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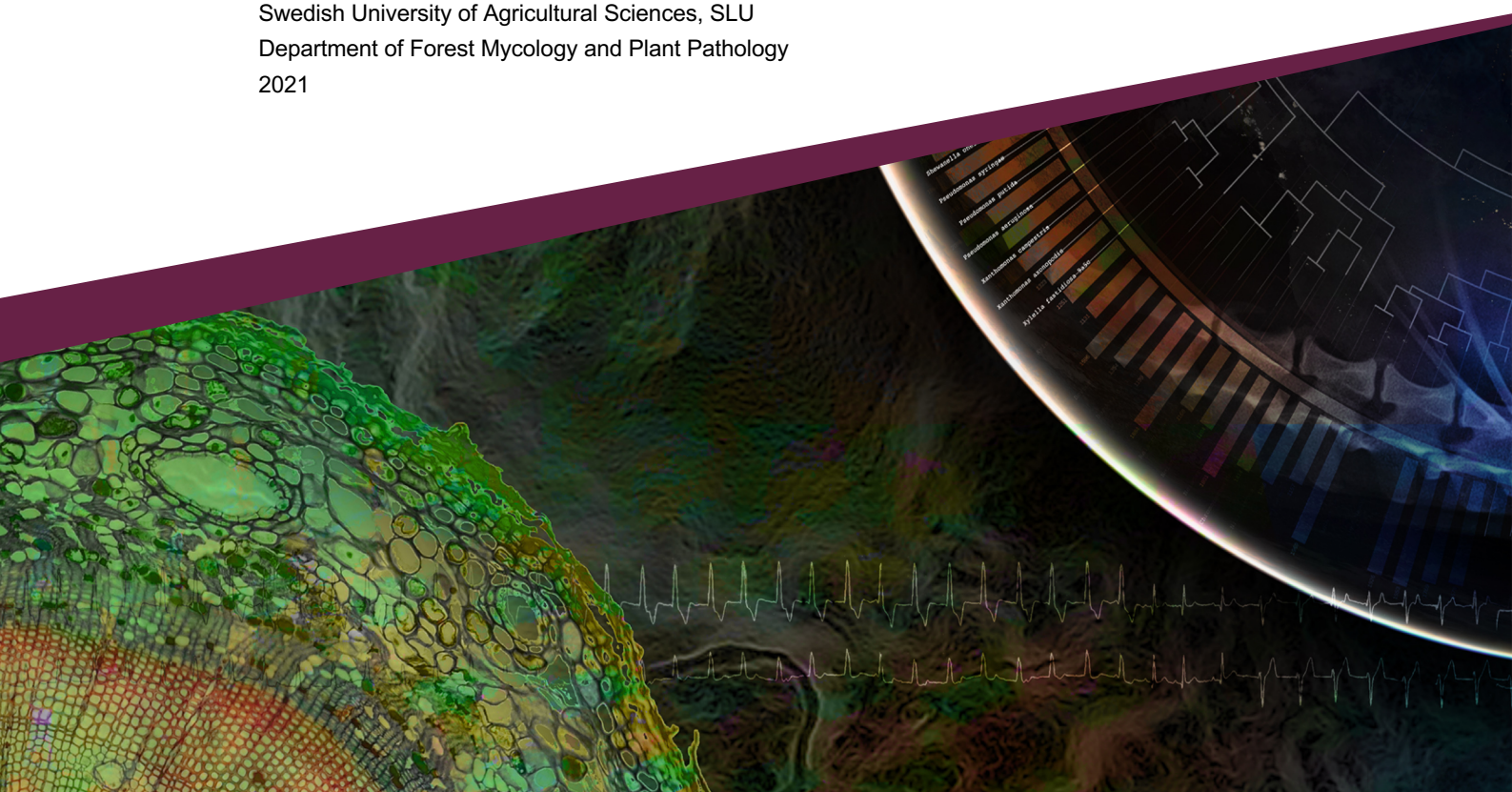

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Summary

The loss of soil organic matter in arable soil has become an increasing problem worldwide, posing a threat to soil fertility and the growing global food demand. Treated and digested sewage sludge is used to bring back organic matter as well as plant nutrients to agricultural soil. In Sweden, ca 39 % of the produced sludge is spread to agricultural fields. However, there are risks involved with the content of metals, organic contaminants, microplastics and pathogens in sludge.

In Sweden, there is a voluntary certification system for municipal sewage sludge quality called Revaq that aims to ensure that sewage sludge used as soil amendment is safe for the environment and has acceptance by the agricultural sector. Certification of sludge involves number of measures, e.g. systematic upstream work to decrease contaminants entering the sewage and annual quality control of sludge, including analyses of 60 trace elements prior to utilization in agriculture. This project investigated the possibility to complement current Revaq analyses by including laboratory measurements of soil microorganisms in sludge amended soil. As targets, we used the overall microbial community composition and the abundance of several functional groups of microorganisms involved in transformation of inorganic nitrogen compounds as well as the activity of these transformations. We assessed both short- and long-term effects of sludge amendment in different soils and with different sludges to evaluate if one or several of targets can be used in routine monitoring or screening tests.

All used targets responded to the sludge amendments in the short-term laboratory screening test, but the response varied between different sludges, and more importantly, the responses were strongly dependent on the soil properties. Microbial community composition was the most sensitive of the tested targets. The measured activities showed positive effects of sludge amendment. The most consistent positive effect across sludges was found on the potential ammonia oxidation, but similar to what was observed for the other targets, the effect was highly soil dependent. The absence of different microbiological responses to sludge treatment in the long-term field samples suggests that the studied sludges did not have long lasting negative effects on the measured microbial activities and functional group abundances in these soils. To conclude, the methodology and microbiological targets used in this study can complement current Revaq tests when assessing sludge prior to use in agriculture. However, choice of reference soil needs consideration before implementing microbiological test for sludge screening.

Sammanfattning

Förlust av organiskt material i åkermark är ett ökande problem över hela världen och utgör ett hot mot markens bördighet och den ökande globala efterfrågan på livsmedel. Behandlat och rötat avloppsslam används för att återföra organiskt material och växtnäringsämnen till jordbruksmark. I Sverige används cirka 39 % av det producerade slammet på jordbruksmark. Det finns dock risker med detta på grund av innehållet av metaller, organiska föroreningar, mikroplaster och patogena organismer i slam.

I Sverige finns ett frivilligt kvalitetscertifieringssystem för kommunalt avloppsslam kallat Revaq som syftar till att säkerställa att avloppsslam som används som jordförbättringsmedel är säkert för miljön och har acceptans i jordbrukssektorn. Certifiering av slam innebär att ett antal åtgärder genomförs, t.ex. ett systematiskt uppströmsarbete för att minska föroreningar i inkommande avloppsvatten till reningsverket och årlig kvalitetskontroll av slam, inklusive analyser av 60 spårämnen före användning i jordbruket. Detta projekt undersökte möjligheten att komplettera nuvarande Revaq-analyser genom att utvärdera laboratorietester för att screena eventuell påverkan på markmikroorganismer vid slamtillsats. Som indikatorer användes sammansättningen och mångfalden av bakterier i mikroorganismsamhället samt mängden och aktiviteten av mikroorganismer i olika funktionella grupper involverade i omvandlingen av oorganiska kväveföreningar. Vi undersökte både kort- och långsiktiga effekter av slamanvändning för att utvärdera om en eller flera av indikatorerna kan användas för rutinövervakning eller i screeningtest.

Alla indikatorerna svarade på slamtillsats i screeningtestet för kortsiktiga effekter, men effekten varierade mellan olika slam och berodde till stor del på markegenskaperna. Sammansättningen av bakteriesamhället var den mest känsliga av de testade indikatorerna. De uppmätta aktiviteterna visade positiva effekter av slamtillsats. Potentiell ammoniakoxidationen uppvisade den mest konsekventa, positiva effekten av de olika slammen, men effekten var liksom för de övriga indikatorerna starkt beroende av jordtyp. Frånvaron av respons när de mikrobiologiska marktesterna användes på fältproverna från försök med långvarig slambehandling tyder på att de studerade slammen inte haft långvariga effekter på de uppmätta mikrobiella aktiviteterna och funktionella grupperna i dessa jordar. Avslutningsvis bedöms screeningmetoden och de mikrobiologiska indikatorerna som används i denna studie kunna komplettera nuvarande Revaq-tester vid bedömning av slam innan användning i jordbruket. Valet av referensjord behöver dock övervägas innan mikrobiologiska test införs för screening av slam.

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Abbreviations

AOA	Ammonia oxidizing archaea
AOB	Ammonia oxidizing bacteria
NMDS	Non-metric multidimensional scaling
OTU	Operational taxonomic unit
PAH	Polyaromatic hydrocarbons
PAO	Potential ammonia oxidation
PDA	Potential denitrification
PCB	Polychlorinated biphenyls
WWTP	Wastewater treatment plant

1. Introduction

The loss of soil organic matter and fertility in arable soil has become an increasing problem worldwide, posing a threat to soil fertility and the growing global food demand [1, 2]. Treated and digested sewage sludge is a resource that can be used to bring back organic matter as well as plant nutrients to agricultural soil [3]. While the production of digested sewage sludge from municipal wastewater treatment plants (WWTPs) is expected to increase, the amount of sludge used in agriculture varies from <5 % to >50 % of the produced sludge among European countries [4]. In Sweden, about 39 % of the produced sludge is spread to agricultural fields. The application of treated sludge as soil amendment could account for most of the nitrogen and phosphorus need for crop production. However, there are risks involved due to the content of heavy metals, organic contaminants and pathogens.

In Sweden, there is a voluntary certifying system for municipal sewage sludge quality called Revaq. It was an initiative by the association Swedish Water (Svenskt Vatten) together with the Federation of Swedish Farmers (LRF) the Swedish Food Federation, with the support by the Swedish EPA and launched in 2008. At present, Swedish Water has 290 municipalities as its members and there are 43 Revaq certified WWTPs producing > 40% of the total sludge in Sweden [5]. The Revaq system aims to ensure that sewage sludge used as soil amendment is safe for the environment and has acceptance by the agricultural sector. To be certified, the WWTPs must show they are actively and systematically working on decreasing contaminants entering the WWTP, and openly report what the sludge contains and how it has been produced. Further, each sludge batch must be traceable. It also involves demands for a number of time-consuming measures, such as sanitation and analysis of *Salmonella* prior to spreading as well as annual analyses of 60 trace elements to ascertain a doubling time not exceeding 0.20 % per year and that levels of trace elements regulated by law (Pb, Cu, Cr, Hg, Ni, Zn) are not exceeded. New Cd from the use of sludge should not be more than from the average mineral fertiliser on the Swedish market.

This project is commissioned by Svenskt Vatten and aims to investigate, whether it is possible to complement the current Revaq analyses for assessing sludge quality

by also including laboratory measurements of soil microorganisms in sludge amended soil. The hypothesis is that the soil microbial community, or subsets thereof, integrates the potential influence of environmentally hazardous compounds. As targets, we used the overall microbial community composition and the abundance of several functional groups of microorganisms involved in transformation of inorganic nitrogen (N) compounds and the activity of these transformations. We assessed both short- and long-term effects of sludge amendment to evaluate if one or several of targets can be used in routine monitoring tests when certifying sludge:

- For the short-term effects, a laboratory assay was developed and used to assess effects of sludge additions on the soil microorganisms and their functions. These results were compared with those obtained from soils with addition of cow and pig manure as well as untreated soil.
- The long-term impact of two Revaq-certified sludges was determined by using two long-term field experiments established in 1981, Petersborg, close to Malmö and Igelösa, close to Lund.

2. Background

2.1. Sewage sludge and sludge spreading in Sweden

Sewage sludge is a complex and semisolid residual material from WWTPs. After a stabilization process of the sewage sludge, such as anaerobic digestion or composting, the sludge can be used as a plant nutrient (containing 4.6 % nitrogen and 2.7 % phosphorus) and organic soil amendment [6]. In addition to being a source of plant nutrients, sludge amendment improves soil physical structure and water holding capacity. In general, soil microbial functions increase by the increased soil organic matter content [7, 8]. However, there is a risk of spreading hazardous compounds such as toxic metals, organic contaminants, drug residues and antibiotics, human pathogens and antibiotic resistant microorganisms into the environment when spreading sludge on arable land [9]. The content of unwanted compounds in the sludge is determined by the quality of influent wastewater to the WWTP. This suggests that proper control over the quality of the wastewater as well as sludge being used as a soil amendment is required.

Treated sludge is widely used as a fertilizer in agriculture as alternative to mineral fertilizers. In Sweden, production of the sewage sludge increased by 3.6 % during years 2016 and 2018, and reached 211 604 tons of dry weight. About 39 % of the produced sludge (82 300 tons of dry weight) is used in agriculture as a soil amendment [6].

2.2. Microbial processes and communities in soil as indicators for soil quality

Apart from screening for *Salmonella*, only chemical analyses for 60 elements are currently performed in the Revaq system to assess sludge quality prior to using it in agriculture. Biological tests could potentially integrate the toxic pressure from multiple compounds. Ecotoxicological tests based on pure cultures of microorganisms, protozoa, vertebrates and invertebrates exist [10-13]. However, their relevance for understanding effects on soil ecosystems has been questioned [14, 15]. Utilizing the organisms present in soil could be a better option, either as

indicator organisms, functions, model groups or the overall microbial community. When different biological tests for soil quality have been compared, tests with soil microorganisms and especially molecular methods for diversity and different functional groups come to the fore [16, 17]. Similarly, the response of soil microbial communities and their activities to sludge amendment could be relevant indicators of sludge quality. Microorganisms play essential roles in various biogeochemical processes and ecosystem services, such as decomposition of organic matter and pollutants as well as cycling of soil plant nutrients. Tests based on different processes in biogeochemical cycles can be interpreted in relation to different soil functions (e.g. [15]). For microorganisms, molecular tools would be ideal for sludge monitoring since molecular quantification and profiling of microorganisms and functional groups can be done in a high-throughput manner.

2.2.1. Nitrogen cycling

Nitrogen (N) cycling processes are important in soil since N availability regulates the rates of key ecosystem processes, such as primary production and decomposition. There are various microbial N transformation processes in soil that determine whether N is available for plants and microorganisms, or capable of leaving the ecosystem by leaching or as gaseous emissions. These losses generate further environmental problems. Excess N in soil that has been leached cause eutrophication in lakes, rivers and coastal waters. Gaseous losses in the form of the very strong greenhouse gas nitrous oxide (N_2O), which also contributes to depletion of the ozone layer, affects the climate.

Results from several studies have suggested that microbial communities involved in N cycling are good indicators of soil quality [18, 19]. Ritz et al [16] compared 183 targets and genetic profiling of bacterial N cycling communities was followed by analysis of the total microbial community and nematodes. The ability of soil to metabolize various N compounds has often been used to test the effects on the microbial community because they have a higher sensitivity than investigating more general functions, e.g. respiration [20, 21]. In addition, nitrification and denitrification activity tests have shown the effect of additives in the soil (pesticides and metals in this case) on both processes [21, 22]. Therefore, this report is mainly focusing on the two N cycling processes - nitrification and denitrification.

Nitrification is either a two-step or a one-step aerobic process. In the two-step process, ammonia is first oxidized to nitrite (NO_2^-) and then to nitrate (NO_3^-) by two very distinct groups of microorganisms. The first step is carried out by ammonia oxidizing bacteria (AOB) and ammonia oxidizing archaea (AOA) (Figure 1). Ammonia oxidation is initially catalyzed by an enzyme ammonia

monooxygenase (Amo) encoded by the *amoA* gene. Ammonia oxidation to nitrite as well as abundance of the *amoA* gene is widely used as enzymatic and genetic markers for studying nitrification [19, 23, 24]. Previous studies have shown that ammonia oxidation archaea are typically more abundant in soil than ammonia oxidation bacteria [25-27].

Denitrification is an anaerobic process where nitrate (NO_3^-) is reduced to either N_2O or dinitrogen (N_2) (Figure 1). The denitrification pathway consists of several steps and is performed by many different microorganisms, with some possessing genes for the complete pathway while others having only part of the pathway [28, 29]. Thus, incomplete denitrifiers can lead to production of the gaseous NO and N_2O as end products. While ammonia and nitrate are readily available for plants and microorganisms, the gaseous compounds NO, N_2O and N_2 are emitted from the ecosystem. Commonly used genetic markers for studying denitrifiers are *nirS* and *nirK* genes (encoding cytochrome-cd_1 and copper-based dissimilatory nitrite reductases (Nir), respectively that catalyze nitrite reduction), and *nosZ*I and *nosZ*II genes (phylogenetically distinct clades encoding nitrous oxide reductase NosZ that catalyze N_2O reduction). Recent studies have shown that microorganisms possessing the *nosZ*I gene are more likely to perform complete denitrification since they also contain *nirS* or *nirK* gene [28]. The *nosZ*II gene is found to be more abundant than *nosZ*I in several environments, including arable land [29, 30].

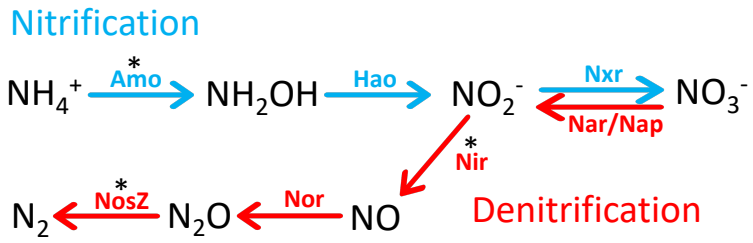

Figure 1. Schematic representation of nitrification (blue) and denitrification (red) pathways. Genes coding for enzymes catalyzing particular chemical reactions in the pathways are presented next to the arrows. The following marker genes encoding for the enzymes denoted with * were used in this study: *amoA* in bacteria and archaea (AOB and AOA, respectively) as proxies for nitrifying microorganisms; *nirS* and *nirK* and *nosZI* and *nosZII* as proxies for denitrifying microorganisms.

2.2.2. Methods used in this study

Three different laboratory approaches for evaluation of effects on the soil microbial community were used in the present study (1) potential activity assays, (2) quantification of specific genetic markers, and (3) investigation of the overall microbial community composition.

In the first approach, assays of microbial activities were carried out to determine potential activities. This approach involves incubations of soil supplemented with a sludge sample. During the specific time set for the assay, the accumulation of a product from the reaction of interest can be coupled to the reaction rate. This approach was used to assess rates of potential denitrification (PDA), potential N_2O production and potential ammonia oxidation (PAO). The results represent "potential activities", i.e. the amount of specific enzymes present, rather than actual activities in the field since the conditions in the assays, such as temperature, carbon source and pH were optimized to not limit the activity. This means that the rate limitation is the amount of active organisms (enzymes) in the soil.

The second approach involves quantification of the specific marker genes known to encode enzymes in the pathways of interest. This can be done by quantitively amplifying the DNA fragments of the specific gene. The results characterize the genetic potential of the microbial communities to perform a particular reaction. This method is very sensitive. Many studies have proven the effect of various factors (type and amount of fertilizer, heavy metals, clay content, pH etc) on the abundance of nitrification and denitrification marker genes in soil [31-33]. Studies showing relationship between nitrification or denitrification marker gene abundance and its corresponding potential activity are more scarce and show contrasting trends [20, 34-37].

The third approach was the investigation of the microbial communities by high-throughput sequencing of the 16S ribosomal RNA gene (16S rRNA). By targeting conserved regions in this gene, overall microbial community composition and diversity can be determined. Increased microbial diversity may indicate a higher number of functions the soil potentially could provide [38, 39]. In addition, increased abundance of specific microbial groups in the community can be related to a particular environmental factor or metabolic function [28, 29, 40-42].

3. Material and methods

3.1. Short-term laboratory incubation experiment

3.1.1. Sampling of soils, and sludges and manures

Two contrasting arable soils collected near SLU Ultuna campus in Uppsala were used in the short-term laboratory experiment – soil A (59° 49.493′, 17° 38.896′), and soil B (59° 49.412′, 17° 38.868′). The upper 20 cm of the soils were sampled by shovel in November, 2019. Soils were homogenized by manual mixing, sieved (4 mm) and stored at -20 °C.

Sludges from four Revaq certified WWTPs were used in the study: Kungsängsverket, Uppsala; Fors, Västerhaninge; Sjölunda, Malmö; and Källby, Lund. Sludge sampling was performed in May and June, 2020, from the sludge stock piles (stored about one year). Prior to sampling, the upper 30 cm sludge crust was removed. In addition to the sludges, stored cow manure mixed with straw and pig slurry mixed with sawdust were sampled from Lövsta farm (Swedish Livestock Research Centre, SLU), Uppsala, in June 2020. Samples were kept cool during transportation from the sampling locations to the laboratory. Samples were homogenized by manual mixing and then stored at -20 °C.

3.1.2. Soil, sludge and manure characteristics

Soil texture and chemical analyses were performed by the Department of Soil and Environment, SLU (Appendix A, Table A1). Both soils, A and B had at sampling similar dry matter content, content of total N, soil organic matter, and soil organic carbon. However, soil A contained higher amounts of fine particles compared to soil B, and had higher pH. Soil water holding capacity also differed, with higher capacity in soil B.

For the sludges, the content of polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), metals (Pb, Cr, Hg, Ni, Cu, Zn, Ag and Sn), plant nutrients (N, P and K), organic matter and pH were analyzed by Eurofins (Appendix A, Table A2). The sludges were characterized by low levels of PCBs, PAH and metals (Pb,

Cr, Hg, Ni, Ag and Sn). All these substances and elements were below detection in cow manure and pig slurry. In addition, the sludges contained higher amounts of P, N, Cu, Zn compared to cow manure and pig slurry, while the two latter contained high amounts of K. Among the sludges, Sjölunda and Källby contained higher amounts of PAHs and Pb compared with the other two.

3.1.3. Experimental setup

Soil (200 g dry weight) and sludge/manure (2.6 - 4.7 g of amendments, corresponding to 12 t of dry weight per hectare) were mixed thoroughly and poured into 500 ml glass bottles. As a control, unamended soil was used. The water content was adjusted to 65 % of its holding capacity with distilled water. Bottles were prepared in triplicate, resulting in 42 bottles in total (2 soils x 7 treatments x 3 replications). The bottles were incubated at 20 °C for 8 weeks. Weight of the mixtures (soil and sludge/manure) were checked several times a week and sterile water was added when needed to maintain the initial water content. After the incubation, the mixtures were stored at 4° C in plastic bags until potential activity analyses (approach 1) were measured. A small portion of each of the soil/mixtures was stored at - 20 °C for DNA extraction (approach 2 and 3).

3.2. Long-term field experiment

To investigate the effects of long-term sewage sludge amendment on microbial potential N-cycling activities, abundance of functional genes, and overall community composition and diversity, samples from the long-term field experiments at Petersborg, close to Malmö, and Igelösa, close to Lund, were collected and studied. Sludge from Sjölunda WWTP and Källby WWTP have been spread at Petersborg and Igelösa, respectively, every four years since 1981 [43].

The experiment has a block design with four blocks. For this study, samples from three different levels of sludge amendment were used: no sludge, half sludge (4 tons dry weight per hectare) and full sludge (12 tons dry weight per hectare). In addition to sludge, mineral fertilizers are used in the fields according to recommendations for the amounts of N, P and K in agricultural practice in Sweden. Samples with two levels of mineral fertilizers were used in this study – no fertilizer, and full fertilizer (NPK 400 kg/ha). Soil samples from the four experimental blocks at Petersborg and Igelösa, respectively, were collected by the field staff at the Rural Economy and Agricultural Society (Hushållningssällskapet), Skåne, in November 2019. The samples collected represented treatments A3 (no sludge, full NPK), B3 (half sludge, full NPK) and C3 (full sludge, full NPK), and in addition C1 (full sludge, no NPK), in the field plan of the experiments [43]. This resulted in 32

samples in total (4 treatments x 4 blocks x 2 locations). According to Andersson (2015), soil organic carbon content is 3-6 % in the Igelösa and 2-3 % in the Petersborg fields, respectively. Samples were stored at 4 °C until analyses of potential activities and in -20 °C for DNA extractions.

3.3. Microbial activity and genetic potential

3.3.1. Potential denitrification and N_2O production

Potential denitrification activity (PDA) and N_2O production were measured by setting up parallel soil incubations with and without the addition of acetylene, respectively. During incubation without acetylene, denitrification occurs and nitrate (NO_3^-) is reduced to both N_2O and N_2. During incubation with acetylene denitrification also occurs, but nitrate is only reduced to N_2O since acetylene inhibits the subsequent reduction of N_2O to N_2.

For both measurements, 20 ml of water was added to 10 g (wet weight) of sample in 100 ml gas-tight bottle. The bottles were sealed and air in the bottles was replaced with N_2 to obtain an oxygen-free environment. 1 ml of a mixture of carbon compounds (1 mM glucose, 3 mM sodium acetate and 1.5 mM sodium succinate, final concentrations) and KNO_3 (3 mM final concentration) was added into the bottles. 10 ml of acetylene was added to the bottles that were subjected to PDA measurement [44], while no acetylene was added to the bottles for measurement of potential N_2O production. The bottles were incubated on a shaking table at 170 rpm at 25 °C for 180 minutes, and 0.5 ml was withdrawn from the headspace every 30 min for analyses of N_2O.The N_2O concentration in the gas samples was determined with a Perkin Elmer Clarus 500 gas chromatograph with and electron capture detector.

The rate of N_2O formation increased with time and the data were fitted to a product formation equation that takes exponential growth into consideration, and, hence, the rate of N_2O accumulation in each bottle was calculated by non-linear regression, representing PDA or potential N_2O production. Denitrification end-product ratio was calculated as the ratio of potential N_2O production rate to PDA.

3.3.2. Potential ammonia oxidation

Potential ammonia oxidation activities (PAO) were measured according to the protocol by Torstensson, 1993 [45]. Briefly, 100 ml of the media (1.5 mM $(NH_4)_2SO_4$, 7.5 mM $NaClO_3$, 0.28 mM KH_2PO_4, 0.72 mM K_2HPO_4, pH 7.2) was added to 25 g (wet weight) of soil sample in 250 ml glass bottle. Chlorate ($NaClO_3$)

is added to stop the oxidation of nitrite to nitrate allowing to specifically determine only the first step in the nitrification process. Bottles were incubated on a shaking table at 170 rpm at 25 °C for 6 h. At 2 h and then every hour, 2 ml soil slurry samples were withdrawn and mixed into 2 ml of 4 mM KCl. The mixture was centrifuged (3 min at 3000 rpm) and filtered through 0.8 μm filter paper. The accumulated nitrite (NO_2^-) in the samples over time were analyzed with AutoAnalyzer 500 (Seal Analytical). The rate of NO_2^- formation, i.e. PAO, was determined by linear regression.

3.3.3. Quantification of 16S rRNA and N cycling marker genes

The 16S rRNA and marker genes for N-cycling bacteria and archaea were quantified. The DNA extraction was done using 0.5 – 1 g (wet weight) soil using a commercial DNA extraction kit (NucleoSpin Soil kit from Macherey-Nagel) according to the instructions provided by manufacturer. The concentration of the extracted DNA was measured with a Qubit fluorometer. Previously published primers and protocols were used for quantitative PCR of 16S rRNA genes as well as different genetic marker genes (*amoA* in AOA *and amoA* in AOB, *nirS*, *nirK*, *nosZI* and *nosZII*) involved in nitrification and denitrification pathways (Appendix B, Table B1).

3.3.4. Microbial community composition analyses

Prokaryotic 16S rRNA genes were subjected to high-throughput sequencing to study the overall microbial community composition in the samples. Samples were prepared to sequencing according to the protocol in the Department of Forest Mycology and Plant Pathology, SLU [46]. MiSeq Illumina with the 2 x 250 bp paired end chemistry was performed by SciLifeLab. Publicly available programs (FASTX-Toolkit, PEAR, USEARCH, VSEARCH, SINA, ARB, FastTree) and databases (SILVA) were used to preprocess and analyze the sequences according to our inhouse pipeline and workflow (e.g. [46]) resulting in OTU tables which were cleaned from the 16S rRNA reads assigned to mitochondria and chloroplasts prior to statistical analyses of the microbial communities.

3.4. Statistical analyses

Statistical analyses and plotting were done in the R environment (R core team, 2013) using packages Vegan [47] and Phyloseq [48]. Two-way ANOVA followed by the Tukey's HSD test were used to analyze the effect of soil type, soil amendments and their interaction on potential microbial activities, gene abundances, and Faith's phylogenetic diversity. The two factors (soil and amendments) were included in the models as main effects. In the laboratory

experiment, soil amendments are different sludges and manures, while in the field experiment soil amendment are different levels of sludges and fertilizer combined. Linear regression with the 95 % confidence values, R^2 and p values were used to find relationships between Faith's phylogenetic diversity and potential activities.

To determine microbial community structures in the soil samples, OTU tables were first rarefied to an equal number of sequences per sample within each of the experiments (32 565 seq/sample in the laboratory experiment, and 56 609 seq/sample in the field experiment) followed by a calculation of dissimilarities (e.g. distances) between the sample pairs. The weighted Unifrac distances were calculated for the samples from laboratory experiment, while the Bray-Curtis distances were calculated for the samples from field experiment. To visualize the distances between samples, non-metric multidimensional scaling (NMDS) ordination analyses were performed together with vector fitting to study the microbial community composition and measured microbiological parameters that are significantly correlating with the separation of the samples. Significant effects of soil type and amendments on microbial community composition were tested using permutational multivariate ANOVA (PERMANOVA) using 999 permutations and the 'strata' option to constrain permutations within the soil type (e.g. for controlling the effect of soil type).

4. Results

4.1. N cycling gene abundances and potential activities

4.1.1. Short-term responses in laboratory experiment

Results from the laboratory experiment showed that after eight weeks of incubation, abundances of most N cycling genes and all potential activities were significantly affected by soil type (Table 1). However, the amendments with sludge/manure and the interactions showed clear effects on several of the microbial parameters studied. The *nosZ*II, AOA, denitrification, N_2O production and the ratio between PDA and potential N_2O production rate, (i.e. end-product ratio), all demonstrated no or only weak effects of amendments.

Table 1. Effects of soil type and amendment on microbial targets in the short-term experiment

Community variable		Soil	Amendment	Soil x Amendment
Gene	*nirS*	***	***	
	nirK	***	***	*
	*nosZ*I	***	***	***
	*nosZ*II		*	
	amoA AOB	**	**	
	amoA AOA	***		*
Activity	Denitrification	***	*	*
	N_2O production	***		
	Denitrification end-product ratio	***		*
	Ammonia oxidation	***	***	***
Diversity	Faith's PD	***	**	*

Main effects and interaction based on two-way ANOVA: $p < 0.001$ ***, $0.001 < p < 0.01$ **, $0.01 < p < 0.05$*

Soil B had 1-2 orders of magnitude higher abundances of 16S rRNA, and genes involved in nitrification and denitrification compared to soil A (Appendix A, Table A3). However, the relative abundances of the genes involved in denitrification (*nirS*, *nirK*, *nosZ*I, *nosZ*II) were in general higher in soil A (Figures 2 A-D). As a general pattern in both soils, *nirK* had higher relative abundance than *nirS*, and

*nosZ*II had higher relative abundance than *nosZ*I, while the AOA and AOB had similar proportions.

Sludge and manure amendment had different effects on gene abundances, and these effects were also dependent on soil type. The relative abundances of denitrification genes tended to increase in most of the sludge amendments compared to the control in soil A, whereas such effect was not detectable in soil B (Figures 2A - D). Sludge amendments increased the abundance of bacterial ammonia oxidizers (AOB) in both soils, whereas the archaeal counterpart (AOA) were unaffected (Figures 2E and F). The abundances of denitrification and nitrification genes in the samples amended with cow manure or pig slurry were very similar to the controls, although some negative effects were observed (Figures 2B, C and F).

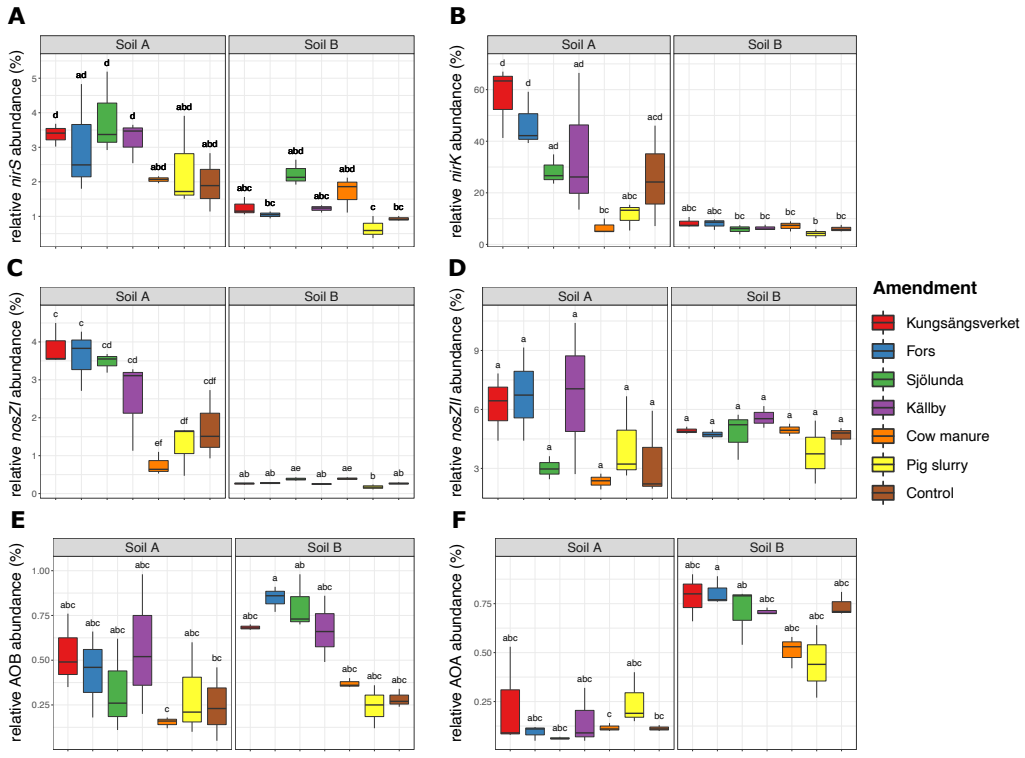

Figure 2. Effects of sludge and manure amendments on nitrogen cycling gene abundances in the laboratory experiment. A – D, abundances of genes from denitrification pathway; **E – F**, abundances of genes from nitrification pathway. Boxplots represent median, 75 % and 25 %, and whiskers 1.5 times of both percentiles of three biological replicates. Letters represent significant differences ($p<0.05$) between groups of samples according to the Tukey HSD test in two-way ANOVA.

Potential denitrification and potential ammonia oxidation rates were in general higher in soil A, while the potential N_2O production rates, and consequently also the calculated denitrification end-product ratio, were higher in soil B (Figure 3).

Effects of the amendments on potential activities were mixed and soil type dependent. The sludges had strong positive effects on ammonia oxidation in soil A (Figure 3D). Sludge from Kungsängsverket had the strongest positive effect on denitrification in soil A (Figure 2A), potential N_2O production in soil B (Figure 2B), and ammonia oxidation in soil A (Figure 2D). Sludge from Fors had positive effect on ammonia oxidation in both soils (Figure 2D), and a slight negative effect on denitrification and potential N_2O production in soil A (Figure 2A and B). Cow manure and pig slurry increased both ammonia oxidation and denitrification in soil B, and decreased the calculated denitrification end-product ratio in soil B.

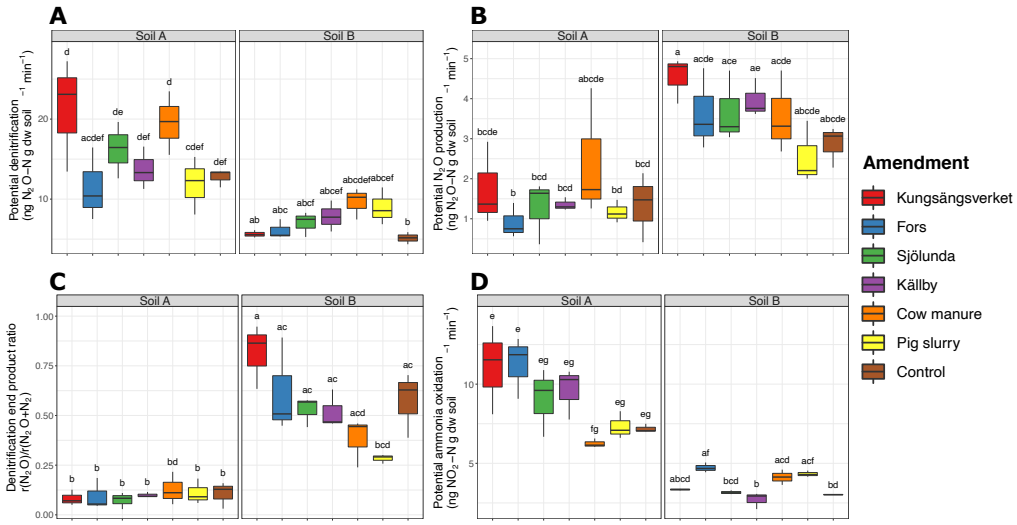

Figure 3. Effects of sludge and manure amendments on nitrogen cycling activities in the laboratory experiment. A, potential denitrification; **B**, potential N_2O production; **C**, denitrification end product ratio; **D**, potential ammonia oxidation**.** Boxplots represent median, 75 % and 25 %, and whiskers 1.5 times of both percentiles of three biological replicates. Letters represent significant differences ($p<0.05$) between groups of samples according to the Tukey HSD test in two-way ANOVA.

4.1.2. Long-term responses in field experiment

The abundances of N cycling genes were strongly affected by soil type in the long-term field experiments at Igelösa and Petersborg (Table 2 and Figure 4), similarly to what was observed in the laboratory experiment. Gene abundances were in general higher in samples from Igelösa compared to those from Petersborg. In addition, *nirK* and *nosZ*II gene abundances were slightly higher than their corresponding counterparts *nirS* and *nosZ*I in both soils (Figure 4A-D). For the ammonia oxidizers, AOB and AOA abundances were similar (Figure 4E and F). The rate of sludge application had no effect on the gene abundances (Table 2 and Figure 4). However, mineral fertilizer addition tended to decrease the AOB, and increase AOA abundances in both soils.

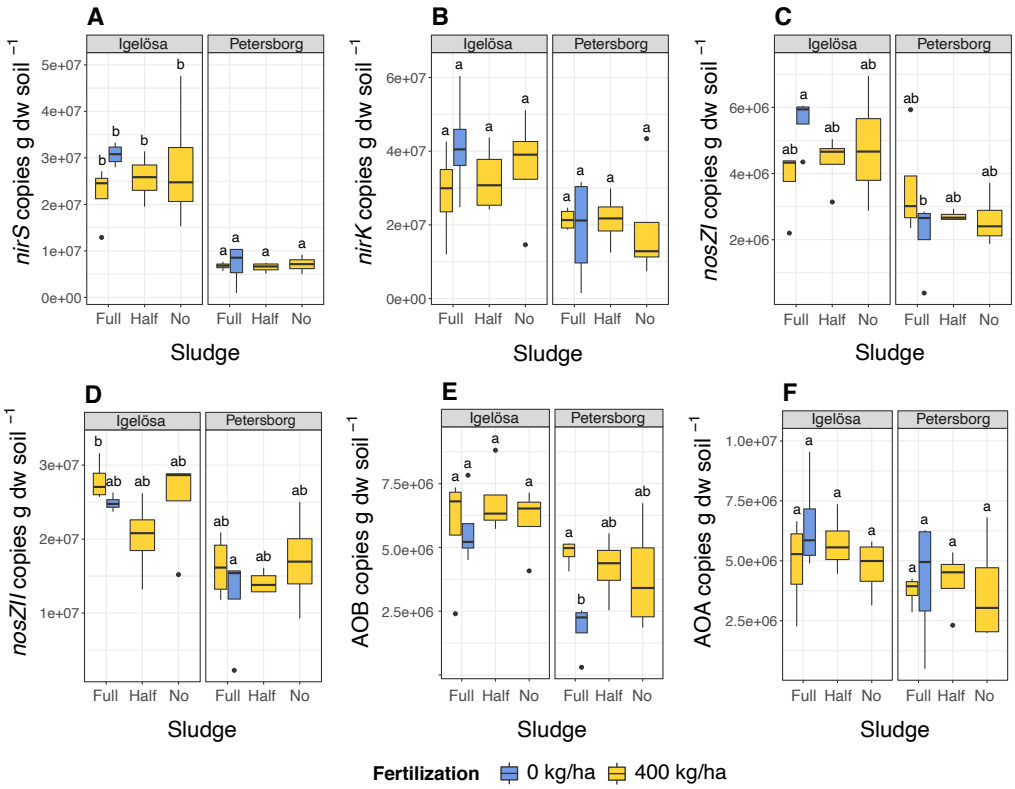

Figure 4. Effects of sludge amendments and mineral fertilizer on nitrogen cycling gene abundances in the field experiment. A - D, abundances of genes from denitrification pathway; **E - F**, abundances of genes from nitrification pathway in soils from Igelösa and Petersborg with different levels of sludge amendments (Full, Half, No) supplemented with NPK fertilizer, and full sludge without fertilizer. Boxplots represent median, 75 % and 25 %, and whiskers 1.5 times of both percentiles of four biological replicates. Letters represent significant differences ($p<0.05$) between groups of samples according to the Tukey HSD test in two-way ANOVA.

Potential activities were only affected by soil type (Table 2 and Figure 5) with PDA, potential N_2O production and PAO rates being higher in samples from Igelösa (Figure 5A, B and D). However, denitrification end-product ratio was higher in samples from Petersborg. Neither the sludge amendment nor mineral fertilizer had a significant effect on the potential activities, although there was an interaction effect of soil and amendment on the potential N_2O production.

Table 2. Effects of soil type and amendment on microbial targets the field experiment

Community variable		Soil	Amendment	Soil x Amendment
Gene	*nirS*	***		
	nirK	**		
	nosZI	***		
	nosZII	***		
	amoA AOB	***		
	amoA AOA	*		
Activity	Denitrification	***		
	N_2O production	***		*
	Denitrification end-product ratio	**		
	Ammonia oxidation	***		
Diversity	Faith's PD	***		

Main effects and interaction based on two-way ANOVA: $p < 0.001$ ***, $0.001 < p < 0.01$ **, $0.01 < p < 0.05$*

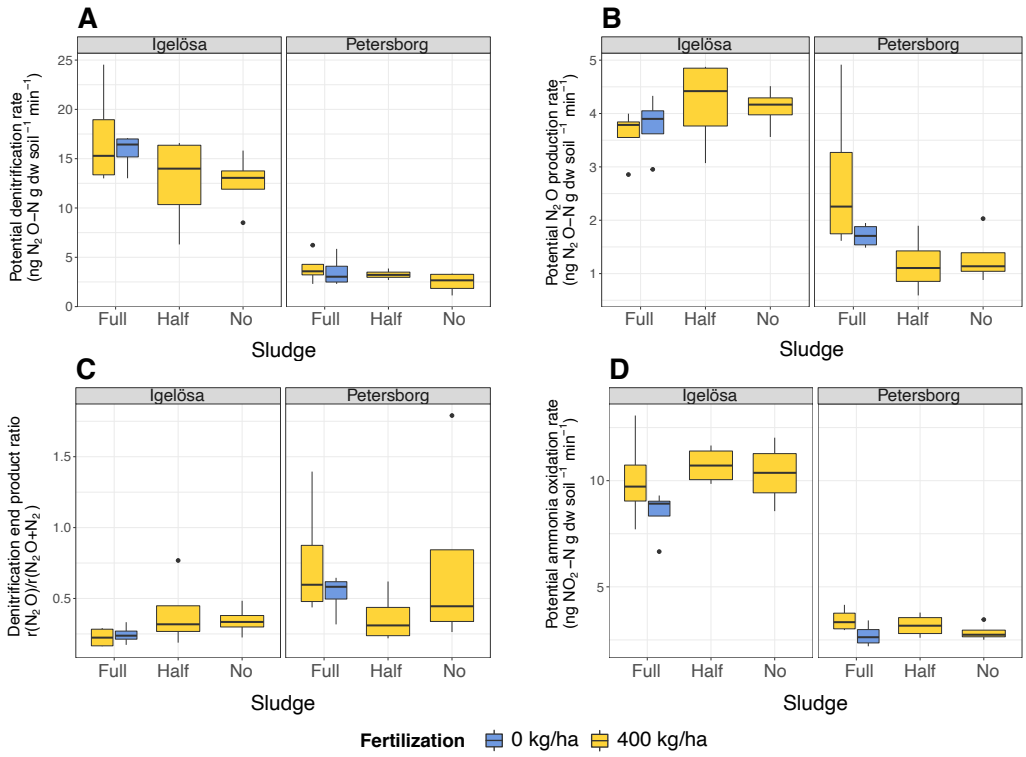

Figure 5. Effects of sludge amendments and mineral fertilizer on potential nitrogen cycling activities in the field experiment. A, potential denitrification; **B**, potential N_2O production; **C**, denitrification end product ratio; **D**, potential ammonia oxidation in soils from Igelösa and Petersborg with different levels of sludge amendments (Full, Half, No) supplemented with NPK fertilizer, and full sludge without fertilizer. Boxplots represent median, 75 % and 25 %, and whiskers 1.5 times of both percentiles of four biological replicates. No significant differences ($p<0.05$) detected between groups of samples according to the Tukey HSD test in two-way ANOVA.

4.2. Microbial community composition and diversity

4.2.1. Short-term responses in laboratory experiment

Microbial community composition in the samples was affected by the soil type (PERMANOVA, $p<0.001$) as well as by the amendment type (PERMANOVA, $p<0.001$). The NMDS analysis revealed two groups of soils clustering according to soil type, with a relatively tight clustering of soil B and a more dispersed cluster of soil A (Figure 6A). Separation of the two soils were driven by higher potential N_2O production, AOA abundance and Faith's phylogenetic diversity in soil B, and higher PAO and PDA with higher proportions of *nirS*, *nirK*, and *nosZ*I genes in soil A. No clear trends induced by the various treatments can be discerned, however, there was a tendency that the control without amendment and the pig slurry and cow manure treated soils formed a group (located on the upper part of the graph). The three treatments seemed to be characterized by a higher phylogenetic diversity (Faiths's PD) as well as higher PDA, and lower AOB and *nosZ*II gene abundances compared to the sludge treated soils (Figure 6A).

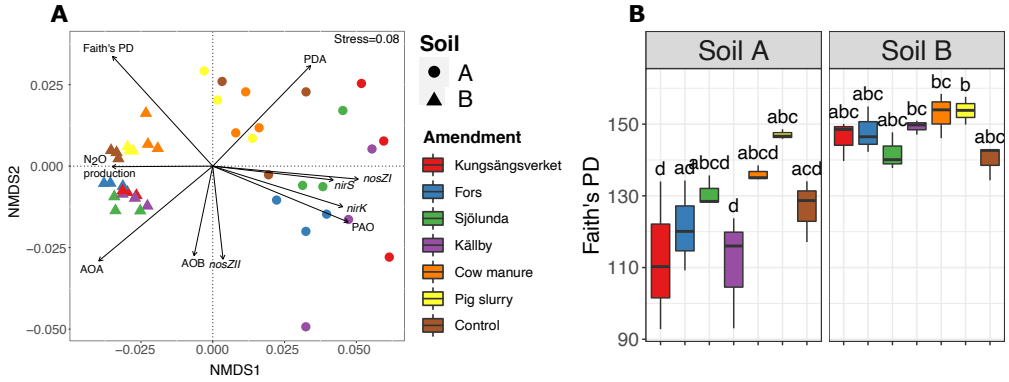

Figure 6. Effects of soil type, and sludge and manure amendments on soil microbial community composition and diversity in the laboratory experiment. A, Microbial community composition in soils A and B (determined by the shape of the symbol) with different amendments (determined by the color of the symbol). Each symbol represents an individual sample, and the dissimilarities between samples are represented by the distances between the position of the samples in the ordination plot. The ordinations are based on NMDS of weighted Unifrac distances of the rarefied OTU abundances from 16S rRNA gene sequence analyses. Arrows indicate different community variables that are significantly correlated with the community structure, and the length of the arrow is proportional to the goodness of the fit (r) of the parameter. **B**, Faith's diversity indices of microbial soil communities with sludge and manure amendments. Boxplots represent median, 75 % and 25 %, and whiskers 1.5 times of both percentiles of three biological replicates. Letters represent significant differences between groups of samples according to the Tukey HSD test in two-way ANOVA.

Phylogenetic diversity (Faith's PD) of the microbial communities was in general higher in samples from soil B compared to those from soil A, and was also affected by the amendment type (Table 1 and Figure 6B). While cow manure and pig slurry amendments tended to increase the phylogenetic diversity in both soils, the different

sludge amendments decreased the phylogenetic diversity in soil A, but a slight increase was observed in soil B.

We also investigated the relationships between the potential activities and phylogenetic diversity of the microbial communities. There were positive relationships between potential denitrification rates and phylogenetic diversity in soil B (Figure 7A), and negative relationships between denitrification end product and phylogenetic diversity in soil B (Figure 7C). It should be noted, however, that the respective relationships in soil A were not significant. In addition, results showed that samples with higher phylogenetic diversity in soil B also demonstrated higher potential ammonia oxidation rates, while negative relationships between potential ammonia oxidation and phylogenetic diversity was observed in soil A (Figure 7D).

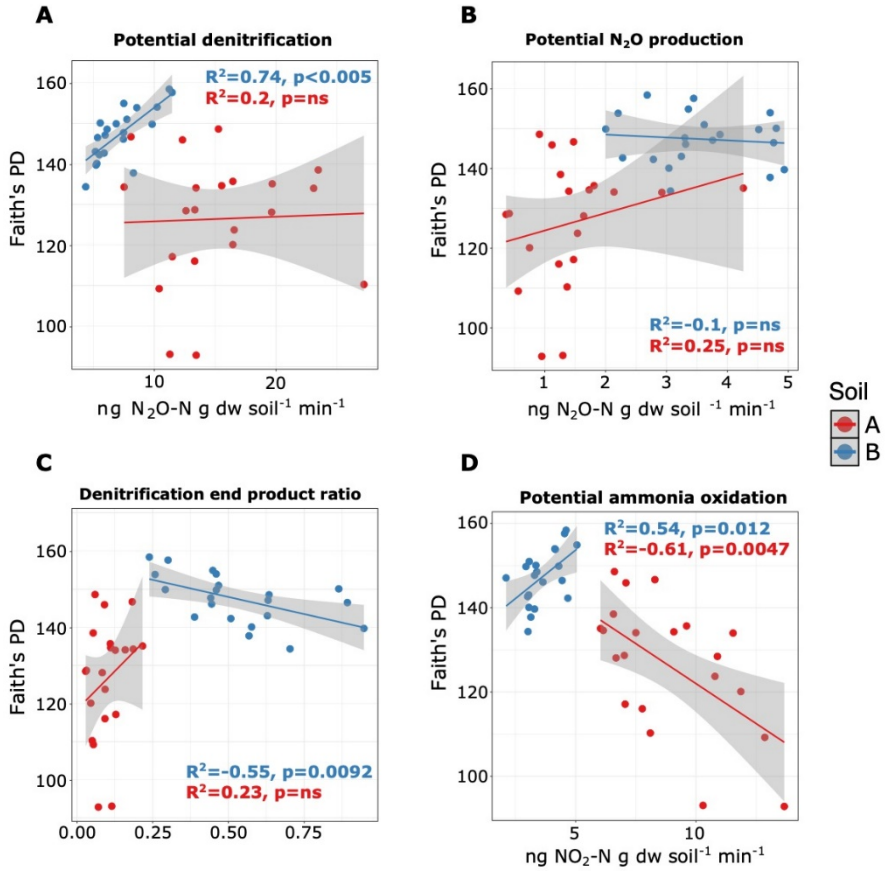

Figure 7. Associations between potential activities and microbial diversity across different amendments in two soils in the laboratory experiment. Linear regression lines and 95 % confidence intervals as shaded areas are presented together with regression lines, R^2 and p values.

All in all, we can summarize, that different soil amendments tended to increase the phylogenetic diversity in soil B, that coincided with higher potential

denitrification potential ammonia oxidation, and smaller proportion of N_2O emitted (denitrification end product ratio). In contrast, sludges decreased the phylogenetic diversity in soil A, but that also coincided with higher potential ammonia oxidation rates.

4.2.2. Long-term responses in field experiment

Microbial community structure in the field samples was affected by the soil type (PERMANOVA, $p<0.001$) and by the different levels of sludge and fertilizer (PERMANOVA, $p<0.01$). The NMDS ordination showed a clear separation of samples based on soil type (Figure 8A), that was associated with higher denitrification, potential N_2O production and ammonia oxidation rates in samples from Igelösa compared to Petersborg. In addition, microbial community composition in samples without NPK fertilizer addition were clearly different from the samples with fertilizer amendment in Petersborg, whereas the effect of the fertilizer was not as obvious in Igelösa (Figure 8A). The NMDS ordination showed some separation of the samples based on different sludge levels in both soils with tendencies of no sludge being located on the lower part of the panel, but it was not significant. The phylogenetic diversity was higher in Petersborg than Igelösa, but there were no effects of sludge or fertilizer addition on the diversity (Table 2, Figure 8B).

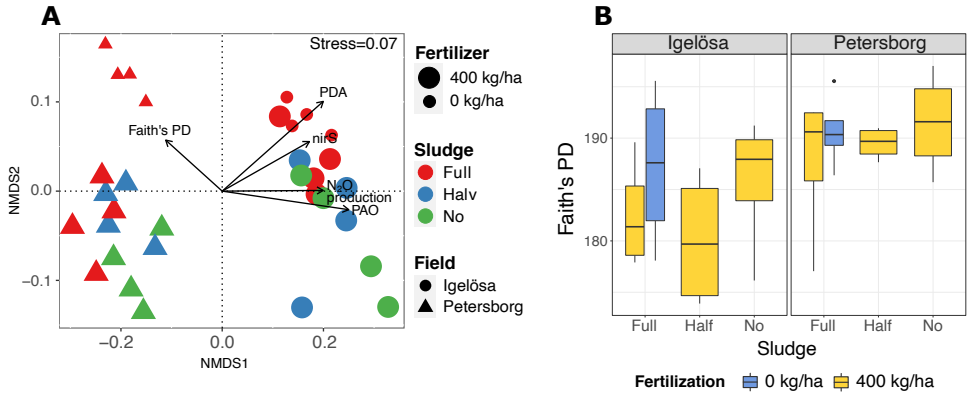

Figure 8. Effects of sludge amendments and mineral fertilizer on soil microbial community composition and diversity in the field experiment. A, Microbial community composition in soils from Igelösa and Petersborg (determined by the shape of the symbol) with different sludge levels (determined by the color of the symbol) supplemented with or without NPK fertilizer (determined by the size of the symbol). Each symbol represents an individual sample, and the dissimilarities between samples are represented by the distances between the position of the samples in the ordination plot. The ordination is based on NMDS of Bray-Curtis distances of the rarefied OTU abundances from 16S rRNA gene sequence analyses. Arrows indicate different community variables that are significantly correlated with the community structure, and the length of the arrow is proportional to the goodness of the fit (r) of the parameter. **B**, Microbial diversities in soils with different levels of sludge and NPK fertilizer. Boxplots represent median, 75 % and 25 %, and whiskers 1.5 times of both percentiles of four biological replicates.

5. Discussion and conclusions

The effect of different soil amendments on the N cycling gene abundances, potential activities and on the overall microbial community composition and diversity was mixed and overruled by the strong effect of soil type. The most distinct effect of soil amendments was observed on the overall soil microbial community composition in both the short-term experiment and the long-term experiment samples.

5.1. Long-term effects

In the long-term field experiment, the main difference in community composition was between the sites rather than between treatments [43]. Nevertheless, the microbial community in the soils receiving a full dose of sludge without mineral fertilizers was the most different compared with the other treatments, including the one with mineral fertilizers without sludge. This suggests that fertilization affected the microbial community composition more than sludge addition. The long-term sludge amendments had no effect on the N cycling gene abundances and potential activities in the field experiments in relation to the fertilized soils. This largely agrees with another long-term field experiment in Sweden with regards to N cycle gene abundances [20], but not potential ammonia oxidation and potential denitrification that were negatively and positively, respectively, affected by sludge addition [49, 50]. In another Swedish field experiment, no effects on these activities could be detected eight years after sludge amendment [51].

Previous investigations of soils at Petersborg and Igelösa (sampled in 1990 and 1994) showed that Petersborg generally had a lower biological activity compared with Igelösa which was attributed to a lower content of organic matter at the latter site [52]. Potential ammonia oxidation rates decreased in Petersborg due to sludge addition while mineral fertilization tended to increase this activity. Generally, potential denitrification at both sites were not affected. These measurements were conducted 9 and 13 years after the experiments started and at that time, the sludges had different chemical properties compared with the currently produced sludges at Källby and Sjölunda. For example, the content of toxic trace metals has decreased

substantially [43]. Nevertheless, the concentration of copper, mercury, and zinc had increased in the soil due to accumulation, but sludge amendment has not increased the uptake of the metals by crops. Other positive aspects of sludge amendment at these sites is the increased crop yield, soil fertility and soil organic matter content [43]. Overall, the results suggest that there are no long-term negative effects of sludge amendments on microbial communities and functional groups involved in N cycling in these soils.

5.2. Short-term effects

In the short-term laboratory experiment, the sludges had stronger effects on the microbial community composition compared to cow manure and pig slurry treatments, which resulted in microbial community structures in sludge treated soils more similar to the control. Major differences between the manures and the sludges were the presence of PCBs, PAHs, metals, P and N in the former and the higher K in the latter. Although sludges from Källby and Sjölunda contained more PAHs and Pb compared to sludges from Fors and Kungsängsverket, the effect of Källby and Sjölunda sludges on microbial community composition was not distinguished from the effect of the other two sludges. Differences in microbial diversity among treatments were not significant from the control, but there was a trend of higher diversity in the manure treated soils compared with sludge addition in one of the soils. It is not possible to relate the microbial diversity to a particular function, but studies have shown higher number of biological functions in soil with increased microbial diversity [38, 39]. In the soil that was affected by sludge addition, a decreased diversity was unexpectedly associated with an increase in ammonia oxidation, whereas the soil with minimal impact of sludge addition in contrast showed increasing ammonia oxidation and denitrification activity with increasing diversity. This highlights that microbial responses to disturbances or other changes are strongly shaped by edaphic properties, including the inherent microbial communities [53].

Apart from changes in community composition, the short-term laboratory experiment could detect effects of sludge addition on gene abundances and potential activities. Sludge amendments were shown to increase the proportion of genes in the community related to the denitrification pathway, particularly in one of the soils. This suggests that the molecular targets and method may be sensitive enough to detect at least the immediate response in the abundances of certain microbial functional groups to sludge amendment. Nevertheless, gene abundances, and potential ammonia oxidation and denitrification rates increased or were similar after sludge addition when compared with those in the control or manure treatments. The contaminant levels in the sludges are low, which could explain that

no negative effects could be detected. Nevertheless, negative effects can also be masked by the positive effects by organic amendments [54]. The positive effects of sludge compared with manure are likely due to the higher contribution of both N and P from the sludge that stimulate microbial activity in the soil. The effect or lack of an effect of amendments on activities and gene abundances should be interpreted with caution, since soil properties may have much larger influence, at least in the long-term.

5.3. Evaluation of targets and screening methods

Among the targets evaluated for sludge screening, microbial community composition captured differences in the samples that were difficult to detect with single targets of specific activities or the abundances of microbial functional groups. Sequencing microbial communities is a high-throughput methodology that is widely used for characterizing microbial communities in complex ecosystems such as wastewater treatment systems [55] and arable soils [56, 57]. The drawback of this methodology is that it requires staff skilled in analyzing sequence data, although open-source bioinformatic platforms like MOTHUR [58] and QIIME [59] are available with tutorials. In addition, interpreting the changes in microbial community composition with possible linkage to microbial functions requires expertise in microbiology.

No differences were found in the sensitivities of the methodologies for measuring potential activities or gene abundances as a response to amendments. Regarding the activities, potential ammonia oxidation rates were more strongly affected by the soil amendments in the laboratory experiment, which indicates that it may also be suitable target for assessment of sludge quality. However, quantification of the gene abundances is more suitable for large-scale screening than potential activities since it requires less manual work per sample, although the operating cost per sample is higher. Further, since gene abundances are based on DNA, it is easily combined with analysis of the microbial community composition.

In the short-term experiment, we used a relevant sludge dose (full dose, 12 tons/ha corresponds to three times an ordinary Swedish sludge dose), which could explain the weak responses and the non-generalizable effects between the two soils. A dose-response approach could potentially be more robust for a screening program since positive or negative trends can be detected instead of only relying to end-point measurements related to a control. The down-side with this approach is that it is more time consuming as it requires more work.

In addition to the selection of appropriate targets and screening methods, the positive effects of sludge were more pronounced in one of the soils in the short-term laboratory experiment. This shows that the targets are sensitive enough to respond to sludge addition in the short-term, but that the response depends strongly on the soil used when screening sludges. Soil texture and organic matter content are important factors determining soil water retention and water holding capacity. These factors, as well as other factors (notably pH) affect microbial activity and growth, thereby influencing the measured targets. Soil type as a primary influence on various soil microbial variables have been demonstrated previously [31-33]. The overriding effect of soil type and differences in responses between soils makes it challenging to compare effects across soils and generalize effects of sludge amendment.

The strong soil effect and the results from the short-term laboratory test show that a reference soil to be used in a standardized screening program must be chosen with care. Further, the selection of controls is important for the interpretation of the results, and dose-response approach should be considered.

5.4. Conclusions

- No negative effects of sludge amendments, neither in the short-term screening assay nor in the long-term field experiment, could be detected for the tested sludges in the specific soils and sites.

- The effect of sludges on gene abundances and potential activities is strongly dependent on the soil factors, and probably also on the inherent microbial communities in soil.

- Positive effects of sludge amendments on gene abundances and potential activities were only observed in the short-term laboratory experiment. This suggests that theses assays and targets can respond to sludge amendment.

- Microbial community composition was the most sensitive of the tested targets. This target integrated effects of sludge amendment and could be useful for screening sludge quality.

- To develop a test system, the selection of a reference soil and controls will be crucial and a dose-response approach should be considered.

Acknowledgements

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6. Appendix A – additional data for the short-term laboratory experiment

Table A1. Soil properties

Table A2. Sludge and manure properties

Table A3. Gene abundances

Table A1. Soil properties. Soil texture and chemical properties of the two soils used in the short-term laboratory incubation experiment. d = particle size diameter in mm.

Soil parameter	**Soil A**	**Soil B**
Clay (d < 0.002; %)	45.5	25.5
Fine silt (0.002 < d < 0.006; %)	10	6.4
Medium silt (0.006 < d < 0.02; %)	8.5	5.8
Coarse silt (0.02 < d < 0.06; %)	11.6	7.2
Fine sand (0.06 < d < 0.2; %)	16.5	22.9
Medium sand (0.2 < d < 0.6; %)	6.6	31.2
Coarse sand (0.6 < d < 2; %)	1.4	1.1
Initial WHC (%)	33.6	43.5
Dry matter (%)	81	84
total N (%)	0.17	0.15
total C (%)	1.69	1.60
organic C (%)	1.67	1.59
SOM (%)	5.0	4.1
pH	8.11	6.53

Table A2. Sludge and manure properties. Chemical properties of sludges from different municipal WWTPs (Kungsängsverket, Sjölunda, Fors, Källby), cow manure with straw, and pig slurry with sawdust. Concentrations of PCBs, PAHs, and metals are given in mg/kg. ND – not detected.

Chemical component	Kungsängs-verket	Sjölunda	Fors	Källby	Cow manure	Pig slurry
PCB 28	0.0013	0.0014	0.0016	0.0014	ND	ND
PCB 52	0.0021	0.0032	0.0025	0.0028	ND	ND
PCB 101	0.0039	0.0039	0.0037	0.0027	ND	ND
PCB 118	0.0017	0.0017	0.0015	0.0013	ND	ND
PCB 153	0.0053	0.0049	0.0050	0.0034	ND	ND
PCB 138	0.0050	0.0048	0.0046	0.0033	ND	ND
PCB 180	0.0026	0.0028	0.0020	0.0017	ND	ND
Fluoranten	0.19	0.94	0.19	0.47	ND	ND
Benso(b)fluoranten	0.077	0.28	0.065	0.16	ND	ND
Benso(k)fluoranten	0.038	0.15	0.034	0.085	ND	ND
Benso(a)pyren	0.056	0.25	0.054	0.13	ND	ND
Benso(g,h,i)perylen	0.056	0.21	0.036	0.088	ND	ND
Indeno(1,2,3-cd)pyren	0.057	0.26	0.06	0.16	ND	ND
Pb	12	24	11	23	ND	3.7
Cd	0.60	0.53	0.80	0.56	0.31	0.47
Cr	21	20	17	20	6.1	11
Hg	0.32	0.39	0.2	0.52	ND	ND
Ni	19	17	21	16	ND	ND
Ag	1.2	1.2	1.2	1.9	ND	ND
Sn	13	11	6.6	15	ND	ND
P	35000	28000	37000	38000	6400	5100
K	1700	2100	2400	2100	44000	39000
Cu	380	470	240	370	33	36
Zn	500	560	570	640	170	210
pH	8.0	8.5	8.6	6.3	8.6	8.8
OM (%)	59.6	51.6	60.7	61.6	59.3	72.1
Kjeldahl N %	4.8	4.0	4.9	3.4	3.0	2.2
NH_4-N %	1.4	1.5	2.5	0.76	0.63	0.41

Table A3. Gene abundances. The range (minimum and maximum values) of abundances of the 16S rRNA gene and N cycling genes across different amendments within soil A and B. Abundances are presented as gene copies per ng extracted DNA.

Gene	Soil A			Soil B		
16S rRNA	1.40×10^{2}	-	1.64×10^{4}	6.78×10^{4}	-	2.21×10^{5}
nirS	4.21×10^{0}	-	3.39×10^{2}	6.94×10^{2}	-	3.06×10^{3}
nirK	6.00×10^{1}	-	8.19×10^{2}	3.87×10^{3}	-	1.19×10^{4}
nosZI	4.97×10^{0}	-	1.05×10^{2}	2.00×10^{2}	-	5.51×10^{2}
nosZII	1.09×10^{1}	-	4.90×10^{2}	3.35×10^{3}	-	5.86×10^{3}
amoA AOB	6.68×10^{-1}	-	3.44×10^{1}	2.12×10^{2}	-	1.12×10^{3}
amoA AOA	1.08×10^{-1}	-	2.90×10^{1}	2.87×10^{2}	-	1.02×10^{3}

7. Appendix B – additional methods

Table B1. Primers and thermal cycling conditions of the qPCR.

Gene	Primers	Primer conc. (µM)	Annealing	Elon-gation at 72 °C	Signal reading	# cycles	Ref.
16S rRNA	515F 926R	0.5	50 °C 30 s	35 s	80 °C, 5 s	40	1, 2
nirS	cd3aFm R3cdm	0.5	65 °C -1 °C/cycle 30 s 60 °C 30 s	30 s	80 °C ,5 s	5 34	3
nirK	876F 1040R	0.5	63 °C -1 °C/cycle 30 s 58 °C 30 s	35 s	80 °C, 5 s	5 34	4, 5
nosZI	1840F 2090R	0.5	65 °C -1 °C/cycle 30 s 60 °C 30 s	30 s	80 °C, 5 s	5 34	6
nosZII	nosZ-II_F nosZ-II_R	2	54 °C 30 s	45 s	80 °C, 5 s	40	7
amoA-AOB	amoA-1F amoA-2R	0.5	55 °C 30 s	40 s	77 °C, 5 s	40	8
amoA-AOA	CrenamoA23f CrenamoA616r	0.5	55 °C 30 s	40 s	77 °C, 5 s	40	9

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