside of the fungal spot showed a uneven border and also no growth in the center of the spot was observed where spore solution and tested solutions were spotted on the paper. The sample with NO_2^- from the NLM in combination with L-aspartic acid showed no growth in the first 3 days, then the growth of dark black spots, which were identified as a *A.brasiliensis* contamination.

T.longibrachiatum (Fig. 3.13 e) showed no inhibition neither in the amount of growth nor in terms of diameter of hyphae growth. Samples containing only one component and H_2O DI or only water showed the building of conidia more in the center of the spot on the filter paper, whereas on samples with acidified nitrite conidia were build more at the edge of the spot.

For *U.maydis* (Fig. 3.13 f) also a inhibition in growth could be observed in the samples containing acidified nitrite. Since spores were collected in this case from an infected corn-

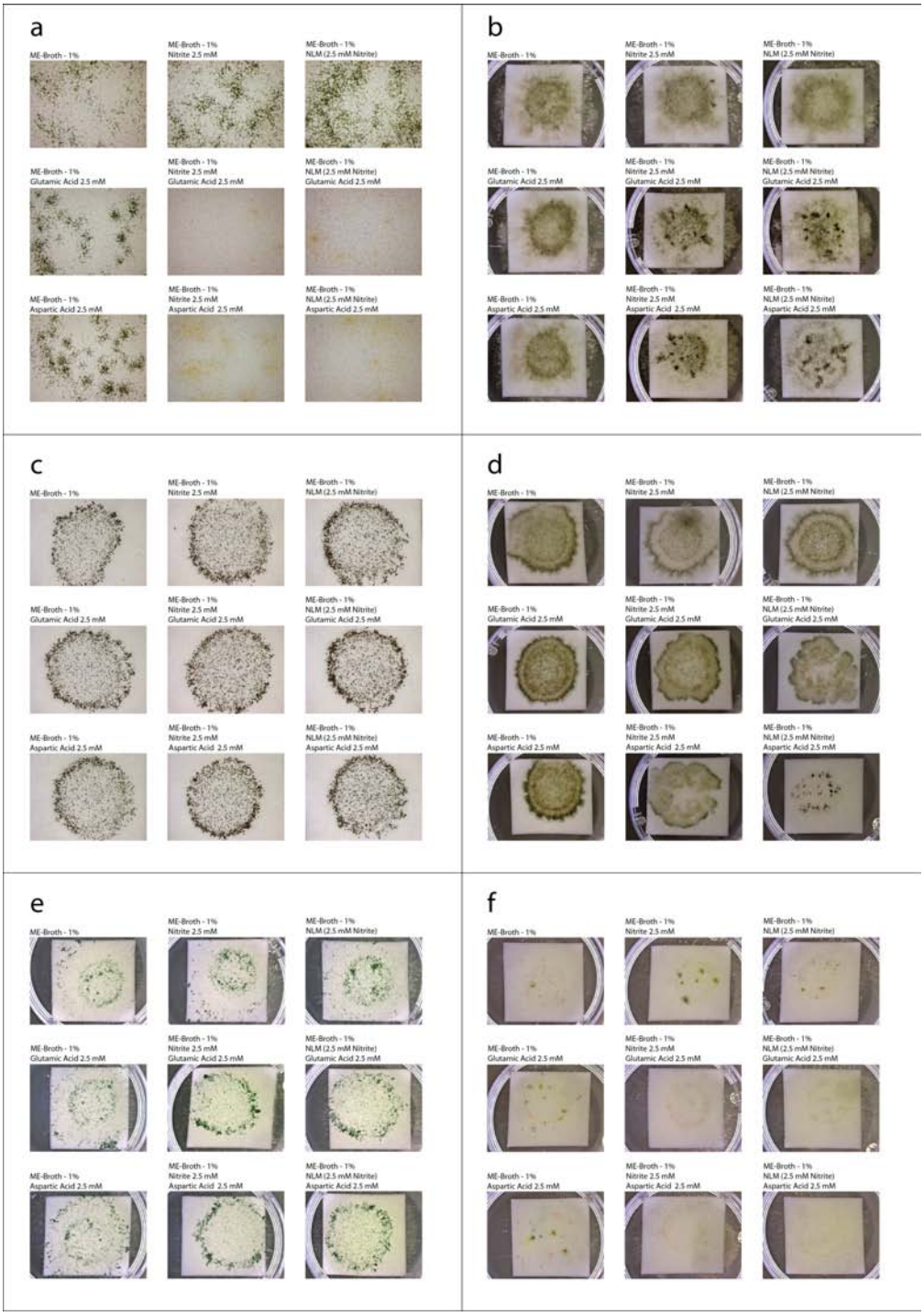

Figure 3.13 Growth test of different fungi spores on filter paper in a artificial moist chamber; $[NO_2^-]$=2.5 mM. Moist chamber was set up as described in section 2.4.3. To spore suspensions of fungi, solutions were added resulting in concentrations noted in the graphic (ME-Broth = malt extract broth; NLM = nitrified liquid manure). Chambers were incubated at 20°C for 5 days and documented by taking pictures under the stereo microscope (Olympus SZ61). **(a)** *Aspergillus nidulans*; **(b)** *Alternaria alternata*; **(c)** *Aspergillus brasiliensis*; **(d)** *Cochliobulus sativus*; **(e)** *Trichoderma longibrachiatum*; **(f)** *Ustilago maydis*

cob, not only *U.maydis* but also other contaminants were pipetted on the filter paper. Nevertheless only mycelium grew on the filter papers containing acidified nitrite whereas the control samples showed different fungi and their spores.

Results of another experiment using higher concentrations of the components (5 mM NO_2^-; 5 mM acidifying amino acid) are shown in figure 3.14. *A.nidulans* was again tested (not shown in graphic) to proof the effect of acidified nitrite on growth inhibition. The same growth behavior than in the experiments before was observed.
Even with higher concentrations of acidified nitrite no reduction in growth could be observed in *A.brasiliensis* (Fig. 3.14 a). Diameter of spots on the filter paper were similar and spores were build also similarly throughout the different approaches.
Growth of *B.fuckeliana* (Fig. 3.14 b) was not affected by the addition of acidified nitrite, growth of the mycelium was even more in the samples containing nitrite and a amino acid.
In contrast *F.graminearum* (Fig. 3.14 c) showed a strong inhibition in growth. In the samples containing acidified nitrite not even mycelium growth could be observed, whereas in the control samples growth occurred (white mycelium of *F.graminearum* is not easily visible on the pictures). Filter papers only containing nitrite as sodium salt as well as from the NLM showed more growth than the other control samples, also pink spots were observed in these two samples.
In addition with *P.eupyrena* (Fig. 3.14 d) a strong effect could be observed too. Filter papers containing acidified nitrite show no growth of the fungus, all control samples were even in fungal growth and diameter of the spot.
Only a few spots of *R.punctiformis* (Fig. 3.14 e) grew in the positive control sample containing only malt extract broth and H_2O DI, even less in the other control samples containing only one of the components. However in the samples with acidified nitrite no fungal growth could be observed.
Also under higher concentrations of the tested solutions *T.longibrachiatum* (Fig. 3.14 f) showed a similar behavior as in the lower concentrated ones. The amount of growth and building of conidia was even throughout the samples, whereas the spots appeared more central in case of the control samples containing only one component and water. In case of acidified nitrite conidia were build more on the outside of the spot even outside of the filter paper.

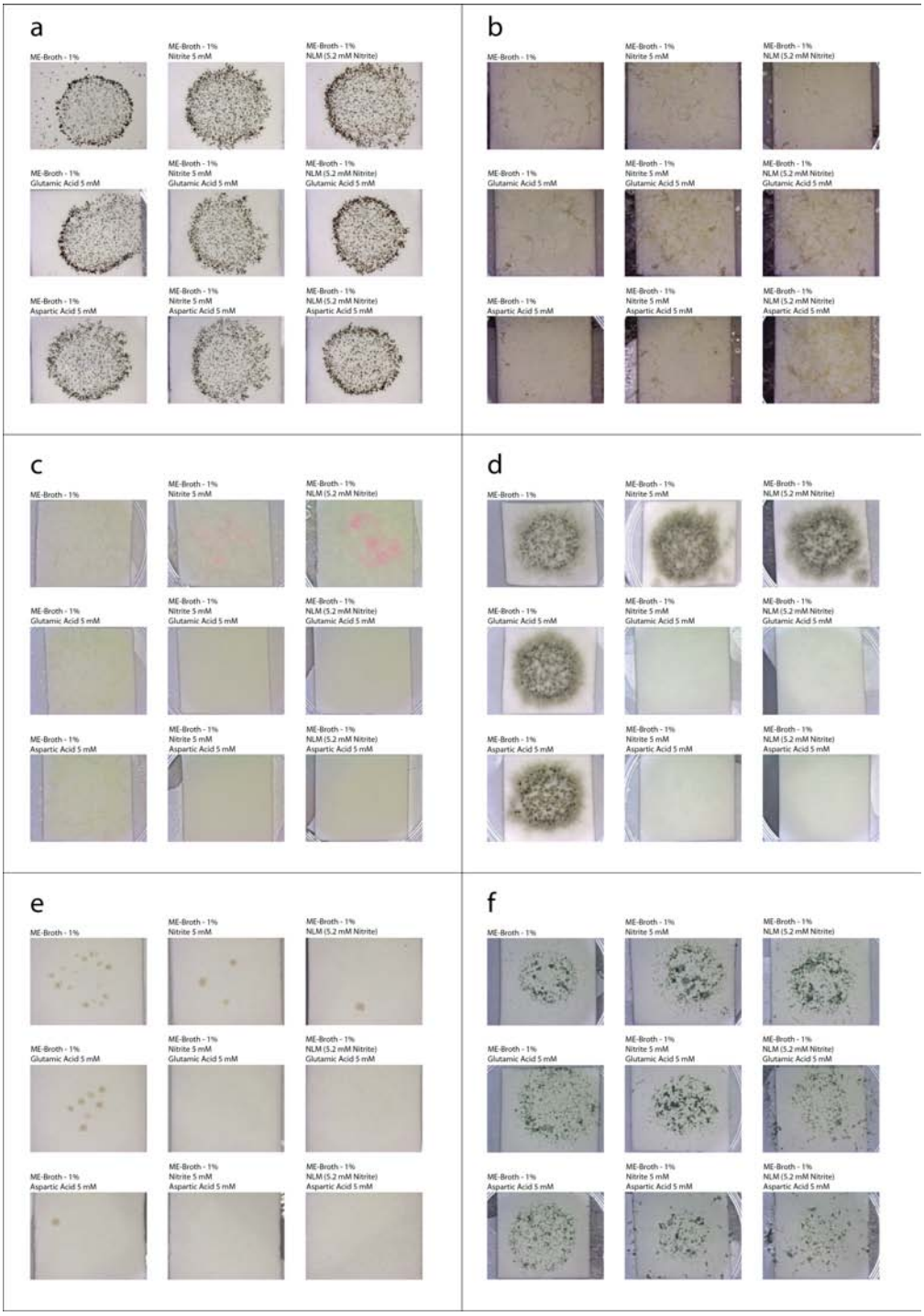

Figure 3.14 Growth test of different fungi spores on filter paper in a artificial moist chamber; $[NO_2^-]$=5 mM. Moist chamber was set up as described in section 2.4.3. To spore suspensions of fungi, solutions were added resulting in concentrations noted in the graphic (ME-Broth = malt extract broth; NLM = nitrified liquid manure). Chambers were incubated at 20°C for 5 days and documented by taking pictures under the stereo microscope (Olympus SZ61). **(a)** *Aspergillus brasiliensis*; **(b)** *Botrytinia fuckeliana*; **(c)** *Fusarium graminearum*; **(d)** *Phoma eupyrena*; **(e)** *Ramularia punctiformis*; **(f)** *Trichoderma longibrachiatum*

Results of the modified approach where agar pieces were placed on the filter papers containing the tested solutions are shown in figure 3.15.

A.solani (Fig. 3.15 a) shows no inhibition in growth all filter papers were evenly occupied by the fungus.

In case of *S.sclerotiorum* (Fig. 3.15 b) a inhibitory effect on growth could be observed in the samples containing acidified nitrite but also the two samples containing just nitrite (derived from sodium salt or NLM) and H_2O DI. Control samples containing just H_2O DI or an amino acid and water showed growth and also dark survival structures of the fungus (sclerotium) were built on the filter paper.

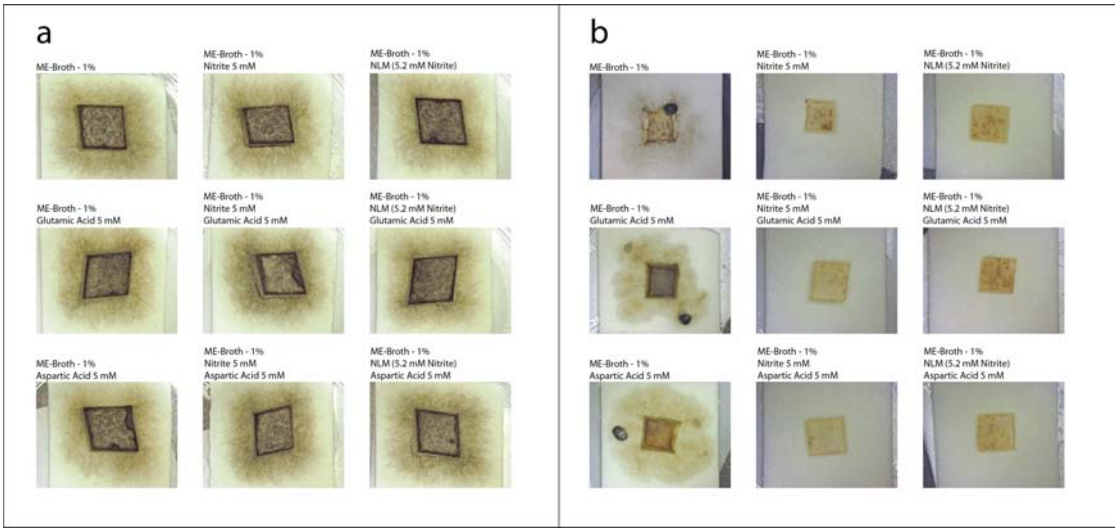

Figure 3.15 Growth test of different fungi on agar pieces placed on filter papers in a artificial moist chamber; [NO_2^-]=5 mM. Moist chamber was set up as described in section 2.4.3. Solutions were pipetted on the filter paper resulting in concentrations noted in the graphic (ME-Broth = malt extract broth; NLM = nitrified liquid manure). Agar pieces were cut out of MEA-plate and placed on top of the filter paper. Chambers were incubated at 20°C for 14 days and documented by taking pictures under the stereo microscope (Olympus SZ61). **(a)** *Alternaria solani*; **(b)** *Sclerotinia sclerotiorum*

4 Discussion

In this thesis we aimed to develop a foliar spray for application on plant canopy with a fungicidal effect based on acidified nitrite. Therefore we established microbial nitrification in batch shaking cultures to convert ammonium from liquid pig manure into nitrite. In combination with L-glutamic and aspartic acid to achieve low pH, growth inhibition of the nitrified liquid manure (NLM) was tested on different fungal plant pathogens. Since we use liquid manure as resource for nitrite production and production of L-glutamic acid by *Cornyebacterium glutamicum* is already well established [50], [51], the developed foliar spray would be also applicable in organic farming.

Nitrogen Quantification Methods in Liquid Pig Manure To monitor the conversion of ammonium in liquid pig manure to nitrite and further to nitrate, a accurate quantification method had to be found. Miranda et al. [52] showed that the used method to simultaneous detect nitrate and nitrite can be applied on biological samples. First measurements of batch samples resulted in negative values for nitrate contents. The subsequently performed experiment (Fig. 3.2) showed that nitrate concentrations are underestimated if nitrite exceeds a concentration of 2.8 mM in the sample. Concentrations in this experiment were chosen in the expected range they were thought to appear in nitrification batches to ensure a suitable method for the further measurements. However, the acidic reduction of nitrate via VCl_3 and subsequent addition of Griess reagent was not applicable in our case, because nitrite concentrations were expected to exceed 2.8 mM in the targeted nitrification process. Colourimetric quantification of NO_2^- and NH_4^+ showed constant parameters for slope and linearity and lead to accurate results.
To solve the problem of the not accurate NO_3^- quantification, two ion selective electrode (ISE)s were set up for a separated detection of nitrite and nitrate. Since results of the electrodes in the above mentioned experiment showed no interferences caused by high nitrite concentrations the ISE was found to be suitable for our purposes. Nevertheless,

also the electrode selective for measuring nitrite was used throughout the experiments to confirm results gathered from the colourmetric method.
Since this was the first use of the ISE measurement system, the protocol at the department of Applied Genetics and Cell Biology had first to be established in the lab. Tests were carried out to adjust sample volume, buffer addition and check stability and comparability of the obtained results over longer time periods. Calibrations of all measurements were compared to find out if ISEs delivered stable mV values over the experiments. Small standard deviations in nitrate measurement (Fig. 3.1) indicate a stable working range of the electrode, whereas the nitrite electrode showed bigger values in standard deviation at lower concentrations in the beginning. Therefore a reassembly of the flow cell was carried out to reduce the sample volume inside of the flow cell. After this reassembly the nitrite ISE showed lower standard deviations over the standard concentrations. In contrast to the manual where 50 μL of sample volume were stated to lead to repeatable results we had to use 250 μL.
Measuring ion concentrations with ISEs demands a time consuming sample treatment to prevent membrane damage of the electrodes and prolong electrode life. The procedure developed for our measurements shows no loss of analyte and thus is suitable for this purpose.
In summary it can be pointed out that the potentiometric quantification of NO_2^- and NO_3^- via ISEs leads to accurate results. For monitoring purposes of batch samples this method turned out to be too time consuming due to sample preparation and manual injection of the samples. For further nitrification approaches a combination of colourmetric NH_4^+ and NO_2^- and potentiometric NO_3^- quantification is recommended.

Nitrification in Batch Cultures Ammonium in liquid pig manure should be converted to NO_3^- over NO_2^- by establishing a nitrification process in batch shaking cultures by addition of a suitable inoculum. No nitrification could be achieved in the batches when Biovin was used as inoculum. Biovin is a composted product produced from grapes after pressing and known to contain around 10^8 colony forming units (cfu) bacterial cells and around 10^5 cfu fungal cells per gram of product. We used this material as inoculum as one could assume that nitrifyers are present among the bacterial community. High nitrate concentrations in this experiment can be explained by the nitrate content of Biovin as a fertilizer. Nitrate level found in the negative control were also found in all other batches

and did not change significantly over the incubation time. Nitrite was not detected in any of the batches, which also supports the conclusion that no nitrification took place since it is the first product of the expected process and should also occur first before any nitrate is build. The decrease of ammonium in the batches with raw undiluted liquid manure and 5-fold diluted liquid manure can be explained by the loss of gaseous NH_3 due to higher pH values. Whereas pH in raw undiluted liquid manure increased from 8.5 to 9.5 at its maximum after addition of Biovin, in 10-fold diluted liquid manure only an increase from 8.3 to 8.9 was measured. NH_4^+ in aqueous solution is in an chemical equilibrium with it's conjugated base ammonia NH_3:

$$NH_3 + H_2O \rightleftharpoons NH_4^+ + OH^- \tag{4.1}$$

The higher the pH in the liquid manure, the more NH_3 is present and due to the high steam pressure of ammonia it evaporates easily from liquids. The pK_S value of NH_4^+ is 9.25 and this indicates that at the measured pH of 9.5 in the undiluted liquid manure even more NH_3 than NH_4^+ is present, thus explaining it's rapid decrease in undiluted liquid manure. Additionally, part of the ammonium and part of the resulting nitrite and nitrate may have been assimilated by the bacterial and fungal community to serve as nitrogen source for growth. In summary in was clear that Biovin is not suitable as inoculum to start a nitrification process in liquid pig manure, not only because no increase in product could be observed but also due to high contents of substrate and product (NH_4^+ and NO_3^- respectively) in Biovin no data from a real negative control could be achieved. It was concluded from the constant values of NH_4^+ in the 10-fold diluted liquid manure over the incubation time, that the loss of NH_3 is minimal in this approach. Therefore this dilution was used in all further experiments to prevent unnecessary loss of substrate. Nitrification is well known to take place in agricultural soils [4]. Consequently we tried to use this conversion process in soil to reach our goal of ammonia oxidation. In the experiments using small soil/sand packed columns to act as a sort of fixed bed reactor (Fig. 3.5), NH_4^+ levels drop fast while NO_3^- increases only in very low levels. One explanation for this can be the binding of positive charged NH_4^+ at clay minerals in the column and the simultaneous leaching of NO_3^- from the soil. The increase of NH_4^+ and the decrease of NO_3^- in later collected fractions supports this explanation. Other reasons for the not successful nitrification may be found in parameters like oxygen limitation and too short retention times of the liquid manure in the column. Although the very fine structured

soil was mixed with sand to half of it's volume the clay formed a compact package in the column allowing no or only limited aeration. The short retention time (approximately 2 hours) would require already very efficient nitrification in the column which clearly is not the case. Theoretically it is possible to implement the process in a continuous system like this as it was shown before by a similar approach [53], but to start the process from the very beginning a batch process still seemed to be more suitable.

Batches inoculated with untreated soil from the field showed no nitrification within the first 17 days (408 h) after inoculation. To shorten the time between inoculation and increase of product concentration, we tried to increase the biomass of the nitrifying community in the soil by feeding the soil with diluted liquid manure as shown before by Cavagnaro et al. [54]. Indeed with soil from the above described soil columns we achieved the first successful increase of NO_2^- and NO_3^- in batches. By using this fed-soil as inoculum a raise of nitrite concentration could be detected around 12 days (288 h) after inoculation (Fig. 3.6). The addition of NH_4^+ at low concentrations to the batches led to a complete nitrification to NO_3^-, whereas NO_2^- concentrations dropped to zero in the end of the process. Calculating the yield from the input of substrate and the end concentrations of product we get values of 132% for the positive control and 141% for the batch with the diluted liquid manure. Since no correction for the evaporating water in the batches was made and incubation time was almost 2 months in this case, the overestimated yield values seem to be plausible. To get more convincing yield values, weighing of the batch cultures should be performed to correct for evaporation.

Our goal was to reach a NO_2^- concentration of 10 mM or more in the processed liquid manure, because antifungal effects where shown by exceeding this value at low pH [27]. As expected the desired nitrite concentrations could not be reached in the first batch cultures as a complete conversion of NH_4^+ to NO_3^- took place. The reduction of nitrate to nitrite in pure culture with *R.terrigena* could not be achieved, although positive controls showed a reduction of nitrate (Fig. 3.9 and 3.10). The addition of different carbon and nitrogen sources to support growth of *R.terrigena* also didn't end in a significant reduction of nitrite in liquid manure. The possible inhibition of *R.terrigena* by high levels of NO_2^- gained from an unfinished nitrification to NO_3^- in the batches were thought to be the reason, but NO_2^- concentrations of 30 mM in positive controls show that the bacterium can handle high nitrite contents. Charles Glass et al. [19] observed an inhibition in denitrification rate in activated sludge related to NO_2^- concentration and pH by the toxic effect of FNA. At pH 6 a threshold inhibitory concentration of 0.02 mg L^{-1}

HNO_2-N was stated. Using the formula from Aslan et al. [55] to calculate the concentration of FNA we get 0.12 and 0.22 mg L^{-1} HNO_2-N for the NLM (pH = 6.28) used in the denitrification experiments, which clearly exceeds the described threshold for inhibition. In contrast the pH in the positive control containing 30 mM NO_3^- increased from 6.2 to 8.8 during the denitrification. Calculated concentrations for FNA at the start and end point are 0 and 0.0017 mg L^{-1} HNO_2-N respectively, thus lower than stated threshold values in literature [22] [19]. However, because NO_2^- accumulation could be achieved in batch cultures, the reduction step in pure culture of *R. terrigena* was not necessary any more.

The nitrifying community can be selectively inhibited by FA and FNA as shown by Anthonisen et al. [15]. Procedures were further investigated to accumulate NO_2^- under high level of NH_4^+ to safe costs for denitrification processes in waste water treatment plants [56], [57]. By adding raw liquid manure to our batches to get NH_4^+ concentrations of 20 mM an accumulation of NO_2^- could be achieved (Fig. 3.7). The moment of the addition was chosen to be when NH_4^+ is still not fully depleted and the increase of NO_2^- is still linear. The slight increase of NO_3^- when NH_4^+ dropped below 10 mM supports the theory of a successful selective inhibition of the nitrifiers. The higher initial levels of NO_3^- can be explained through the accumulation of nitrate in the fed soil by continuous feeding with raw liquid manure. Also the earlier start of nitrite formation can be explained by a higher biomass of nitrifiers in the inoculum. Calculated yield for NO_2^- was 80%, for NO_3^- 9%. Actual yield may be a little lower, because no correction for evaporation was done. However a concentration of 24 mM NO_2^- could be achieved in 28 days (672 h) of incubation, which is more than twice of the goal defined in the beginning.

Additionally we were interested in product building rates of nitrite and nitrate to describe the process of nitrification in more detail. According to the data shown in figure 3.8 and table 3.2 product building rates are closely related to substrate consumption rates. Willers et al. [58] tested digested pig slurry from a large scale plant on nitrification rates at different temperatures based on the decrease of ammonium. At 20°C a volumetric nitrification rate of 1.4 mM h^{-1} was measured which is 14 fold higher than the maximum decrease of NH_4^+ observed in our batches (0.1 mM h^{-1}). Of course the nitrifiers of Willers where taken out of aerated tanks from a plant whereas our nitrifying community developed in the batches. Furthermore the nitrification rates increased with every addition of substrate and a further increase is expected by setting the right process parameters. In the biofilm reactor of Hwang et al. [53] a maximum ammonium removal rate of 6.4 mM h^{-1} was achieved showing the potential for improvement.

Fungal Growth Inhibition of Acidified Nitrite As soon as successful accumulation of NO_2^- in the liquid manure batches was achieved we wanted to test the fungicidal effect of the NLM at low pH values on different fungi. Earlier tests carried out by Thorsten Schinko [27] showed a inhibition of growth on *A.nidulans* at NO_2^- concentrations of 10 mM and pH of 5. These tests where carried out in aspergillus minimal medium and concentrations of nitrite and pH were set in the medium by adding nitrite salt and buffer. Due to the relatively low concentrations of NO_2^- in NLM and small volumes generated to that time point, the combinations of solutions were plated (Fig 3.11) or spotted (Fig 3.12) on the agar. In both cases, no inhibition in growth of the fungus can be explained by the diffusion of NO_2^- into the agar and the consequent decrease in concentration. By assuming an equal diffusion of NO_2^- into the agar and a agar volume of 20 mL in the plates the end concentration of nitrite for the plates with plated solutions dilutes to 0.1 mM, which is too low for any inhibitory effect since pH of the MEA is 5.6. Consequently we wanted to develop a simple test for fungicides which imitates field conditions as good as possible. Since cellulose is a main part of plant cell walls [59] we chose the material for our filter paper test. A 1% malt extract broth was chosen to be a minimum substrate for the fungus and tested solutions were pipetted on the spores to imitate fungicide application in the field. This growth test set-up can be used to easily compare the effect of different components on fungal growth in a quick and time saving manner.

Antimicrobial effects of acidified nitrite is attributed in literature to different mechanisms. Zhou et al. [22] pointed out three different mechanisms which are thought to be partly responsible for an inhibitory effect on microbes, explained in more detail in the introduction section. Mortensen et al. [24] supported the hypothesis, that FNA acts as an uncoupler of energy generation and growth, by proofing a rapid decrease of the intracellular pH in *Debaryomyces hansenii* and *Candida zeylanoides* due to diffusion of FNA into the cell and dissociation of the acid inside of the cell. Two external pH values where tested, 5.5 and 4.5, where growth of *C. zeylanoides* was inhibited in both cases when nitrite was added, whereas *D. hansenii* was inhibited just at pH 4.5. This shows that different species react differently on stress caused by acidified nitrite, which also fits to the different results observed in the fungal growth tests (Fig. 3.13, 3.14 and 3.15). Furthermore it underlines the statement of Zhou as well as Mortensen, that the inhibitory effect caused by acidified nitrite cannot be attributed to a single mechanism discovered so far, it seems to be an interplay of different ones. However, the presence of FNA seems to be a key factor in these mechanisms, which is a function of NO_2^- concentration, pH

and temperature in the environment. In our tests nitrite concentration was 2.5 and 5 mM which is comparable to values used by Mortensen (4.3 mM) and also pH values where similar. By increasing the nitrite concentration in our tests we could increase the proportion of FNA and thus may achieve inhibitory effects on more fungi species. Nevertheless our tested concentrations showed sufficient inhibition on *A.nidulans*, *U.maydis*, *F.graminearum*, *P.eupyrena*, *R.punctiformis* and *S.sclerotiorum* representing a promising outcome for the development of a foliar spray with fungicidal effects out of nitrified liquid pig manure.

As additional benefit this product may have nutritional effects as well as it may contain amino acids, organic nitrogen, carbon and sulfur and other components. This aspect has not been tested yet during this thesis but analytics will be launched in the lab. Even more so, the product could be amended with trace elements and other products feeding the plant and stimulating the pathogen defense system.

5 Appendix

5.1 Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst und keine anderen als die im Literaturverzeichnis angegebenen Hilfsmittel verwendet habe. Ich versichere, dass ich alle wörtlichen und sinngemäßen Übernahmen aus anderen Werken als solche kenntlich gemacht habe.
Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

5.2 Statement

I hereby declare that I have written this thesis independently and without assistance and I did not use resources other than those specified in the list of references. I certify that I have indicated all figuratively and literally meanings acquired from other works as such. However, if a violation of the copyright should be disclosed, I request to contact me.

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5.4 Abbreviations

AMO	ammonia monooxygenase
AOB	ammonia oxidizing bacteria
Asp	L-aspartic acid
cfu	colony forming units
FA	free ammonia
FNA	free nitrous acid
Glu	L-glutamic acid
H_2O DI	deionised water (desalted, ion-exchange, autoclaved)
HAO	hydroxylamine oxidoreductase
ISA	ionic strength adjustment buffer
ISE	ion selective electrode
MEA	malt-extract agar
NLM	nitrified liquid manure
NOB	nitrite oxidizing bacteria
NXR	nitrite oxidoreductase
RT	room temperature

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