Impact of Organic Waste Residues on Structure and Function of Soil Bacterial Communities

With Emphasis on Ammonia Oxidizing Bacteria

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2006

Acta Universitatis Agriculturae Sueciae
2006: 20

ISSN 1652-6880
ISBN 91-576-7069-2

Tryck: SLU Service/Repro, Uppsala 2006

Abstract

Nyberg, K. 2006. Impact of Organic Waste Residues on Structure and Function of Soil Bacterial Communities - With Emphasis on Ammonia Oxidizing Bacteria. Doctoral dissertation.
ISSN 1652-6880, ISBN 91-576-7069-2

The recirculation of biologically treated organic waste back to soil will help to promote sustainable agricultural development. However, although the use of such waste residues as fertilizers generally is beneficial for the soil ecosystem there is concern regarding their content of pollutants.

In this thesis, the effects of biologically treated organic waste residues on the structure and function of ammonia oxidizing bacteria (AOB) and total bacteria communities in agricultural soils were investigated using molecular fingerprinting methods in combination with potential activity measurements. The AOB perform the rate-limiting step in the nitrification process and are considered to be sensitive to environmental disturbances.

Using AOB activity as an indicator, different organic waste residues were demonstrated to contain organic compounds that inhibit this group of organisms. Furthermore, it was possible to identify a correlation between the degree of inhibition of AOB activity and the concentration of phenols. However, in these studies no link between activity inhibition and shifts in community structure was seen.

The possibility to link effects on bacterial activity to community structure was further studied by targeting the active AOB by BrdU incorporation. In this way, short-term effects of a pollutant (4-ethylphenol) on the active AOB community structure were identified, which could not be detected on the total community.

Effects of different fertilization regimes on the structure and function of AOB became more evident when a long-term field study was investigated. A link between AOB community structure and metabolic function was detected in a soil subjected to different fertilization regimes for 50 years. In addition, a low AOB diversity was detected in soil treatments in which the total bacterial community also showed signs of stress. Thus, the decrease in AOB activity and diversity also indicated a stressed soil environment.

In conclusion, this thesis has demonstrated that pollutants in biologically treated organic waste residues can have negative impacts on the structure and function of AOB communities in soil. However, potential risks must be compared with the benefits arising from the use of organic waste residues in agriculture.

Keywords: ammonia oxidizing bacteria (AOB), soil, organic waste, fertilizers, community structure, stress

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Contents

Appendix

Papers I-V

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Nyberg, K., Sundh, I., Johansson, M. & Schnürer, A. 2004. Presence of potential ammonia oxidation (PAO) inhibiting substances in anaerobic digestion residues. *Applied Soil Ecology* 26:107-112.

II. Levén, L., Nyberg, K., Korkea-aho, L. & Schnürer, A. Phenols in anaerobic digestion processes and inhibition of ammonia oxidising bacteria (AOB) in soil. *Science of the Total Environment* (In press).

III. Nyberg, K., Schnürer, A., Sundh, I., Jarvis, Å. & Hallin, S. Ammonia oxidizing communities in agricultural soil incubated with organic waste residues. *Biology and Fertility of Soils* (In press).

IV. Enwall, K., Nyberg, K., Bertilsson, S., Cederlund, H., Stenström, J. & Hallin, S. Long-term impact of fertilization on activity and composition of bacterial communities and metabolic guilds in agricultural soil. (Submitted).

V. Nyberg, K., Schnürer, A., Sundh, I. & Hallin, S. Stress response of replicating ammonia oxidizing populations in arable soil. (Manuscript).

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My contribution to each paper has been as follows

I. Performed major part of the laboratory work, analysis of the results and writing of the manuscript.

II. Took part in planning the study, participated in the writing of the manuscript and in analysing the results. Performed minor part of the laboratory work.

III. Took part in planning the study and took major part in analysing the results and writing of the manuscript. Performed all the laboratory work.

IV. Performed all laboratory work regarding AOB, participated in analysing the results and did a minor part in writing of the manuscript.

V. Took major part in planning the study and writing of the manuscript. Performed all the laboratory work.

Introduction

In order to achieve a sustainable development, we must rely more on renewable resources instead of further depleting our environment. To create sustainability, we must recognize that waste is a resource that should be recycled and reused. If handled and treated correctly, waste can be a source of energy to complement other renewable energy sources so that the use of fossil fuels can be reduced. In addition, the organic waste fractions contain high amounts of plant nutrients that ought to be re-circulated back to agricultural fields. This is especially important with regard to phosphorous, which is a limited resource.

During intensive cropping, nutrients and organic matter are continuously removed from the agricultural system. Returning nutrients and organic matter to the soil will therefore help to maintain and improve its fertility. Contrary to mineral fertilizers, the use of organic fertilizers supplies both nutrients and organic matter to agricultural land. Manure is a widely accepted and commonly used fertilizer in agriculture, but there are other alternatives that can be used either exclusively or as a complement to manure. One example of this is the organic fraction of biologically treated source-separated urban waste. During biological treatment, the nutrients in the waste are released and concentrated in a residue. As these residues contain nitrogen and phosphorous they have the potential to function as fertilizers.

However, it is important to assure the quality of biologically treated organic waste before it is used as fertilizer. Both farmers and consumers will have great difficulty in accepting a fertilizer that cannot be guaranteed to be free from contaminants. In addition, unacceptable levels of pollutants may pose a risk for the future fertility of the soil, and this is not consistent with sustainable agriculture. The fertility of a soil ecosystem depends on its microbial community. As many soil microorganisms are in immediate contact with the physical and chemical soil environment they can function as suitable biological indicators of introduced pollutants (Brookes, 1995). Thus, valuable information on soil health can be obtained by studying the effect of biologically treated organic waste residues on soil microorganisms.

In this thesis, the ammonia oxidizing bacteria have been used as indicator organisms in exploring effects of different waste residues. The ammonia oxidizing bacteria (AOB) are a group of bacteria essential for global nitrogen cycling, as they perform the first step in the nitrification process, where ammonia is oxidized to nitrate via nitrite. The AOB have been shown to be sensitive to environmental disturbances and are therefore often used as indicator organisms to study different kinds of stress in soil (Hastings et al., 1997, Stephen et al., 1999, Phillips et al., 2000, Chang et al., 2001, Oved et al., 2001).

Aim and outline of the thesis

The objective of this thesis was to explore how organic waste residues affect the structure and function of bacterial communities in agricultural soil, with focus on the ammonia oxidizing bacteria. The waste residues investigated in this thesis were mainly products originating from urban organic waste, such as source-separated municipal household waste and sewage sludge, although manure and chemical fertilizers were also included as controls.

Initially, the impact of different digestates, produced by anaerobic digestion of a variety of organic wastes, on the activity of both the total bacterial community in soil and the AOB was studied (**I**). In order to isolate effects of specific organic compounds, the digestates were subjected to an extraction procedure prior to soil amendment. The effects of the extracts were then compared to the effects caused by dried digestates. The results showed the presence of substances inhibiting AOB activity, although no such effects were detected when the whole residual material was studied. Two parallel projects were then initiated to further investigate inhibition of AOB activity. The aim of the first project was to identify the substances that caused inhibition of AOB (**II**). The results demonstrated a clear correlation between high phenol content in the digestates and inhibition of AOB activity. In the second project, possible links between inhibition of AOB activity and shifts in AOB community structure were studied. Even though clear differences in activity were noted after amendment of different organic waste residues, no community shifts could be observed (**III**). Possible links between AOB activity and community structure were further investigated in a long-term fertilization trial in which soil had been fertilized with manure, sewage sludge and two kinds of mineral fertilizers continuously for 50 years (**IV**). A clear impact of the different fertilization regimes was detected on both the activity and community structure of AOB and total soil bacteria. Finally, the possibility for detecting short-term stress response caused by phenol was explored by targeting the active AOB community in soil (**V**). The active fraction of AOB showed a stress response that could not be detected when the total AOB community was studied.

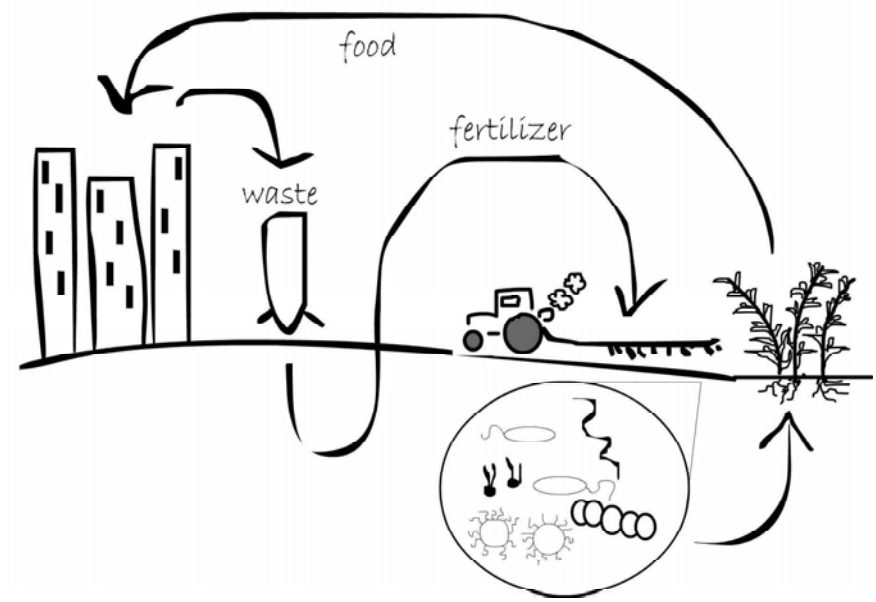

Fig. 1. A schematic overview of the flows of organic wastes between urban and rural areas that promote a sustainable society.

Organic waste in circulation

The origin of organic waste

Organic waste is produced both in urban and rural areas. Animal manure is produced in agriculture, which is naturally returned to arable land. Both manure and garden composts are commonly used as fertilizers and these products are often regarded as safe. However, the quantity of organic waste produced in urban areas has increased steadily throughout the past century, as a result of increased population size and changes in life style. Due to urbanisation and globalisation, food is often consumed far from where it was produced. As a result of this, a natural recipient for the waste has been lost. In addition, the recipients have no control over what chemicals are used by the producers, and there may also be a higher risk for the spread of pathogens together with the waste. Also, urban waste consists of a complex mixture of different types of waste, which makes it very difficult to directly re-circulate it back to agriculture. Therefore, this waste has often been deposited on landfills or combusted.

Since 2005, deposition of organic waste in landfills is prohibited in Sweden. As part of the political vision for sustainable development, the nutrients in biologically treated organic waste should be utilized as a resource and be re-circulated back to agricultural land (Agenda 21 and governmental bills 1996/97:172, 1997/98:145). Hence, many municipalities in Sweden and other European countries have been encouraged to find methods to re-cycle the nutrients from urban areas back to the land. One obvious alternative is to use biological waste treatment. In Sweden, it is common to treat sewage sludge biologically, but the large-scale treatment of other types of municipal organic wastes (e.g. household waste, manure, slaughterhouse waste, industrial waste etc.) is still under development. Presently there are about a dozen large-scale anaerobic digesters (The Swedish Biogas Association, 2005) and approximately double that number of composting reactors (The Swedish Association of Waste Management, 2005) in full operation in Sweden today treating mixed organic waste material. Approximately 400 000 metric tons of organic waste is biologically treated each year in Sweden, not including sewage sludge (The Swedish Association of Waste Management, 2005). Biological waste treatment is likely to increase in the near future due to the recent prohibition on deposition of organic waste.

Biological treatment of organic waste

Biological waste treatment can be defined as the degradation of organic waste by microorganisms under controlled conditions. The degradation can be performed either aerobically, by composting, or anaerobically through anaerobic digestion (Fig. 2). During composting, microorganisms degrade the organic material in the presence of oxygen, which leads to the production of CO_2, H_2O and a substantial amount of heat, whereas in anaerobic digestion the organic material is converted to energy-rich CH_4 and CO_2, so called biogas. Residual products are formed in both treatment processes, which consist of released mineral nutrients and undegraded organic material. The production of biogas from the anaerobic digestion process has major environmental benefits, since the methane gas can be used as vehicle fuel, for heating or for electricity production. Thereby the anaerobic process not only covers its own energy requirements but also, if optimised, provides a surplus of energy for alternative uses.

Anaerobic digestion is most commonly used to reduce the volume of sludge produced at municipal sewage treatment plants. However, the use of anaerobic digestion is increasing, since the process is also well suited to degrade a variety of other types of organic wastes. Most common is the treatment of animal manure, slaughterhouse waste and source-separated municipal household waste (The Swedish Association of Waste Management, 2005). Composting is suitable for degrading drier fractions of organic waste. In Sweden, it is commonly used at small scales for treating garden waste and for treating source-separated municipal household waste in large-scale plants (The Swedish Association of Waste Management, 2005). The composting process does not function well if the substrate has a low carbon content. Therefore an external carbon source such as straw or park and garden waste is often added in order to increase the C:N ratio.

Both composting and anaerobic digestion greatly reduce the volume of the original waste into nutrient rich residues. Even if the original raw waste can be more or less the same, the residues from the two types of process are very different. They differ in both structure and nutrient content as a consequence of differences in management strategies. In addition, degradation pathways as well as degradation efficiency are dependent on the presence or absence of oxygen. (Bouwer & Zehnder, 1993, Baker & Herson, 1994). In the following text, residues from composting will be called compost and residues from anaerobic digestion will be called sewage sludge when the source is wastewater treatment or digestates when the source is other types of organic waste.

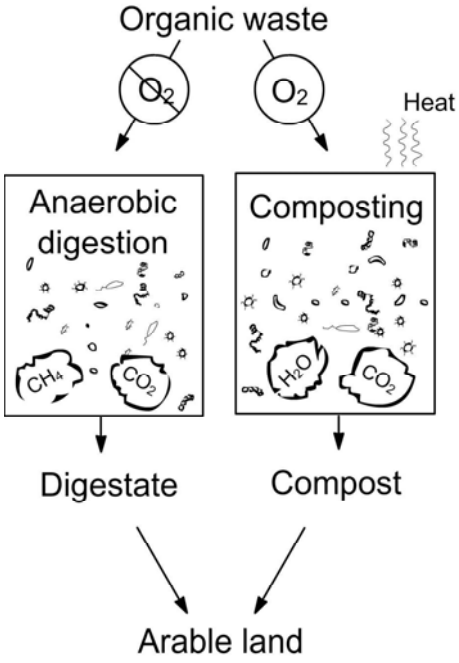

Fig. 2. Organic waste can be treated either aerobically by composting or anaerobically by anaerobic digestion. Both methods utilize the vast degradation potential that microorganisms possess in order to degrade mixed organic wastes. The original waste is converted into nutrient-rich residues, digestate and compost, which can be used as fertilizers on arable land.

Organic waste residues as fertilizers

Compost, digestates and sewage sludge all have high contents of plant nutrients. Therefore such residues have the potential to function as an excellent complement to manure and commercial fertilizers in agriculture, and to contribute in maintaining the organic matter content of soil. In general, the addition of organic matter is beneficial for soil fertility, since it will improve soil structure, increase water-holding capacity and stimulate microbial activity (Ritz, Wheatley & Griffiths, 1997, Simek *et al.*, 1999, Marinari *et al.*, 2000). Increased microbial

activity will in turn lead to a further release of substantial amounts of plant nutrient from both the added residue and the soil organic matter in itself.

One major difference between aerobically and anaerobically produced residues is that compost often contains lower amounts of nitrogen, since substantial losses of NH_3 can occur during composting. Therefore, it is not likely that compost alone can function well as a complete fertilizer. However, amendment with compost will gradually build up a pool of organic nitrogen in soil (Odlare, 2005). As this pool increases, NH_4^+ will eventually be released, which could be sufficient to increase the crop yields. Presently, compost must be complemented with mineral nitrogen in order to function as a fertilizer, although it has been reported to function well as a soil conditioner (Svensson, Odlare & Pell, 2004). During 2004, 136 000 metric tons of compost was produced, and this was mainly used as a soil conditioner (The Swedish Association of Waste Management, 2005).

In contrast to composts, digestates and sewage sludge have greater potential as fertilizers, since they contain large amounts of NH_4^+. Only in intensive grain cropping might there be a need to complement the digestate with mineral nitrogen (Svensson, Odlare & Pell, 2004). A few field trials have evaluated the effects of fertilization with digestates on crop yield. These studies have shown that the use of anaerobically digested municipal household waste will increase crop yields compared to unfertilised soil, but not to the same level as mineral fertilizers (Rivard *et al.*, 1995, Svensson, Odlare & Pell, 2004, Odlare, 2005). In 2004, 211 000 metric tons of digestate was produced in Sweden, of which 93 % was re-circulated back to agricultural land (The Swedish Association of Waste Management, 2005).

Pollutants in organic waste residues

Before urban organic waste residues can be used in agriculture on a large scale it is important that their quality can be guaranteed, with respect to the content of heavy metals, organic pollutants and pathogens. This is important in order to prevent disturbance to the soil ecosystem and avoid health risks arising from the production of food and feed. In 1999, a voluntary certification system for anaerobic digestates and composts was launched (Report from The Swedish Association of Waste Management, 99:2). In order to be certified according to this system there is an obligation to meet certain standards and declare the contents of plant nutrients, heavy metals and pathogens. However, in the present certification system there is no requirement regarding organic chemicals in the residues.

Sewage sludge often contains large amounts of both heavy metals and organic pollutants. Therefore the Swedish Farmers Association (LRF) advises against the use of sewage sludge in food cropping systems (LRF:s policy and goal nr 42323). However, many studies have investigated the effects of sewage sludge application in agriculture and several of them have reported no negative effects on soil microbiology even though metal-rich sludge was applied (e.g. Chandler, Brookes & Harding, 1995, Johansson, Stenberg & Torstensson, 1999). However, sewage

sludge will probably not gain acceptance as a fertilizer in agriculture due to uncertainties concerning the effects of accumulation of heavy metals on the soil ecosystem in the long-term. As with sewage sludge, compost can also be polluted with heavy metals, originating from anthropogenic activities in urban areas, since the initial waste is sometimes mixed with urban park and garden waste prior to composting (Odlare, 2005). This is unfortunate, but can hopefully be addressed by choosing other types of external carbon source.

Other types of urban organic wastes do not suffer from problems associated with metal contamination. It has for instance been shown that anaerobic digestion residues that originate from source-separated organic household waste contain only small amounts of heavy metals (Eklind *et al.*, 1997). However, organic waste may contain a variety of organic pollutants (e.g. dioxin-like compounds, phthalate esters, phenolic compounds, pesticides and PCBs). Many of the organic pollutants are difficult to avoid since they originate from pesticide traces on fruit and vegetables, or are formed during the degradation of larger organic compounds. Several of these pollutants resist degradation and will end up in the residual product after biological treatment of the waste (Grossi, Lichtig & Krauss, 1998, Angelidaki, Mogensen & Ahring, 2000, Nilsson, 2000, Nilsson, Kylin & Sundin, 2000, Engwall & Schnürer, 2002, Olsman *et al.*, 2002, Levén & Schnürer, 2005). At present, it is not known how common such compounds are in different types of biologically treated organic waste residues and what effects they have on the soil environment when applied with the residues to agricultural land.

Organic pollutants applied to soil with organic residues

Phenols are a group of compounds that are commonly found in the environment and they have a variety of both natural and industrial sources such as pesticides, plant material, manure etc. They are also common degradation products from many kinds of organic material. Therefore it is not surprising that they can be found in biologically treated waste residues. Many phenols are toxic at low concentrations and have a persistent nature and are as a result often considered priority pollutants even though they may have natural sources (Alonso *et al.*, 1998).

In this thesis, chemical analysis of anaerobic digestates from seven Swedish large-scale biogas processes revealed the presence of many different phenolic compounds (Fig. 3). Phenol concentrations in some of the digestates were almost at the maximum limit in the guidelines for contaminated soil issued by the Swedish Environmental Protection Agency (4 μg g^{-1} dry weight soil for phenol and creosol; **paper II**).

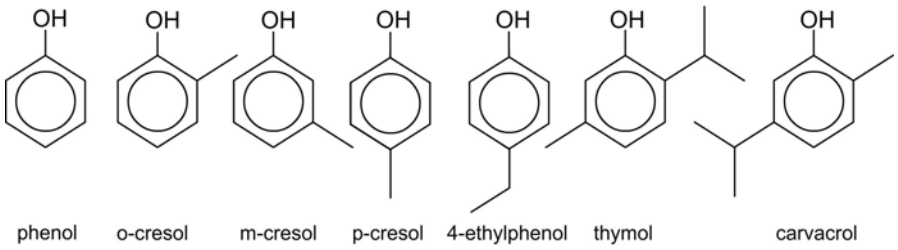

Fig. 3. The main phenols found in digestates from anaerobic digestion processes degrading mixed organic wastes.

The presence of phenols in biologically treated waste residues is unwanted due to their toxic properties, which will make the residues unattractive as fertilizers. However, when the degradation of phenols in soil was investigated (**paper II**) they all appeared to be rapidly degraded in soil. This would imply that the phenols found in the investigated digestates would not constitute an environmental risk if applied in agriculture. On the other hand, the degradation study performed in this thesis (**paper II**) was conducted with pure phenols, so it is still not clear how the phenols behave when added together within a waste residue matrix.

It is not a simple matter to evaluate the risks for the soil ecosystem resulting from organic pollutants present in waste residues used as fertilizers. By identifying the pollutants present in the residues, their fate and degradation patterns in soil can be investigated. Such information will be of importance for estimating the long-term effects on the soil ecosystem. For instance, if the pollutants are rapidly degraded or transformed to non-toxic intermediates they will probably not pose any long-term threats. But they might influence soil fertility if they have characteristics that allow accumulation in soil.

Microorganisms in soil

Microbial diversity in soil

Even though microorganisms only comprise 1-3% of the soil organic matter (Jenkinson, 1988), they are critical for both soil structure and fertility. The microbial biomass itself constitutes a minor pool of nutrients in soil, but their role in the decomposition of organic matter leads to the release of substantial amounts of bound nutrients. In addition, microorganisms are key players in all major biogeochemical cycles, such as carbon, nitrogen and sulphur turnover, and have a critical role in many environmental processes, i.e. climate change and degradation of pollutants (Forney, Zhou & Brown, 2004, Curtis & Sloan, 2005).

Soil is a highly complex and heterogeneous environment and its microbial diversity is immense, with as many as 10^9 bacterial cells per gram soil (Torsvik, Goksoyr & Daae, 1990, van Elsas & Rutgers, 2005). As more and more quantitative studies are being performed within the field of microbial ecology, an

understanding is developing of just how diverse the soil ecosystem really is. The large diversity of bacterial populations in soil presents a challenge for all who study bacterial communities.

There is also great diversity among the different functions that are performed by microorganisms. Due to the complex living environment that nature offers, regarding different habitats and possible combinations of physical and chemical conditions, microorganisms have evolved to fulfil a huge variety of different metabolic functions. A functional redundancy is often found in microbial systems, as many functions can be performed by several taxonomically different groups of bacteria. This can be regarded as a safety component for the soil ecosystem, as any given function can still be performed even after a change in the community structure. Thus, functional stability does not necessarily imply a stable community structure (Nannipieri *et al.*, 2003). However, it is important to investigate and understand shifts in community structure, since stresses or disturbances that result in reduced microbial diversity or quantity in soil could imply a risk for a deterioration in the ability to perform a certain function as well as to withstand perturbations in a long-term perspective (Girvan *et al.*, 2005).

Measuring microbial diversity and activity in soil

Structural diversity

Microbiologists have faced limitations in their exploration of microbial communities in environmental samples due to the large number of microorganisms that are unculturable in the laboratory. It has been estimated that 95-99% of the total amount of soil bacteria are unculturable (Torsvik, Goksoyr & Daae, 1990, Amann, Ludwig & Schleifer, 1995). The rapid progress in molecular biology has led to the development of numerous cultivation-independent methods that enable microbial ecologists to further study the diversity of microorganisms directly in environmental samples. Depending on which approach is chosen, microbial community structure can be analysed at different resolution levels.

The extraction of DNA from environmental samples and the use of polymerase chain reaction (PCR) to amplify ribosomal RNA (rRNA) genes, as reviewed by Head, Saunders & Pickup (1998), has greatly facilitated studies of microorganisms in the environment. PCR-amplified rRNA fragments can be studied by different fingerprinting methods (Fig. 4). Two such methods that have been used in this thesis are denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP; **paper III-V**). With DGGE, PCR-fragments of the same size but differing in sequence can be separated and visualized as individual bands on a polyacrylamide gel with a gradient of increasing denaturant, due to differences in melting behaviour of the DNA (Muyzer, De Waal & Uitterlinden, 1993). Individual bands can be excised from the gel, re-amplified and then sequenced. This can provide information regarding the identity of different populations within a community (Ranjard, Poly & Nazaret, 2000). In T-RFLP, fluorescently tagged PCR-products, digested with restriction enzymes, are separated according to size by electrophoresis and

different populations are visualized as peaks in electropherograms (Marsh, 1999, Osborn, Moore & Timmis, 2000). With rRNA based approaches one generally has the option to either target the total bacterial community or a specific group, depending on the PCR-primers that are used (Head, Saunders & Pickup, 1998).

Even though most molecular fingerprinting methods are based on nucleic acids, RNA is not the only macromolecule that can be used as a target in microbial ecology (Tunlid & White, 1992, Pinkart *et al.*, 2002). In particular, the phospholipid fatty acid (PLFA) composition of cell membranes is frequently targeted in microbial ecology studies (Bååth, Frostegård & Fritze, 1992, Ibekwe & Kennedy, 1998, Peacock *et al.*, 2001, Debosz *et al.*, 2002; **paper III**). PFLA analysis does not provide information at the level of species, but rather groups the microbial community, as different subsets of organisms have different signature lipids in their membranes (Tunlid & White, 1992). One benefit of the PLFA method is that it can also be used to estimate the microbial biomass, since phospholipids are rapidly degraded in soil after cell death (Balkwill *et al.*, 1988, Pinkart *et al.*, 2002).

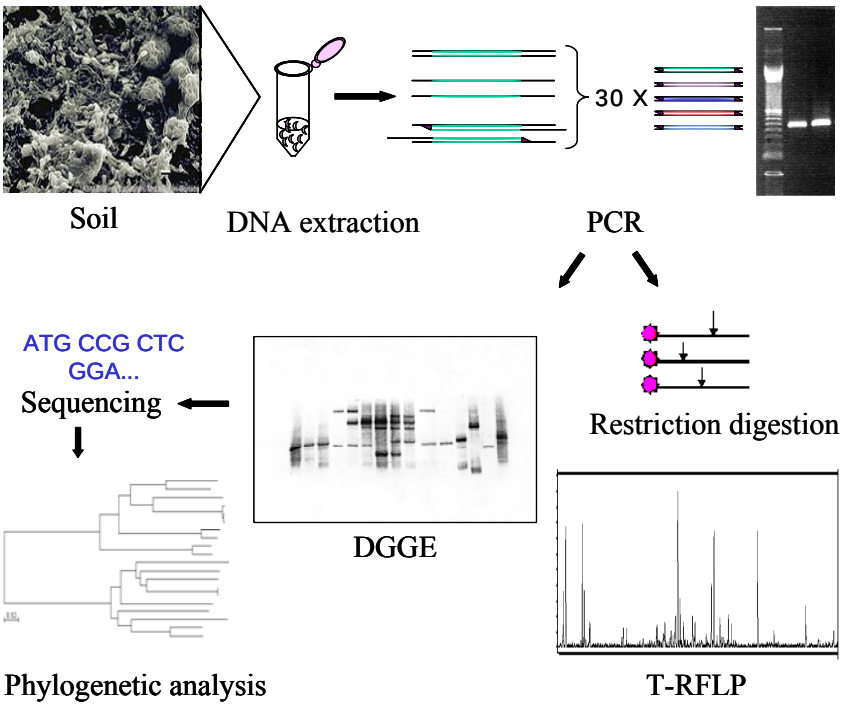

Fig. 4. Schematic drawing of the approach that was used in this thesis to investigate the community structure of ammonia oxidizing and total bacterial communities in soil. DNA is extracted directly from the soil and is amplified by PCR. The PCR-products can then be studied by fingerprinting methods such as DGGE or T-RFLP.

Functional diversity

Community fingerprinting based on rRNA genes or PLFAs gives little information on soil functional diversity. One way to study microbial functions is by activity measurements, or by the use of potential enzymatic activities for specific functions, such as the turnover of carbon and nitrogen. Some examples of proposed microbial indicators for soil health are potential ammonia oxidation, potential denitrification, basal respiration and substrate induced respiration (SIR; Torstensson, Pell & Stenberg, 1998, Stenberg, 1999). The potential ammonia oxidation method, which has been used throughout this thesis (**paper I-V**), is assayed as accumulated nitrite in soil slurries that contain optimized amounts of ammonia, chlorate and buffer (Belser & Mays, 1980, Torstensson, 1993, ISO, 2004). SIR (**paper I & IV**) is measured as the increase in basal respiration caused by the addition of glucose, and is determined by monitoring the conductivity change in KOH caused by CO_2 trapping (Stenström, Stenberg & Johansson, 1998, Pell, Stenstöm & Granhall, 2005). The ratio between basal respiration and SIR, the metabolic quotient (qCO_2), which reflects the efficiency of heterotrophic microorganisms to convert organic carbon into microbial biomass, has been used as an indicator of stress caused by heavy metals and pesticides in soil (Chandler, Brookes & Harding, 1995, Jones & Ananyeva, 2001, Renella *et al.*, 2005). A high qCO_2 value can be regarded as an indicator of increased stress (Anderson & Domsch, 1985).

Potential activities are cheap and rapid to measure, and have previously been used in several soil toxicity studies (Johansson, Pell & Stenström, 1998, Pell, Stenberg & Torstensson, 1998, Johansson, Stenberg & Torstensson, 1999). Integrated approaches to study the effects of contaminants on soil microorganisms are especially valuable. Methods at different levels of resolution can be combined, for instance the ammonia oxidation assay that targets a small and sensitive group of bacteria and SIR, which is a robust measure of the activity of all aerobic bacteria that respond to glucose (Torstensson, Pell & Stenberg, 1998).

Targeting the active microorganisms

It is a challenge for microbial ecologists to link functional diversity to structural diversity. Elucidating these links would help to improve understanding of ecosystem functions (Tilman *et al.*, 1997, Hulot *et al.*, 2000, Loreau & Hector, 2001, Worm *et al.*, 2002). Several methods have been employed to target the active microorganisms and to link community structure with corresponding functions, e.g. targeting mRNA for functional genes (Nogales *et al.*, 2002, Bürgman *et al.*, 2003) or stable isotope probing (SIP; Radajewski *et al.*, 2002, Rangel-Castro *et al.*, 2005). One experimental strategy used in this thesis (**paper V**) is bromodeoxyuridine incorporation and immunocapture. In this method, soil is incubated with the thymidine analogue 5-bromo-2'-deoxyuridune (BrdU), which is thereby incorporated into the DNA of growing cells. The BrdU-incorporated DNA can then be separated from the total DNA by immunocapture with antibodies against BrdU. The BrdU method has previously been used to investigate bacterial communities in soil (Borneman, 1999, Yin *et al.*, 2000,

Artursson & Jansson, 2003) as well as in marine and freshwater environments (Urbach, Vergin & Giovannoni, 1999, Pernthaler *et al.*, 2002, Warnecke *et al.*, 2005).

The use of an indicator organism

Specific effects of perturbation can be difficult to detect because of the vast diversity of bacteria in soil and significant functional redundancy. By targeting either a specific function or a smaller taxonomical group of bacteria, as a key species or indicator organism, this complexity can be reduced (Øvreås & Torsvik, 1998, Nannipieri *et al.*, 2003). This would facilitate the study of specific effects of perturbations on the microbial components in soil. The ideal case is that of an ecologically important biological function carried out by a specific group of bacteria. The organism group responsible for nitrification is a good example of this.

Ammonia oxidizing bacteria in soil microbial ecology

Nitrification in terrestrial environments

Nitrification is a central part of the global nitrogen cycle (Fig. 5). It involves the bacterial oxidation of reduced inorganic nitrogen to nitrate (NO_3^-). The reduced nitrogen can be oxidized either heterotrophically or autotrophically, although autotrophic nitrification is considered to be the most significant form. Autotrophic nitrification consists of a two-step oxidation process carried out by two groups of bacteria, in which ammonia (NH_3) is oxidized first to nitrite (NO_2^-) and then to NO_3^-. The first step is performed by ammonia oxidizing bacteria and the second by nitrite-oxidizing bacteria. Both groups of bacteria are strict aerobes with oxygen as their terminal electron acceptor, and they utilize the reduced nitrogen as an energy source and CO_2 as a carbon source (Prosser, 1989).

Ammonia (NH_3) is released to soil when organic material is mineralized or, as in agriculture, by fertilization with inorganic ammonium or urea. At neutral pH it is present as ammonium (NH_4^+), which is relatively stable in soil since it can attach to negatively charged clay particles. Due to nitrification, NH_4^+ is transformed into the negatively charged NO_2^- and NO_3^- ions, which are much more mobile in soil. This can result in leaching of NO_3^- to surrounding waters, which in turn can lead to eutrophication of surface water or groundwater. In addition, nitrification can result in gaseous losses of nitrogen from the soil environment due to the production of NO, N_2 and N_2O. N_2O is both a greenhouse gas and an ozone destructing agent (Conrad, 1996). Thus, a high nitrification rate can have several environmental drawbacks, especially in agricultural environments with large inputs of nitrogen fertilizers.

Autotrophic ammonia oxidizing bacteria

Autotrophic ammonia oxidizing bacteria (AOB) perform the first step of the nitrification process, the oxidation of NH_3 to NO_2^-. AOB are aerobic, gram negative and chemolitho-autotrophic bacteria. They have a relatively long generation time, ranging between 11 and 50 hours in pure cultures (Prosser, 1989). Their slow growth is due to bioenergetic problems. The use of NO_3^-/NH_3 couple as electron donor gives a low production of ATP. Furthermore, most of the energy produced must be used for the fixation of CO_2 via the Calvin cycle. AOB are also quite poor competitors for ammonia, and are therefore difficult to study with conventional microbiological cultivation techniques (Prosser & Embley, 2002). Therefore, the use of culture-independent methods, such as the PCR-based methods described above, has revolutionized our knowledge of AOB communities in the environment. In this thesis, denaturing gradient gel electrophoresis (DGGE) (Muyzer, De Waal & Uitterlinden, 1993) of 16S rRNA AOB fragments has been used to study the AOB community composition in environmental samples (**paper III, IV &V**). This is a well established approach that has been used frequently in other studies (Stephen *et al.*, 1998, Kowalchuk *et al.*, 2000, Bäckman *et al.*, 2003, Cébron *et al.*, 2004).

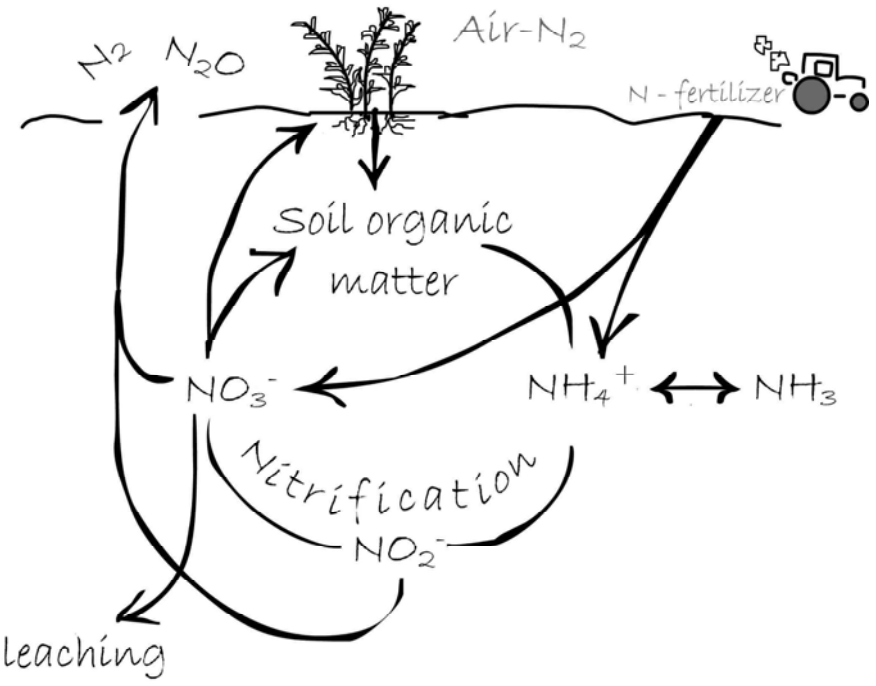

Fig. 5. The nitrification is a central part of the global nitrogen cycle. This drawing gives a simplified picture of the nitrogen cycle as it occurs in soil.

Studies of 16S rRNA of AOB have revealed a strong relationship between function and phylogeny. All known isolates of AOB belong to the two phylogenetic groups of *β*- and *γ*-Proteobacteria (Head, Saunders & Pickup, 1998).

However, only AOB belonging to the *β*-Proteobacteria have been found in terrestrial environments (Fig. 6). The *β*-Proteobacterial AOB are generally divided into two genera, *Nitrosospira* and *Nitrosomonas*, which can then further be divided into nine separate clusters (Purkhold *et al.*, 2000, Purkhold *et al.*, 2003). However, it is difficult to perform such subdivisions, because all known nitrosospiras are very closely related phylogenetically (Purkhold *et al.*, 2003).

AOB as indicators

Because of their environmental and economic importance, AOB have been extensively studied and have become somewhat of a model system in molecular microbial ecology (Kowalchuk & Stephen, 2001). Ammonia oxidizing communities have been studied in a wide variety of environments such as agricultural soils, forest soils, grasslands, sand dunes, sediments, marine environments and activated sludge processes (Kowalchuk *et al.*, 1997, Mendum, Sockett & Hirsch, 1999, Webster, Embley & Prosser, 2002, Bottomley *et al.*, 2004, Bernhard *et al.*, 2005, Hallin *et al.*, 2005, O'Mullan & Ward, 2005).

As mentioned above, the activity of AOB can be studied as part of an integrated approach to assess soil health (Torstensson, Pell & Stenberg, 1998). The activity of AOB has been reported to be more sensitive to different pollutants than other microbial parameters (van Beelen & Doelman, 1997) and has therefore been used as a indicator of environmental impact in soil toxicity studies (Remde & Hund, 1994, Pell, Stenberg & Torstensson, 1998, Nielsen *et al.*, 2004). The sensitivity of AOB can be explained by their complex metabolic cell machinery, from the combination of being both lithotrophic and autotrophic, and their narrow taxonomic origin (Pell & Stenström, 2005). The advantage of using AOB as an indicator is that they are a monophyletic group of bacteria that are the major performers within their functional guild. Therefore, if a pollutant has negative effects on AOB it is likely to be detected as a reduced ammonia oxidation rate.

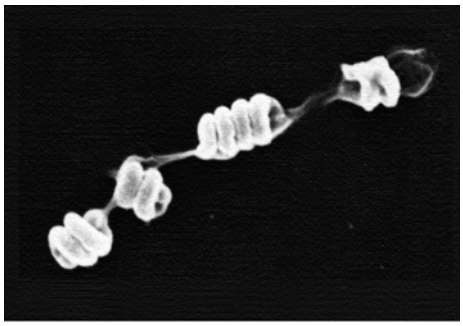

Fig. 6. A scanning electron photomicrograph of *Nitrosospira* sp. isolated from arable soil, Norway. The micrograph is taken and kindly provided by Ågot Aakra.

Impact of organic residues on bacterial communities in soils

Total bacterial response to organic waste residues

It is commonly reported that amendments of organic waste residues and manure to agricultural soil have major effects on biomass and activity of soil microorganisms (Ritz, Wheatley & Griffiths, 1997, Pascual, García & Hernandez, 1999, Simek *et al.*, 1999, Debosz *et al.*, 2002, Petersen *et al.*, 2003). The amount of easily degradable carbon in the residues directly stimulates the heterotrophic bacterial community in soil, which can be measured as an increase in activity. Different fertilization regimes have been shown to also affect the total microbial community structure (Peacock *et al.*, 2001, Enwall, Philippot & Hallin, 2005). However, it has proven difficult to link changes in microbial activity to changes in community structure.

In this thesis, laboratory incubations were performed with soil amended with dried organic waste residues as well as organic extracts of the residues (**paper I & III**). The total microbial community, measured by both SIR rates (**paper I**) and PLFA concentration and composition (**paper III**), remained largely unchanged compared to the unamended controls. Similarly, Debosz *et al.* (2002) found that incubations with household compost and sewage sludge had no effect on the total amount of PLFA in soil. In contrast, Frostegård *et al*. (1997) stated that a detected shift in PLFA-based community structure caused by manure amendment was correlated to the dissolved organic carbon released from the manure. Factors that could explain why no changes in community structure were detected in this thesis (**paper III**) are time of sampling and total incubation time. All these experiments were performed as laboratory incubations over 2 weeks to 3 months, and it is possible that the chosen incubation times were to low for shifts to occur, or that only temporary shifts occurred after amendment.

Table 1. The treatments studied in the Ultuna long-term soil organic matter experiment. The table is modified from Table 1 in paper IV

Treatment	Fertilizer regime	pH[1]
A	Unfertilized (without crop)	5.47 ± 0.16
B	Unfertilized	5.63 ± 0.05
C	Calcium nitrate $Ca(NO_3)_2$	6.26 ± 0.04
D	Ammonium sulphate $(NH_4)_2SO_4$	3.97 ± 0.14
J	Solid cattle manure	6.02 ± 0.09
O	Sewage sludge	4.68 ± 0.03

[1]When the experimental field site was established in 1956 the soil pH was 6.5

Suitable field experiments are needed in order to study the long-term effects of different fertilization regimes on soil bacterial communities. Unfortunately, field experiments that include urban waste residues, other than sewage sludge, are not common. One long-term agricultural field experiment is located at Ultuna campus, situated south of Uppsala, Sweden (as described in Kirchmann, Persson & Carlgren, 1994; Fig 7). This site was established in 1956, in order to investigate the impact of different fertilization regimes on crop yield, soil carbon and nitrogen content, pH and soil structure. The experimental site comprises four replicated blocks, each consisting of 15 plots that are treated with different fertilizers, e.g. sewage sludge from the municipal wastewater treatment plant in Uppsala, solid cattle manure and the inorganic fertilizers $Ca(NO_3)_2$ and $(NH_4)_2SO_4$. This field experiment therefore offers a good opportunity to study the long-term effects of at least one type of urban waste residue (sewage sludge) and enables us to compare it with manure and inorganic fertilizers. In this thesis (**paper IV & V**), soil samples from the four treatments mentioned above, as well as two unfertilized treatments with and without crops, have been investigated. These treatments are described in Table 1.

Total bacterial activities were investigated by measuring basal respiration and substrate induced respiration (SIR), and the metabolic quotient (qCO_2) was used as an indicator of stress. A high qCO_2, which indicates increased stress (Anderson & Domsch, 1985), was observed after long-term amendment of sewage sludge (**paper IV**). These results were in concordance with previous studies by Fernandez, Bettiol & Cerri (2005) who also detected high qCO_2 in soil fertilized with sewage sludge. One explanation for these results could be the large concentrations of Cd which have been found in the sludge-amended soil in the Ultuna experiment (Bergkvist *et al.*, 2003). However, a high qCO_2 was also detected in the plots fertilized with $(NH_4)_2SO_4$ (**paper IV**). These two fertilizers have in common that continued application has caused a drastic decrease in the soil pH (Table 1). Thus, it is likely that the enhanced stress detected in these two treatments is correlated to a low pH in addition to pollutants added in the sewage sludge (**paper IV**). Correlations between pH and qCO_2 have also been reported by Blagodatskaya & Anderson (1998).

Fig. 7. Two of the four blocks in the Ultuna long-term soil organic matter experiment, showing the differences in growth in the differently fertilized field plots. Photographed and kindly provided by Karin Enwall.

As observed in previous studies (Enwall, Philippot & Hallin, 2005), the activity and community structure of total soil bacteria from samples from the Ultuna field experiment did not correlate with each other. Differences in activity caused by the fertilization regimes did not give rise to corresponding shifts in T-RFLP patterns (**paper IV**). However, a pH effect was detected. The treatments with the most distinct T-RFLP patterns were the ones with low soil pH (fertilized with $(NH_4)_2SO_4$ and sewage sludge). No clear differences in community structure were seen among the other treatments (cattle manure, $Ca(NO_3)_2$ and unfertilized with and without crops; **paper IV**). Both Girvan *et al.* (2003) and Larkin, Honeycutt & Griffin (in press), have proposed that soil type is the primary determinant of the bacterial community composition in arable soils, and not different amendments or management practices. However, after 50 years of differing management it can be argued that the fertilization regimes have completely changed the soil conditions, which can explain the shifts that were observed.

Effects of organic waste residues on soil AOB

Effects on AOB activity

It is difficult to evaluate effects on soil microbes of organic pollutants present in a mixed organic waste residue, because of the general stimulation that will occur due to the vast amount of easily degradable carbon applied together with the waste. However, in this thesis, an extraction method was used that made it

possible to isolate the organic fraction from complex waste residues and thereby study their effects separately (**paper I**). The extraction procedure was performed by shaking dry residues with acetone and hexane, and was designed to extract a wide range of organic compounds. The organic extract could then be concentrated and added to soil in laboratory incubation studies. With this approach, it was demonstrated that anaerobic digestates and swine manure can contain organic substances that inhibit AOB activity, and that such inhibitions were not detected when the whole waste fraction (unextracted) was investigated (**paper I, II & III**). It appears as if the particulate material added together with the initial unextracted waste will cause a positive response, overshadowing underlying negative effects. Since AOB are autotrophic bacteria and thus not directly dependent on organic carbon the stimulation of their activity after application of whole residues is probably due to direct or indirect additions of nitrogen (**paper I & III**).

Phenols were identified as the probable cause of AOB activity inhibition after amendment of waste residues extracts (Fig. 3; **paper II**). The degree of ammonia oxidation inhibition after amendment with anaerobic digestates was clearly related to the concentration of phenols in the digestates (Fig. 2 in **paper II**). It was revealed that digestates with high phenol concentration inhibited AOB activity to a great extent whereas digestates with low phenol concentration did not. In addition, dose response tests on potential ammonia oxidation activity with pure phenols supported the hypothesis that it was the phenols in the digestates were the cause of inhibition. Interestingly, the EC_{50} was lower after amendment with digestate extract than after amendment of pure phenols, indicating synergistic effects caused by a combination of phenols acting together in the residues. Another likely explanation is that other pollutants present in the digestates also could be responsible for the enhanced degree of AOB inhibition.

Effects on AOB community structure

Even though major inhibitory effects on AOB activity occurred after amendment with both waste residue extracts and pure phenols, no changes could be detected on AOB community structure when the total AOB DNA was analysed by as DGGE fingerprinting (**paper III & V**). This is in accordance with other studies in which changes in activity of AOB have not been followed by subsequent shifts in DGGE patterns (Phillips *et al.*, 2000, Chang *et al.*, 2001, Avrahami, Conrad & Braker, 2002, Ibekwe *et al.*, 2002). As ammonia oxidizers grow slowly, both incubation time and temperature are important factors, especially in laboratory incubation studies (Avrahami, Conrad & Braker, 2002, Avrahami, Liesack & Conrad, 2003). Therefore it is possible that changes in AOB community structure would have occurred if a prolonged incubation time had been used.

However, even though no changes in DGGE banding pattern were detected, a significant inhibition of activity is a clear indication that the bacterial community was affected in some way. A decreased AOB activity could be caused by either a shift in AOB community structure or reduction in their abundance. That a variation in AOB cell concentrations can occur without it being detected as a shift in DGGE fingerprint has previously been reported by Phillips *et al.* (2000).

Another possible reason for the lack of change in the DGGE banding pattern is that DNA may be preserved in dead cells, or is present as naked DNA that is slowly degraded outside dead cells (Blum, Lorenz & Wackernagel, 1997). Thus, such DNA can be picked up during extraction and PCR amplification of environmental samples, generating bands on the DGGE. The difficulties in distinguishing between DNA from viable and non-viable cells can be a problem when short-term impacts of potential stress agents on community structure are studied.

In this thesis, DNA from replicating AOB cells was separated from the total DNA by using a BrdU-immunocapture approach. Thereby, the possibility to detect short-term effects on AOB communities by studying the active AOB specifically was explored (**paper V**). Soil from two of the treatments from the Ultuna long-term experiment, described above, was used (Table 1). The soils were chosen due to their differences concerning activity and diversity, as well as in soil properties (as shown in **paper IV**). The soil fertilized with cattle manure (J) had a higher AOB activity and diversity, while the sewage sludge-amended soil (O) had lower activity and diversity. The soils were supplemented with a stress agent, 4-ethylphenol, at a dose estimated to drastically inhibit AOB activity. The soils were then incubated together with BrdU for 2 weeks.

By using the BrdU method, it was shown that the replicating AOB were not as divers as the total AOB community, as fewer bands were visible on the DGGE gel. Furthermore, with this method effects on the active community structure could be detected even after a short incubation time (2 weeks), although the total AOB community remained unchanged (Fig. 8; **paper V**). Interestingly the two different soil types reacted differently to the stress agent. In the soil with initially high diversity (J) a shift occurred in response to 4-ethylphenol amendment. In the low-diversity soil, however, the DGGE bands were the same after stress, only weaker, indicating a lower AOB abundance. It is possible that the sewage sludge amended soil was less resistant to 4-ethylphenol stress because of the low initial diversity.

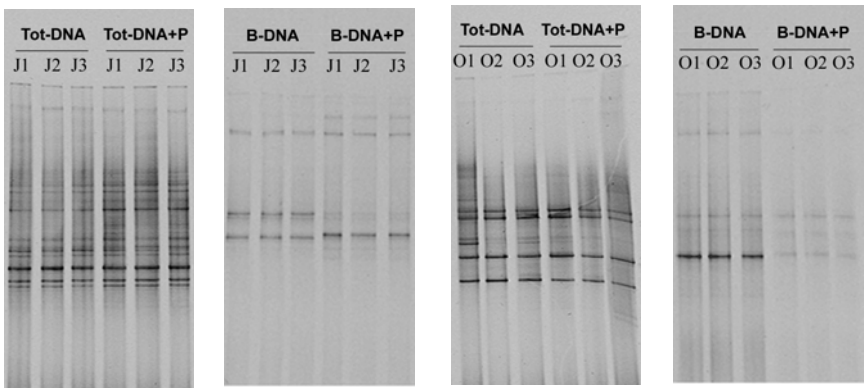

Fig. 8. Differences in DGGE banding pattern of specific AOB 16S rRNA fragments when comparing total AOB DNA (Tot-DNA) and BrdU incorporated AOB DNA (B-DNA). The two soils have been stressed by amendment of 4-ethylphenol (+P). The picture is modified from Figs. 2 & 3 in paper V.

By using the BrdU-immunocapture approach it was possible to detect short-term changes in the community structure after stress (**paper V**). This is, to our knowledge, the first time that the incorporation of BrdU into replicating AOB cells in soil has been reported. Short-term effects have previously been difficult to identify in conventional studies of total extractable DNA. However, it is important to note that there is a possibility that not all bacteria can incorporate BrdU into their DNA to the same extent (Borneman, 1999, Urbach, Vergin & Giovannoni, 1999, Artursson, Finlay & Jansson, 2005). In a study by Artursson, Finlay & Jansson (2005) no bacteria with G/C rich genomes were detected in BrdU incorporated DNA. However, other studies have not reported any similar problems with BrdU incorporation (Pernthaler *et al.*, 2002, Warnecke *et al.*, 2005). The results presented in this thesis would be difficult to interpret if differences in the ability to incorporate BrdU existed within the group of ammonia oxidizing bacteria. However, we assume that AOB will incorporate BrdU to a similar extent, because they are so similar phylogenetically.

Long-term effects of organic fertilizers on AOB

Even though AOB are phylogenetically close they do possess physiological differences, for example with respect to substrate preferences, pH tolerance and response to different environmental factors. The community composition of AOB has been shown to respond to different N inputs, liming and temperature (Hastings *et al.*, 1997, Oved *et al.*, 2001, Avrahami & Conrad, 2003, Bäckman *et al.*, 2003). In agriculture, fertilization can strongly alter the properties of the soil and thus the habitat for the AOB. For instance, many fertilizers decrease the soil pH, and pH is a factor that impacts the growth and activity of AOB (Prosser & Embley, 2002). In addition, different groups of AOB appear to be adapted to different soil pH (Stephen *et al.*, 1998, Kowalchuk *et al.*, 2000). However, so far it appears difficult to generalize connections between environmental conditions and differences in AOB community structure.

In this thesis, the long-term effects of fertilization on the AOB communities were studied in soil samples from the Ultuna experiment (Table 1; **paper IV**). It is not surprising that effects on the activity and community structure of the AOB were found (**paper IV & V**) as the different fertilization and agricultural management treatments in this experiment have had a clear impact on many soil properties such as pH, carbon and nitrogen content (Enwall, Philippot & Hallin, 2005). Most obvious was the discovery that the potential activity was drastically lower in the low pH plots, i.e. the ones fertilized with $(NH_4)_2SO_4$ and sewage sludge, than in the other treatments (Fig. 1 in **paper IV**).

An unusually low AOB diversity was also found in these low pH treatments. Especially in the soil plots fertilized with $(NH_4)_2SO_4$, as only a single DGGE band was found in this treatment (Fig. 6 in **paper IV**). Also, a slightly smaller number of bands were detected in the sewage sludge treated plots compared with the other fertilizer treatments. It has been reported that low pH can favour specific

Nitrosospira strains (Stephen *et al.*, 1998, Laverman *et al.*, 2000). In contrast, AOB diversity was higher in the plots fertilized with manure, which also exhibited the largest potential ammonia oxidizing activity. Thus, DGGE results and the potential activities from the long-term field experiment appeared to be linked.

The DGGE results and the low potential activity in the $(NH_4)_2SO_4$ and sewage sludge treatments can be explained either by a low diversity of AOB or by a reduction in the number of AOB cells in the soil. However, studies of the copy number of functional gene amoA, from the same DNA extracts as used in this thesis, showed that the low activity in the plots fertilized with sewage sludge was not correlated to a low number of AOB cells in the soil, although this was the case in the plots fertilized with $(NH_4)_2SO_4$ (unpublished results). These results indicate that the causes of reduced AOB activity may differ. It can be caused by either a decrease in cell quantity or a reduction in bacterial diversity.

Concluding remarks

In this thesis, the ammonia oxidizing bacteria (AOB) have been used as an indicator of environmental disturbance, and by this approach effects on both their structure and function was seen after amendment of biologically treated organic waste residues. However, it is not fully understood how effects on the AOB correlate to and affect the function of the total soil ecosystem. Therefore it can be difficult to know how to interpret an AOB activity inhibition. If the AOB are to function well as indicators, negative effects on their activity should be interpreted as a sign of a potential risk for disturbance of other soil bacteria, which would over time also be of concern for the future soil fertility. Contrary to this, if the AOB are the only bacteria that are affected, the risks involved for the soil ecosystem should be small. In agricultural systems a low nitrification activity is even considered beneficial, as it will prevent nitrogen from being leached from the soil.

However, it is obvious that biologically treated waste residues can be polluted by a complex mixture of compounds that are unwanted in potential fertilizers. In this thesis, phenols were identified in digestates, and previous studies have reported the presence of other kinds of organic pollutants in biological waste residues. What type of pollutants that will be present in biologically treated waste residues will depend on the type of waste that is being treated, as well as on the process management. Therefore, it is not easy to give any general recommendations. However, if there is to be a future acceptance for biological waste residues as fertilizers in agriculture, there is a need for a well functioning certification system. The one that is used today must be extended to also include organic pollutants. Perhaps one way is to list a few key pollutants that can be screened for as part of a routine analysis of the waste residues.

Even though the identity of the pollutants present in waste residues will affect what impact they will have on the soil environment, it is not the only factor that needs to be considered. Another aspect, which has been emphasized in this thesis, is the importance of the state of the soil at the time of impact, as different soil types appear to have different resilience to pollutants. If soil microorganisms already are suppressed by other kinds of stress it is likely that they will be less capable to withstand further impact caused by pollutants in amended fertilizers. Therefore, further knowledge of microbial communities in soil and how they relate to other soil properties is needed. It is also important to consider that the pollutants in organic waste residues will be added to the soil within a matrix of organic matter and inorganic nutrients. This will probably influence what effect the pollutants will have on the soil ecosystem, as compared to if they are added as pure substances. The addition of organic material and nutrients to soil will stimulate the overall microbial activities, which generally is beneficial for the soil and will increase its ability to withstand perturbation.

Many questions still remain on how the utilization of biologically treated organic waste residues in agriculture affects the quality and fertility of the soil ecosystem. The results from this thesis have shown that long-term field studies can facilitate the study of shifts in microbial structure and function. However, it is still largely unknown how such shifts in structure and function of soil microorganisms correlate to the function and fertility of the total soil environment. Such correlations pose a future challenge to explore.

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Acknowledgements

There are a number of people who directly or indirectly have contributed to the completion of this thesis, which I would like to thank:

Först och främst vill jag tacka min handledargrupp för det goda samarbete vi haft under den här tiden och för ert engagemang i mig och mina projekt. Det har varit en lärorik resa och jag är väldigt glad för de erfarenheter och den personliga utveckling som min doktorandtid har gett mig.

Anna Schnürer, ett stort tack för stöd och vägledning under mina år på mikrobiologen, från exjobbet fram till idag. Att du trott på mig och min förmåga och för att du ständigt motiverat mig under den här processen.

Sara Hallin, tack för all den inspiration du givit mig. Din drivkraft har betytt väldigt mycket för mig, speciellt under det senaste året.

Ingvar Sundh, för all värdefull hjälp jag fått från dig, ifrån initierande av projekt till skapandet av manuskript.

Åsa Jarvis, för hjälp och visdom under mina första år som doktorand.

Alla medlemmar i POP-gruppen, vilken bra grupp vi har varit! **Maria Erikson**, speciellt tack för all hjälp med mitt sista projekt, det hade aldrig gått utan dig. **Karin**, **Ingela, Lotta** och **Kristin**, för alla glada stunder på lab, på skrivrummen och på konferenser här och där i världen. Det har helt enkelt aldrig varit tråkigt att jobba med er! **Micke Pell**, tack för alla genomläsningar av min avhandling.

Jag vill även tacka **Harald** och **Leticia,** för alla roliga samtal i korridorerna samt all hjälp med allt som har med jord i rör och burkar att göra.

Elisabeth Börjesson, för hjälp rörande extraktioner, kemikalier och analyser.

Kalle Svensson, som introducerade mig till nitrifikationsmätningar och till FIAn.

Alla nuvarande och tidigare medarbetare på institutionen för Mikrobiologi som gemensamt bidragit till den goda stämningen där. **Ingemar** för all teknisk hjälp, **Sture**, **Anki** och **Barbro** för all administrativ hjälp. Ett stort tack även till alla som hänger i fikarummet och som piggar upp vardagen på alla möjliga sätt, det är många som passerat genom åren, ingen nämnd ingen glömd…

Tjejerna som också ses utanför jobbet, **Karin E**, **Åsa K**, **Klara**, **Torun**, **Mia**, **Lotta J**, **Ingela**, **Helena** och **Anna-Ida**, för alla roliga middagar och kalas. Snart får vi ha vinprovning igen!

Jag vill även passa på att uppmärksamma mina vänner utanför mikrobiologen, en speciell tanke går till mina globe trotter kompisar Kerstin och Teresia (kram).

Ett stort tack även till min kära familj, **mamma, pappa, Ingrid** och **Thomas**, för allt.

Extra stort tack till **Ingrid** och **Magnus**, det är alltid kul att hänga med er, vad vi än hittar på. Tack för alla gånger som ni tar hand om mig, bjuder på middag och tar med mig ut i skogen!

Martin, Jag saknar ord att beskriva vad du betyder för mig. Tack för allt du gjort för mig, speciellt under den här senaste mest hektiska tiden, och tack för att du finns! ♥