Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences

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– Development and evaluation of method, and nitrification potentials in sewage sludge before and after application to soil

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Master´s thesis • 30 hec • Second cycle, A2E
Master of Agriculture, Crop and Soill Science
Examensarbete/Sveriges lantbruksuniversitet,
Institutionen för mikrobiologi, 2016:1 • ISSN 1101-8151
Uppsala 2016

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Credits: 30 hec
Level: Second cycle, A2E
Course title: Independent Project in Biology - Master's thesis
Course code: EX0565
Programme/education: Master of Agriculture, Crop and Soil Science

Place of publication: Uppsala
Year of publication: 2016
Cover picture: Caroline Jöngren
Title of series: Examensarbete/Sveriges lantbruksuniversitet, Institutionen för mikrobiologi
no: 2016:1
ISSN: 1101-8151
Online publication: http://stud.epsilon.slu.se

Keywords: Sewage sludge, nitrification potentials, PAO, sanitations treatment, storage treatments, nitrous oxide, *Nitobacteriacea*, *Nitrosomona*

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Abstract

Sewage sludge (SS) contains valuable plant nutrients applicable to soil as fertilizer. Application to arable land is though controversial as SS can contain contains such as human pathogens, heavy metals and xenobiotics. Post-treatment processes of SS such as long term storage which can reduce pathogenic bacteria before application to soil implicate a risk of nitrogen (N) losses via emissions of the greenhouse gas nitrous oxide (N_2O) and leaching of nitrate (NO_3^-). Nitrification is the microbial process that contributes to the turnover of these nitrogen compounds, however measurement of nitrification in long-term stored SS has never been done before. The hypothesis of nitrification activity in stored SS was the underlying reason for the questions: Is there any measurable nitrification activity in stored SS and how does it affect N2O emissions? How does different sanitation-treatments affect the nitrification in stored SS? Hence, the objective of this study was to develop an assay for assessment of potential ammonium oxidation rate (PAO) in SS, i.e. the turnover rate of ammonium oxidizing bacteria (AOB) constituting the first step in nitrification assayed in an optimal environment. Another objective was to measure the PAO in fresh and stored SS with different sanitation-treatments, and in soil after SS application. Also, by literature study evaluating the PAO in stored SS and its contribution to N_2O emissions.

Fresh dewatered and stored SS from the wastewater treatment plant Kungsängsverket in Uppsala was used in the development of the new PAO assay. The development was based upon the existing PAO method developed for soil. In the PAO assay a slurry (a sample with liquid substrate) is incubated during standardized conditions from which samples are taken over a time period. The sample is analyzed for nitrite content by spectrophotometric detection, which requires samples to be extracted and centrifuged. The rate of increasing NO_2^- content over time is defined as PAO. The assay development involved four steps: (1) Spectrophotometric scanning of SS extracts, (2) nitrite recovery analysis (3) refinement of extraction and centrifugation, and (4) optimization of sample size for a reliable PAO. The modified PAO assay compared to that used for soil resulted in reduced sample size and centrifugation of extracts amount. This resulted in a sample size with sufficient nitrification activity and minimized coloration of the extract. Accumulation of NO_2^- over time was analyzed using Flow Injection Analysis and demonstrated a stable and linear process. This demonstrated a stable PAO assay and nitrification activity in stored SS.

The new PAO assay was used analyzing SS from a pilot study with four different sanitation treatments in combination of storage for one year with and without cover. The result showed that long term stored thermophilically digested SS (TC) had significantly higher PAO activity than the other treatments using mesophilically digested SS, probably a result of increased access of oxygen (O_2) due to a more porous texture of the thermophilically digested SS. Mesophillically digested SS treated with ammonia (MAC) showed a low PAO activity as a high concentration of ammonia indicated inhibition of nitrification. The PAO rates of TC and MAC follow the results of N_2O emissions from the same treatments in a previous study and indicate that inhibition of nitrification lowers the amount of substrate available for processes driving N_2O emissions. SS application to soil did not change the intrinsic PAO of the soil, however some inhibitory effect was indicated with strongest effect displayed by MAC.

This study shows the PAO can be measured in SS and the developed method enables e.g. analysis of sanitation treatment effects in SS. However, further development is needed to ensure a reliable assay as the PAO activity indicated to decrease with increased sample amount, probably an inhibitor effect of increasing levels of contaminants.

Sammanfattning
Spridning av avloppsslam på jordbruksmark kan återföra värdefulla växtnäringsämnen till odlingssystemet. Det anses dock kontroversiellt då slammet kan innehålla föroreningar såsom sjukdomsalstrande bakterier, tungmetaller och organiska föreningar. För att minska innehållet av sjukdomsalstrande bakterier kan slammet hygieniseras genom bl.a. långtidslagring. Under lagringstiden riskerar man dock att förlora kväve (N) genom emissioner av lustgas (N2O), en potent växthusgas, och läckage av nitrat (NO3-) till grundvattnet. Nitrifikation är en mikrobiell process som påverkar förekomsten av dessa kväveföreningar, men mätningar av nitrifikation i lagrat avloppsslam har inte gjorts tidigare. Frågeställningarna som låg till grund för denna studie formulerades som: Kan nitrifikationsaktivitet påvisas i lagrat avloppsslam och i så fall kan den förklara observerade emissioner av N2O? Hur påverkas nitrifikationen av olika hygieniseringsmetoder? Målet var att utveckla en metod för att bestämma den potentiella ammonium-oxidationshastigheten (PAO) i avloppsslam, d.v.s. hastigheten av det första oxidationssteget i nitrifikationsprocessen under optimala förhållanden. Ytterligare mål var att bestämma PAO i färskt och lagrat avloppsslam med olika hygieniseringsmetoder liksom i jord efter tillsats av slam. Vidare utvärderades med hjälp av litteraturen huruvida PAO skulle kunna vara en bidragande orsak till avgång av N2O från lagrat slam.

Under utvecklingen av PAO metoden användes avvattnat färskt och lagrat avloppsslam från Kungsängsverket i Uppsala. Metodutvecklingen baserades på en etablerad metod för bestämning av PAO i jord. Vid mätning av PAO inkuberas en slurry (ett prov plus vätskesubstrat) under standardiserade förhållanden varefter prover av nitrit tas ut för analys under en tidsperiod. För att möjliggöra spektrofotometrisk bestämning av nitrit extraheras och centrifugeras proverna innan analys. Hastigheten av ackumulerad nitrit definieras som PAO. Metodutvecklingen utfördes i följande fyra steg: (1) Spektrofotomerisk karaktärisering av vattenextrakt från slam, (2) analys av känd mängd nitrit som satts till slamextrakt, (3) förfining av extraktions- och centrifugeringsförfarandet och (4) test av optimal provmängd för säker bestämning av PAO. PAO-metoden för slam jämfört med den för jord modifierades genom att minska provmängden i testet. Även mängden extrakt som provtogs minskades för att tillåta en högre centrifugeringshastighet. Dessa modifieringar innebar att bakgrundsfärgen på slamextraktet kunde reduceras och en tillräcklig nitrifikationsaktivitet bibehölls för tillförlitlig bestämning. Ackumulation av nitrit analyseras med Flow Injection Analysis och nitritackumulationen över tid visade en stabil rätlinjig process. Resultatet påvisade att den nya PAO metoden för slam fungerade och att nitrifikationsaktivitet förekommer i lagrat avloppsslam.

Den nya PAO metoden användes för att analysera slam från en pilotstudie där slam behandlats med fyra olika hygieniseringsmetoder och sedan lagrats under ett år med och utan täckning. Resultatet visade att termofilt rötat slam (TC) hade signifikant högre PAO än mesofilt rötat slam. TC hade en mer porös struktur än det mesofilt rötade slammet som troligen möjliggjorde ökad syresättning och ledde till ökad PAO. Mesofilt rötat slam behandlat med ammoniak (MAC) visade låga PAO värden vilket tyder på att den höga halten av ammoniak inhiberade nitrifikationen. PAO värdena från TC och MAC gick i linje med N_2O mätingar från samma behandlingar i en tidigare studie, d.v.s. inhibering av nitrifiktionen tyder på minskad mängd substrat för N_2O-bildande processer vilket leder till låga N_2O missioner. Inblandning av hygieniserat slam i jord förändrade inte jordens PAO värde signifikant.

Slutsatsen från studien är att det är möjligt att mäta PAO i avlopsslam och att den nyutvecklade PAO metoden möjliggör analys av effekter av t.ex. olika hygieniseringsmetoder för slam. Dock krävs ytterligare utveckling för att säkerställa metodens tillförlitlighet då PAO värden tenderade att minska med ökade provmängder, troligen en inverkan av ökande koncentrationer av föroreningar i slammet.

Abbreviations

AOB	-	Ammonium oxidizing bacteria
FMS	-	Fresh dewatered mesophillically digested sewage sludge
M	-	Mesophilically digested sewage sludge stored without cover
MC	-	Mesophilically digested sewage sludge stored with cover
MAC	-	Mesophilically digested sewage sludge treated with ammonia (by incorporation of 1.5% urea) stored with cover
PAO	-	Potential ammonium oxidation
TC	-	Thermophilically digested sewage sludge stored with cover
SMS	-	Stored dewatered mesophillically digested sewage sludge
SS	-	Sewage sludge
WWTP	-	Wastewater treatment plant

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1. Introduction

High-yield crop production on arable land requires high input of nitrogen (N) and phosphorus (P). After harvest, the nutrient rich crops are refined into various food products consumed in urban areas. The residual plant nutrients are conveyed via excreta into the sewer pipes ending up in the sludge of the wastewater treatment plant (WWTP). In this linear route of plant nutrient use, the soil has to be replenished with nutrients to maintain its fertility. This is most often done by adding mineral fertilizers. As the concentrated plant nutrients in the wastewater and sewage sludge risk to leak into the environment, this frequently cause eutrophication of lakes and water courses. Hence, it is obvious that the linear way of handling plant nutrients and wastewater is not sustainable. Ideally, plant nutrients should be recycled back to the arable land from the sewage systems.

WWTP are designed to reduce the content of organic material, P and N in effluent to prevent spreading to sensitive environments, thus the focus is not to produce an end product for reuse as fertilizer. The solid end product from WWTP, so called sewage sludge (SS), can contain digested organic material, P and N, as well as other macro and micronutrients, but also contaminants such as human pathogens, heavy metals and xenobiotics (Odlare et al., 2014; Börjesson et al., 2014). Application of SS on arable land is beneficial to the soil system as it can improve its structure (Kätterer et al., 2014) and supply plant nutrients (Odlare et al., 2014). Nevertheless, low content of heavy metals in the SS has to be ensured before it can be used as fertilizer. In addition, treatment practices to minimize spreading of human pathogens are needed.

Treatment practices to lower the content of pathogens in SS have been suggested by the Swedish Environmental Protection Agency (SEPA), including thermophilic anaerobic digestion, incorporation of urea for ammonia treatment and storage for at least one year (Naturvårdsverket, 2002; Naturvårdsverket, 2013). However, long-term storage (Flodman, 2002; Jönsson et al., 2014) as well as application of SS to arable soils (Thangarajan et al., 2013; Jönsson et al., 2014; Rodhe and Pell, 2005) may result in emission of nitrous oxide (N_2O) and methane (CH_4). N_2O is a potent greenhouse gas (GHG) with high global warming potential and long residence time in the atmosphere (IPCC 2013). Around 13% of the GHG-emissions in Sweden year 2012 originated from the agriculture sector, including sewages sludge used as fertilizer, of which N_2O represented 63% (Naturvårdsverket, 2014). These findings emphasize the need for better knowledge of the microbial processes and regulating factors behind N_2O production in long-term stored SS and in soil fertilized with SS.

Emission of N_2O is a result of the microbial processes nitrification and denitrification. Nitrification is the aerobic process in which specialized lithotrophic bacteria oxidize ammonium (NH_4^+) first to nitrite (NO_2^-) and then further to nitrate (NO_3^-) (formula 1) (Wunderlin et al., 2012). Denitrification is conducted by a range of organotrophic bacteria under anaerobic conditions where NO_3^- is reduced in several steps to the end product dinitrogen gas (N_2) (formula 2) (Firestone and Davidson, 1989). Shifts in aerobic and anaerobic conditions in soils has shown to stimulate production of N_2O, an intermediate product of both processes (Clemens et al., 1997). Factors affecting the amount of N_2O emitted from a soil or water ecosystem are the availability of mineral N, easy accessible carbon, oxygen, water, temperature and pH (Thangarajan et al., 2013). Nitrifying and denitrifying bacteria are indigenous inhabitants (Firestone and Davidson, 1989) of soils and crucial players

in biological wastewater treatment processes (Wunderlin et al., 2012), hence most likely inhabitants of SS as well.

$$\text{Nitrification: } NH_3 \rightarrow \begin{matrix} & & N_2O \\ & & \uparrow \\ N_2O & & NO \\ \uparrow & & \uparrow \\ NH_2OH & \rightarrow & NO_2^- \end{matrix} \rightarrow NO_3^- \qquad (1)$$

$$\text{Denitrification: } NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad (2)$$

In a study by Jönsson et al, (2014) N_2O and CH_4 emissions were investigated in a pilot-scale storage of thermophilic and mesophilic digested and dewatered SS, with and without cover and addition of urea. The different treatments produced NO_3^- and emitted N_2O. This lead to the hypothesis that nitrification could be the underlying mechanism for the N_2O emissions. To our knowledge nitrification activity has previously not been investigated in stored SS. The main reason for this could be the lack of methods for analyzing this property in SS.

One way of estimating the capacity of a soil to nitrify is to analyze the potential ammonium oxidation (PAO) rate. PAO is defined as the capacity of nitrifying enzymes in an optimal environment to oxidize NH_4^+ to NO_2^-, which can be assayed in an aerated soil slurry with excess of NH_4^+ buffered to pH 7.2 (Belser and Mays, 1980). Ammonium oxidizers are highly specialized bacteria sensitive to environmental changes, also making the PAO assay a valuable tool in analyzing the soil functioning and to test toxicity of chemical substance added to the soil (Pell et al., 1998; Pell and Torstensson, 2003). In addition, PAO capacity of a soil indicates risks for N_2O emission. Thus, developing a PAO assay for SS would provide a tool for studying how the ammonia oxidizing capacity in stored SS is affected by digestion at different temperatures prior to storage as well as by urea treatment and covering during storage. Finding a combination of treatment methods lowering the nitrification activity during storage would contribute to minimize risks of N_2O emissions and NO_3^- leaching from SS handling.

1.1. Aim

The overall aim of this work was to develop a PAO method for assaying nitrification activity in fresh and stored sewage sludge. Specific aims were to (1) develop and optimize a PAO-assay for sewage sludge (2) run the new PAO-assay on fresh sewage sludge and sludge stored using different treatment methods, (3) measure PAO activity in soil applied with sewage sludge, and (4) discuss whether nitrification in sewage sludge could contribute to N_2O emission.

The objective was to provide a reliable tool for measuring nitrification activity in SS. Also, from a previous study, evaluate if nitrification activity is affected by different sanitation-treatments in stored SS and if the nitrification contributes to the measured N_2O emissions.

The study aims to address these following research questions:

- Is there any nitrification activity in fresh and stored sewage sludge?
- If any nitrification, is it measurable?
- Does different sanitation-treatments of stored sewage sludge affect the nitrification?
- Does nitrification contribute to N_2O emissions from stored sewage sludge?

2. Background

The importance of recycling plant nutrient stands clear in maintaining sustainable food production, however, several factors throughout the wastewater treatment chain obstruct a fully acceptable SS fertilizer product for agriculture use. The following background section aims to provide understanding of the complexity of the processes behind a final SS fertilizer product. First, an overview of the wastewater treatment process is given. Then, the nitrification process in both SS and soil is reviewed with focus on its potential to be source of N_2O emissions. Finally, a review of different techniques for assessment of nitrification rates in both soil and the WWTP is given.

2.1. Sewages sludge - production and application to soil

2.1.1. Wastewater treatment

Municipal wastewater treatment plants (WWTP) receive sewage water from households and industries, and are primarily designed to rid the water from dissolved organic carbon, P and N enabling discharge of the water to the environment without disturbing the recipient ecosystem. In order to obtain clean wastewater various physical, biological and chemical techniques are applied.

In the first step of the WWTP, called primary or mechanical treatment, the wastewater is conveyed through a grid to separate unwanted material such as paper and cloths. In addition, heavy particles are removed by sedimentation. Chemicals can also be added in this step to facilitate separation of the particles.

In the second step, after the pretreatment the wastewater is lead to a basin for biological treatment. Here microorganisms are stimulated to mineralize and reduce dissolved organic carbon and by combined nitrifying and denitrifying activities reduce the nitrogen (Bitton, 2005). The microbial activities are parts of the natural energy and carbon metabolism of the microorganisms where the most efficient biological process is created by mixing the water to allow close contact between suspended material, nutrients and microorganisms. As nitrifying and denitrifying bacteria prefer aerobic and anoxic conditions, respectively, alternating zones of air blown into the basin from beneath and non-aerated zones are created in the so called activated sludge process (Pell and Wörman, 2008).

In the final step chemicals, typically salts of Al, Ca, Fe or Mg are added to precipitate orthophosphate ions (PO_4^{3-}). The stable precipitates are allowed to sediment and therefore become part of the sewages sludge. Removal of P can also be achieved biological, by the so called Bio-P process, in which conditions favorable to poly-P accumulating bacteria are created. One benefit with the Bio-P process is that it produces less sludge than chemical precipitation as the bacterial uptake of phosphorus increases the density of the SS. Chemically precipitation increases the hydrophilic character of SS as which restrain an effective dewatering process (Bitton, 2005; Pell and Wörman, 2008).

Throughout the wastewater treatment process, from the sewer pipes to the final sludge storage and application to arable land, N_2O and CH_4 two potent GHG are emitted. N_2O and CH_4 have global warning potentials 265 and 84 times higher than carbon dioxide in a 100 year period (IPCC 2013). In 2012, N_2O emissions in Sweden corresponded to 6.3 million tonnes CO_2-equivivalents which was mainly emitted from the agricultural sector including soil management and fertilization with organic substrate, such as sewage sludge (Naturvårdsverket, 2014). From storage of SS, emission rates of GHG in the range of 2-17 kg

CO_2-equivivalents per person and year was reported from three different studies (Akerman et al., 2010; Flodman, 2002; UKWIR, 2013). Furthermore, from a study of pilot-scale storage the emissions of N_2O were 0-44 kg CO_2-equivalents per square meter (Jönsson et al., 2014).

Heavy metals, organic pollutants (xenobiotics), medical residues and human pathogens are unwanted substances frequently disposed into toilets and sinks of which some will end up in the sewage sludge. This contamination poses a problem as the value of the recycled plant nutrients is constrained. The concentration of most heavy metals in SS has decreased and will probably continue to decrease in the long perspective (Naturvårdsverket, 2013). As opposed to this trend copper is increasing due to its release from the numerous water pipes made of copper. Also more than 250 organic chemical compounds have been detected in Swedish SS and a few of them in concentrations over mg/kg dry weight (Naturvårdsverket, 2013). Among the chemicals detected are medical residues and some antibiotics that may affect the biological treatment processes in the WWTP (Heberer, 2002). Bacteria such as *E. coli* and *Salmonella* are present in SS, the former occurring naturally in the human guts, however, both bacteria may be pathogenic and, hence, SS should not be used as fertilizer in agriculture in ways that risks contaminating food.

2.1.2. After-treatment

The settled SS produced in the WWTP is treated further to obtain a stable product with less water, improved texture and reduced content of pathogens. This process includes thickening, where polyelectrolytes are added to allow the soild fraction of the sludge to separate from the water, followed by dewatering by centrifugation or belt pressing. Further stabilization and sanitation can be done by anaerobic digestion before adding additional polyelectrolytes and dewatering to achieve a SS dry weight of 25-30% before final storage. The treated and stable SS can be used as e.g. filling material in road embankments, soil improvers and fertilizer or be deposited in landfills if highly contaminated (UppsalaVatten, 2013; Bitton, 2005). To be approved for use on arable land the SS has to be sanitized and fulfill issued limit values of contaminants (Naturvårdsverket, 2013; SvensktVatten, 2015).

Thermophilic (50-60°C) digestion reduces levels of pathogenic bacteria such as *Salmonella* and certain *E.coli*, while mesophilic digestion (20-40°C) has a rather low sanitation effect (Vinnerås, 2013). The digestion process most likely have a negative effect on nitrifying bacteria as the most common genera of *Nitrosomonas* and *Nitrobacter* are sensitive to heat (Jiang and Bakken, 1999) and anaerobic conditions. The digestion process, which is regulated by the specific retention time of the feedstock and temperature, is an approved sanitation treatment method. Normally the digestion has to be combined with long term storage of the produced digestate with a minimum of six months to fulfill hygienic limit value of SS for agricultural use (Naturvårdsverket, 2013; SvensktVatten, 2015). The sanitation effect can be further improved by ammonia (NH_3) treatment which can be achieved by adding urea. Treatment of the SS with urea in high concentrations will result in release of NH_3 which will reduce the numbers of pathogenic bacteria and possibly also nitrifying bacteria (Anthonisen et al., 1976; Vinnerås, 2013). Though, long term storage of SS fulfills the sanitation requirements and could lead to increased emissions of the GHG (N_2O, CH_4, CO_2) and thus further aggravate the GHG burden from the agriculture sector, either during storage itself or during land-spreading when used as organic fertilizer (Jönsson et al., 2014). Hence, a good after-treatment includes a lot of thinking in order to design a safe system without environmental side effects.

2.1.3. Land application of sewage sludge

Around 200 000 tonnes dry weight per year of SS is produced in Sweden but only 25% is spread on arable land even though that 84% is approved for arable land application (Naturvårdsverket, 2013). If all SS produced were used as fertilizer this would recycle around 6 000 tonnes P and 8 000 tonnes N back to the Swedish arable land, which would equal around 3 kg P and 4 kg N per ha (KSLA, 2012). The total consumption of mineral fertilizer in Sweden was year 2013/2014 around 12 000 tonnes P and 180 000 tonnes N (Jordbruksverket 2015).

The amount of P and N available to the plants of the total content in SS is thought to be restricted by chemically precipitated P (Krogstad et al., 2005; Linderholm, 1997) and N bound in organic material (Rigby et al., 2009). However, field- and lab experiments have demonstrated increased P values in soils after several years of SS application, and partly increased water-soluble phosphorus especially from SS with biological precipitated P using Bio-P process (Andersson, 2012; Otabbong, 1997). Heterotrophic microorganisms deriving energy and carbon by degrading organic material to produce new biomass. This result in N bound in organic material being mineralized (NH_4^+) but will be immobilized when the population of microorganisms continue to grow. However, as the fraction of easily degraded carbon in the organic material of SS is low after anaerobic digestion, which increase the biological stability of SS, the mineralization of organic N can be restricted. (Stinner et al., 2008). The result of the microbial immobilization of N will be, in short term, less N available to the plants, thus restricting the value of SS as short-term N fertilizer (Jezierska-Tys and Frac, 2008). Most N bound in the organic material of SS will be gradually mineralized and released over several years depending on soil moisture, pH, temperature, soil type and microbial immobilization (Rigby et al., 2009; Borjesson et al., 2014). Urea treated SS can, however, increase the amount of plant-available N (Vinnerås, 2013).

Application of SS per hectare is regulated according to Swedish Environmental Protection Agency (SEPA) regulation (SNFS1994:2MS:72), which consider the soil and SS content of P, N and heavy metals. The structure of the SS allows spreading by a dry-manure spreader and after application it has to be incorporated by tillage into the soil within a certain time frame, based on the same regulation as that for other organic fertilizers as issued by the Swedish Board of Agriculture (SJVFS2004:62). Sewages sludge certified by REVAQ ensures a certain quality making it more suitable for agricultural use. The REVAQ certificate is owned by The Swedish Water and Wastewater Association and is accepted by farmers' federations and food processing industries such as The Federation of Swedish Farmers (LRF), The Swedish Food Federation (Livsmedelsföretagen) etc. The REVAQ system requires analysis and tracking of 60 metals and *Salmonella* in the SS and that application on arable land follows existing rules and regulations regarding allowed rates of metals and P (SvensktVatten, 2015).

Sewage sludge should probably be principally regarded as a P fertilizer product but as it contains well digested carbon, it will also improve the structure of compact and poorly structured mineral soils. Application of SS will increase the carbon content in the topsoil, improve water infiltration and decrease the soil bulk density which leads to improved plant growth and yield, and increases the microbial biomass and activity (Kätterer et al., 2014; Jezierska-Tys and Frac, 2008; Thangarajan et al., 2013). However, disadvantages with arable spreading of SS involve risk of contaminants being accumulated in the soil and further on taken up by plants and transferred to food products. The concerns of the public regarding health risks and environmental long-term effects have made many food-processing companies and mills restricted in accepting crops grown on land with SS applied (Lantmännen, 2015).

In a long-term field trial at two locations with application of SS since 1981 the SS content of heavy metals have decrease over the years (Andersson, 2012). However, the content of copper, zinc and mercury in the soil has increased whereas the content of cadmium increased significantly at one location, but not at the other. This emphasizes the importance of considering the soil background concentrations of cadmium when compare results. In the field-trial there was no significant accumulation of the heavy metals in harvested crop compared to control with no SS application (Andersson, 2012). Accumulation of selected xenobiotics were detected in sugar-beets from the same field trial, although this was seen only in treatments with SS applied at rates three times higher than limited application rates (Hörsing et al., 2014). Increased levels of mainly copper and zinc in soil were also shown in long-term field trial study by Börjesson et al. (2014), the plant uptake of heavy metals was however low.

2.2. Nitrification

Nitrogen is essential for all living cells as it constitutes part of DNA and proteins. In plants, N also has an important role in the photosynthesis as it constitutes structural elements in the porphyrin group of the chlorophyll. In soil, N is bound mainly in organic material and therefore must be mineralized and transformed by microorganisms before taken up by the plants or other organisms.

Mineralized NH_4^+ and NO_3^-, are the forms mainly taken up by the plants, which implicate the important role of soil microorganisms in making organic N available to the roots. However, the ratio between carbon and N in soil determines the amount plant available N. Generally, a C/N ratio in soil or organic fertilizer less than 20 will stimulate mineralization and if higher than 20 immobilization will dominate. If the organic material is rich in N and exceeds the microbial need it will be mineralized and left available to other organisms (Robertson and Groffman, 2007).

Surplus of mineral N in soil may lead to that NH_4^+ is first oxidized to nitrite (NO_2^-) by ammonia-oxidizing bacteria (AOB) and then to nitrate (NO_3^-) by nitrite-oxidizing bacteria (NOB) (Robertson and Groffman, 2007). The nitrification is mainly conducted by bacteria within the family *Nitobacteriaceae* which are principally autotrophic and chemiolitotrophic, meaning that they derive their carbon from CO_2 and energy from the oxidation of N (Tolli and King, 2005). Nitrifying bacteria are mainly obligate aerobes, as oxygen is used as terminal electron acceptor in their respiration. In respiration, protons builds up charge over membranes in the cell which drives the so-called electron transport phosphorylation of ADP to energy rich ATP. A large quantity of NH_4^+ has to be oxidized by the AOB to obtain energy for deriving the reducing power needed to fix CO_2 into organic carbon by the Calvin cycle. In the oxidation process protons are released (formulas 3 and 4) to the environment causing lowering of pH. The oxidation of NO_2^- to NO_3^- by NOB yields less energy than the NH_4^+ oxidation, which implies slower growth (McGill, 2007). In addition, ammonia-oxidizing archaea (AOA) has also been discovered and found to be abundant in agricultural soils (Leininger et al., 2006; Kelly et al., 2011). Also heterotrophic fungi has been reported to substantially contribute to nitrification in forest soils (Stams et al., 1990).

The first step of ammonia oxidation is catalyzed by the membrane bound enzyme ammonia mono-oxygenase and produces hydroxylamine (NH_2OH) (formula 3). The hydroxylamine is then further oxidized in a second step by the enzyme hydroxylamine oxido-reductase to NO_2^- (formula 4) (Bolan et al., 2004). This first step is mainly conducted by the AOB genera

Nitrosomonas which is common inhabitant of soils, sediments and water environments (Prosser, 1989; Koops and Pommerening-Röser, 2001; Kowalchuk and Stephen, 2001) and also the most pronounced genera of AOB in WWTP (Ahn, 2006). Other genera of AOB are *Nitrosococcus*, *Nitrosopira*, *Nitrosovibrio*, and *Nitrosolobus* (Ahn, 2006).

$$NH_4^+ + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (3)$$

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

In the nitrite oxidation by NOB, NO_2^- is oxidized to NO_3^- by the enzyme nitrite oxidoreductase (formula 5) (Bolan et al., 2004). This step is mainly conducted by the genera *Nitrobacter,* but also by other genera *Nitrospira*, *Nitrospina*, *Nitrococcus* and *Nitrocystis* (Ahn, 2006) may be common.

$$NO_2^- + H_2O \rightarrow NO_3^- + 2e^- + 2H^+ \quad (5)$$

During ammonium oxidation production of nitric oxide (NO) and N_2O is possible and these pathways are complex involving e.g. multiple enzymes with redundant function and overlapping pathways (Stein, 2011). Production of N_2O occurs when oxygen is limiting (Poth and Focht, 1985) or in presence of high concentrations of NO_2^- (Firestone and Davidson, 1989; Stein, 2011). The resulting NO_2^- may be used as electron acceptor instead of oxygen leading to nitrite reduction, which show the ability for nitrifying bacteria to denitrify, so called nitrifier denitrificaiton (formula 6) (Wrage et al., 2001).

$$NH_3 \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow N_2O \rightarrow N_2 \quad (6)$$

Nitrous oxide (N_2O) can also be produced trough hydroxylamine oxidation (formula 7) (Stein, 2011) by heterotrophic nitrification bacteria, however these are less significant for N_2O emissions than autotrophic nitrification.

$$NH_3 \rightarrow NH_2OH \rightarrow NO \rightarrow N_2O \quad (7)$$

It should also be noted that N_2O can also be a significant end product in denitrification, where NO_3^- normally is stepwise reduced to NO_2^-, NO, N_2O and finally N_2 (formula 2). As denitrification bacteria are heterotrophs, a complete reduction of nitrate requires sufficient amount of a simple carbon source as well as anaerobic environment (Firestone and Davidson, 1989).

The most important factor regulating nitrification is the supply of NH_4^+. The availability of NH_3, the actual substrate for AOB, is depending not only on the concentration of NH_4^+ but also on the ammonia-ammonium ion equilibrium which is pH dependent (pKa = 9.25) implying that environments with high pH are favorable to these bacteria. Other factor regulating the process is the production rate of ammonium via mineralization, which will increase if N rich substrates like proteins is available. Nitrifying bacteria have to compete for the N with other microorganisms and plants assimilating N, and by ammonia volatilization (Norton and Stark, 2011). Nitrifying bacteria are weak competitors for nitrogen and will have access to NH_4^+ only if the supply in soil exceeds the plant uptake and the demand of heterotrophs. It is known that an increase of NH_4^+ in soil accelerates nitrification if no other factor is limiting, however this can differ between soils (Stark and Firestone, 1996). Some ammonia-oxidizing bacteria, *Nitrosomonas* and *Nitrosopira* are sensitive to high

concentration of ammonia (Norton and Stark, 2011), which has also been shown by Anthonisen et al. (1976) and Smith et al. (1997). Generally nitrifying bacteria are sensitive to environmental changes such as high temperatures (Jiang and Bakken, 1999) and high concentrations of heavy metals (Subrahmanyam et al., 2014; You et al., 2009). Nitrite, the product of ammonium oxidation, does also have a general toxic effect to microorganisms (Anthonisen et al., 1976). High concentration of both NH_4^+ and NO_2^- has therefore to be considered during experimental design of measuring nitrification rates, e.g. by reducing the sample amount.

Nitrifiers are obligate aerobic bacteria, which, in submerged soils where oxygen is limiting, leads to decreased nitrification (Norton and Stark, 2011). Hence, nitrifying bacteria are less active in anaerobic environments but can live in the root zones of submerged plants to which oxygen is supplied via diffusive or convective mechanisms through the plant or in submerged soils where oxygen can be trapped in micro aggregates (Robertson and Groffman, 2007). It has also been shown that ammonia oxidizers via nitrifier-denitrification can use nitrite as electron acceptor when oxygen is limiting (Poth and Focht, 1985).

The optimum pH for nitrification is in the range of 7.5 - 8 (Prosser, 1989). Shammas (1986) discussed that both *Nitrosomonas* and *Nitrobacter* can still be active at pH around 6, however with lowered nitrification efficiency. Autotrophic nitrification activity have been determined in soils with as low pH as 3 (De Boer and Kowalchuk, 2001) and up to pH 10 (Sorokin et al., 2001; Sorokin, 1998). Dry condition will decrease the activity of ammonium oxidation mainly due to dehydration effect on the microbial metabolic activity and cell physiology as well as restricted substrate availability (Stark and Firestone, 1995). The optimal temperature for nitrification is around 25-30°C (Koops et al., 1991). It has been shown that nitrification bacteria can adapt to different temperature and moisture regimes (Mahendrappa et al., 1966) and be active at soil temperatures as low as 2-10°C (Avrahami and Conrad, 2005; Avrahami et al., 2003).

Different natural or anthropogenic chemicals can inhibit nitrification. Applying specific inhibitors in e.g. agriculture allows means to retard losses of N and emissions of NO_3^- and N_2O from nitrification and denitrification. Calcium carbide is one product that can be incorporated in soil which when reacting with water produces acetylene (C_2H_2). C_2H_2 inhibits nitrification as well as the last step in denitrification, i.e. the reduction of N_2O to N_2 (Robertson and Groffman, 2007). Another product, with the same function as calcium carbide is the active substance nitrapyrin (2-chloro-6-(trichloromethyl)pyridine) (Goring, 1962).

2.3. Methods for determination of nitrification activity in soil

Measuring the nitrification rate, i.e. consumption of NH_4^+ or production of NO_3^- in soil or activated sludge can be used to identify potential sources of N_2O emissions. Analyzing N_2O itself can also be done to indicate bacterial activity but will not give answer to the actual underlying mechanism of production as N_2O may be formed by both nitrifying and denitrifying bacteria.

Disturbance of the soil ecosystem could be caused by presence of toxic substances or restricted access to factors needed by the microbes. The activity of the nitrification bacteria have been used in test systems for evaluating toxic effects of chemicals (Pell et al., 1998; Pell and Torstensson, 2003; Jezierska-Tys and Frac, 2008). Due to the importance of nitrification, regulating N availability to roots and causing environmental pollution, several methods for measuring its activity have been developed. The nitrification rate without addition of extra

mineral N estimates the natural changes in the *in situ* NH_4^+ or NO_3^- pool over time, while the potential nitrification, i.e. activity of all nitrifying enzymes present in the system can be measured at saturated substrate conditions by adding surplus of NH_4^+. Nitrification rate can be measured in the field as well as in the laboratory depending on experimental objectives (Norton and Stark, 2011).

Changes in NO_3^- pool size over time due to nitrification can be measured using ^{15}N isotope trace or dilution techniques. In the tracer technique the N in NH_4^+ is labeled with ^{15}N and added to the system, and from the following decrease in source pool of $^{15}NH_4^+$ and increase in product pool of $^{15}NO_3^-$ the nitrification rate can be calculated. The tracer technique has its weaknesses in that, (1) adding NH_4^+ can increase the accessible substrate and increase the actual nitrification rate, thus overestimating the rate, (2) other non-labeled N sources of mineralized NH_4^+ can be included in the nitrification substrate which underestimates nitrification rate, and (3) immobilization of NO_3^- by cells will underestimate the flow of ^{15}N into the product pool (Norton and Stark, 2011).

In the isotope dilution technique ^{15}N-labled NO_3^- is added to the product pool and throughout the nitrification the product pool will be diluted by the non-labeled $^{14}NO_3^-$. The rate of dilution of the ^{15}N can be measured and used to calculate the gross nitrification rate. Difficulty with the dilution technique is that it implies uniform distribution of the isotopes, which is more or less impossible to achieve due to the heterogeneity of the soil matrix. Another problem is that it seems like that the nitrifying enzymes display isotopic preference, i.e. the enzymes prefer ^{14}N before ^{15}N which will underestimate the nitrification rate. The method is also based on the assumption that nitrification and NO_3^- consumption is constant through the incubation period (Norton and Stark, 2011) .

Nitrification inhibitors like C_2H_2 and nitrapyrin can also be used to measure the nitrification rate by blocking the nitrification. The nitrification rate can be estimated using two soil samples, one with and one without added inhibitor. From the change in NO_3^- concentration, measured before and after the incubation in inhibited and non-inhibited soil samples, the nitrification rate can be calculated. As the two samples are compared this method is based on the assumption that the inhibitor blocks the nitrification completely and that the NO_3^- consumption are the same in both soil samples (Norton and Stark, 2011).

Potential ammonium-oxidation rate (PAO) is defined as the rate of the nitrifying enzyme activity in a sample under non limited substrate conditions (NH_4^+) in an aerobic environment with favorable temperature and pH to the bacteria. PAO can be used to estimate the nitrifying capacity of a soil and has been used as indicator of soil quality. The PAO assay is a rapid method with incubation times of 6 h, which prevents AOB to display growth in number. The method has been used in e.g. dose-response tests to assess toxicity of pesticides in soil (Pell et al., 1998; Pell and Torstensson, 2003) and to study resources of ammonium in soil (Bollmann, 2006). The PAO assay is based on a method by Belser and Mays (1980), that after some modification has become ISO standard (ISO, 2012). In the assay, 25 g soil sample is blended into a substrate consisting of ammonium sulphate (0.04 mM), potassium phosphate buffer (0.2 M) at pH 7.2 to form a slurry. The nitrite oxidation, i.e. the second step of nitrification is inhibited by adding sodium chlorate (15 mM) which results in NO_2^- accumulation during the assay. Samples from the soil slurry are taken after 2 h and then every hour during a total incubation period of 6 h. The samples are added to potassium chloride (4 M) to stop the ammonium oxidation. The sample is then filtered and the filtrate at the different times is

analyzed calorimetrically for NO_2^-. The rate of ammonium oxidation in the assay can theoretically be described by the following formula (formula 8):

$$P_{PAO} = P_0 + K_{PAO}t \quad (8)$$

where P_{PAO} is the concentration of NO_2^- at time t, P_0 is the concentration of NO_2^- at start and K (or PAO) is a rate constant. Thus, PAO can be determined by linear regression of obtained data in the assay (Pell and Torstensson, 2003). The workflow of the PAO assay is described in a picture (Fig. 2) in Material and Methods.

2.4. Methods for determination of nitrification activity in WWTP

Nitrification is a crucial step in wastewater treatment as it is prerequisite for N removal by denitrification. A common strategy to estimate nitrification activity in the activated sludge process of a WWTP is to monitor the decrease of substrate (NH_4^+) or increase in end-product (NO_3^-). Though this is a straight forward and simple method it does not give accurate information on nitrification activity as several other biological N transformation processes may take place simultaneously. Another method suggested is based on measurement of oxygen utilization rates after stepwise addition of first the AOB inhibitor $NaClO_3$ and then the NOB inhibitor allylthiourea (ATU) (SurmaczGorska et al., 1996). This method allows simultaneous assessment of ammonium oxidation and nitrite oxidation, i.e. both steps of nitrification.

To our knowledge there is no method available for determining nitrification activity in fresh or stored SS. The PAO assay, or similar, have been used for measuring nitrification potential in agricultural soils (Laanbroek and Gerards, 1991; Stoyan et al., 2000), paddy soils (Bodelier et al., 2000) and sediments (Bodelier et al., 1996), and could fulfill the needs for determination of nitrification activity in SS. In addition of being simple it makes sense monitoring the first step of nitrification as it is this step that may emit N_2O. However, as PAO has been developed for measurements in soil the method probably has to be adapted to SS to ensure a reliable result. One crucial problem could be the high content of colored and colloidal organic substances in SS that may interfere with the wavelength used in the spectrophotometric detection of NO_2^- (Moorcroft et al., 2001; Davis et al., 1999). Another problem could be that added chemicals (salts) to the wastewater treatment process for P precipitation and the addition of polymeric electrolytes in the post treatment of the sludge may affect the affinity of the chemicals in the assay. This could result in stronger binding of the chemicals used in the PAO assay or the produced end-product NO_2^-, which could cause biases in both the process rate and the following colorimetric analysis. Yet another factor that must be considered is the amount of sample needed to yield optimal rates of activity, which most likely differs to that of soil. Hence, it is obvious that before applying any existing method for measuring nitrification activity in treated and stored SS the method must undergo a thorough optimization to be proved reliable.

3. Material and methods

The workflow of the development of a new method for assaying PAO of processed SS was organized in 4 steps (Fig. 1). The development was based on some critical sections (1, 5, 6 and 7; Fig. 2) of the PAO assay workflow in able to receive qualitative results. After each development step, decisions were made leading to the design of a new experiment, performed in the following step. In Step 1 the absorbance spectrum was scanned using different amounts of SS to determine the optimal sample size not interfering (color and opacity) with the wavelength used for spectrophotometrical analysis of NO_2^- (Section 1 and 6, Fig. 2). Step 2 comprised NO_2^- recovery analysis of known amounts of NO_2^- added to extracts of different amounts of SS to see if the analysis protocol as that used for soil could be applied (Section 1, 5 and 6, Fig. 2), and in Step 3 further NO_2^- recovery analysis were done to evaluate if different centrifugation- and extraction methods could lower disturbances and interference of SS coloration and particles (Section 4 - 6, Fig. 2). In Step 4, PAO assay of different amounts of SS were performed to evaluate if SS possess any AOB activity, and if so to determine what sample amount yields highest specific rate (Section 1 and 7, Fig. 2). Results from the Step 1 - 4 were evaluated and compiled into a new recommended PAO protocol for SS. In Step 5 the new protocol was used to assess the PAO activity of fresh and stored sewage sludge and Step 6 included PAO assay of soil applied with SS (Fig. 1).

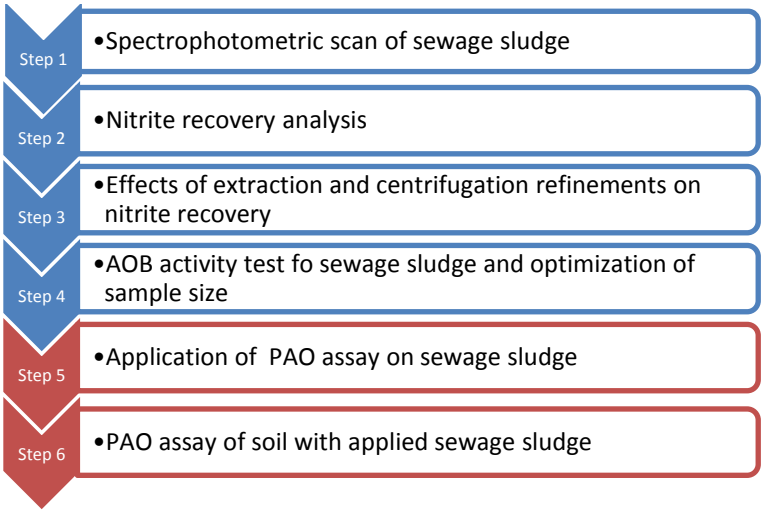

Figure 1. Four-step strategy (Step 1 - 4, blue box) for development of a potential ammonium oxidation (PAO) assay for sewage sludge (SS), and application of the new developed PAO assay for assessing PAO in different sewage sludge (Step 5, red box) and in soil applied with sewage sludge (Step 6, red box).

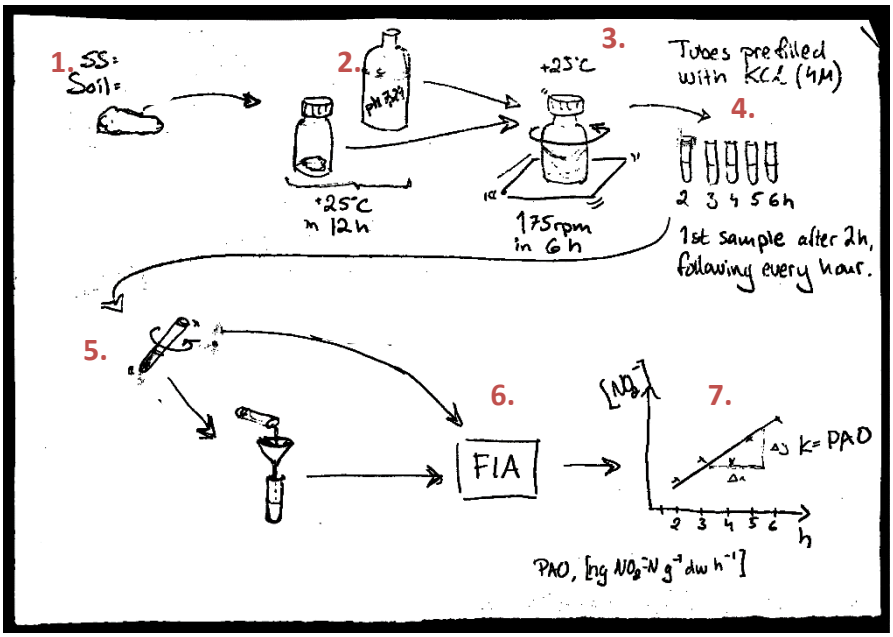

Figure 2. Schematic picture over the workflow of the PAO assay in 7 sections (after ISO15685:2012): 1) Sample amount of soil or sewage sludge (SS), 2) Incubation of samples and buffer substrate in +25°C for 12h, 3) Blending substrate and sample to from a slurry, place on a shaking board for 6 h in +25°C, 4) Collecting five slurry samples over the 6 h period, first sample after 2 h, following four samples every hour, 5) Centrifugation alone, or centrifugation and filtrating of slurry samples to receive extracts with reduced amount particles, 6) NO_2^- detection in extracts by spectrophotometric analysis using Flow Injection Analysis (FIA), 7) The rate of increasing NO_2^- content over time is defined as PAO (ng NO_2^--N g^{-1} dw h^{-1}).

3.1. Sewage sludge

The PAO assay development Step 1 - 4 used fresh dewatered mesophilically digested sludge (FMS) from the Kungsängen WWTP, Uppsala (Appendix 1, Table 1), and 7 month stored mesophilically digested sludge (SMS) from Kungsängen WWTP stored at Hovgarden land-fill site, Uppsala (Appendix 1, Table 2). In the WWTP phosphorus removal was achieved by application of $FeCl_3$ (PIX-111; Kemira Kemi AB, Helsingborg, Sweden), and the polyelectrolytes Zetag 7557 (BASF, Ludwigshafen, Germany) and Superfloc C-498 (Kemira Kemi AB) were used as flocculation agents in dewatering of the SS. The FMS was collected directly from the dewatering centrifuge running at the WWTP and the SMS were collected from the surface layer of a non-covered sludge pile. The SS was collected in March 7, 2014 and samples for development Step 1-3 (Fig. 1) were stored at +2°C for 2 - 4 weeks, while samples for Step 4 were frozen in -20°C for 4 weeks.

In Step 5 and 6 the SS used, originated from a pilot study applying four different storage methods (Jönsson et al., 2014). The SS were dewatered mesophilically digested sludge (37.5°C) from Kungsängen WWTP and dewatered thermophilically digested sludge (53°C) from Sunne WWTP. The P removal at Sunne where achived by $AlCl_3$ (Ekoflock 90, Akzo Nobel, Amsterdam, Netherlands) and Polymer Sedifloc 1060C (3F Chimica Americas/US Polymers Inc., Aberdeen, US) for dewatering. Representative and thoroughly mixed samples for assay of PAO in the present study were collected from all treatments of the pilot storage study before (start) and after (end) the storage period of 375 days, 15 September 2011 - 7

September 2012. The temperature during storage followed the outdoor temperature (Jönsson et al., 2014). Samples from top and bottom layer after the storage period were also assayed. Samples of un-stored SS (start) and stored SS (end) were frozen in -20°C for 2 years and 8 months and 1 year and 8 months respectively (Stenberg et al. 1998a).

The purpose of the pilot experiment of Jönsson et al. (2014) was to evaluate the different storage methods regarding emission of greenhouse gases (GHG) during one year. Briefly, the experiment was set up in a randomized complete block design with four treatments and three blocks run for 357 days. The storage tanks consisted of cylindrical high-density polyethylene, 2 m x 1.63 m (hight x diam.) and the cover consisted of plastic tarpaulin and were attached in a way to prevent precipitation from entering the stored sludge. Each tank was filled up to approximately 1.3 m with sludge. The results showed that storage of SS will contribute to the emission of GHG and that the amounts emitted depend on the design of storage. For more details on chemical characteristics on SS and results see Appendix 2, Table 3, and (Jönsson et al., 2014).

The SS from the pilot study applying four different storage methods are described bellow.

1) Mesophilically digested sewage sludge stored without cover, i.e. no cover (M)
2) Mesophilically digested sewage sludge stored with cover (MC)
3) Mesophilically digested sewage sludge treated with ammonia (by incorporation of 1.5% urea by weight) stored with cover (MAC)
4) Thermophilically digested sewage sludge stored with cover (TC)

3.2. Soils

Soil was used as control in all steps of the method development (Step 1 - 6). For this purpose, the 0 - 20 cm top layer of two arable soils were collected.

Clay soil (Soil I) was collected from a field trial at Brunnby experimental farm, Västerås in central Sweden (59° 37'N, 16° 33'E). The soil was slightly dried and then sieved through a 5-mm screen. The soil was then portioned into polyethylene bags and stored at -20°C until use. The soil was previously characterized to have PAO of 4.8 ng NO_2-N g^{-1} dw min^{-1}, which was considered to be of median activity among Swedish soils (Stenberg et al., 1998b). This soil was used as control in experimental Steps 1 - 5, for further details on the soil see Appendix 3.

Clay soil (Soil II) was collected from a field trial north of Uppsala (59° 53'N, 17°32'E). The soil was transported to the laboratory on the day of sampling where it was portioned into polyethylene bags and frozen at -20°C until use. When use the soil was thawed and sieved through a 2 mm screen prior the experiment. The soil is the same soil as that used in a field experiment reported by Jönsson et al. (2014). The soil was used in experimental step 6, for further details on the soil see Appendix 3.

3.3. Analysis of nitrite

Flow Injection Analysis (FIA) is an automatized photometer method conveniently used for analysis of large number of samples (Karlberg, 1989). The NO_2^- in SS and soil samples in Step 2 - 6 were analyzed by spectrophotometric detection using FIAstar 5000 (Foss - Techator AB, Höganäs, Sweden) provided with a 5027 Sampler and the method cassette NO_2^-/ NO_3^- according to application note ASN 5200. In this application NO_2^- concentrations in the range 0.01 - 1 mg NO_2^--N l^{-1} could be analyzed using a 40 µl sample loop and carrier solution of 2 M KCl with the pump speed of 40 rpm. The instrument was continuously re-calibrated every

11th sample using the standard of 0.1 mg NO_2^--N l^{-1}. Carrier solution, extraction solution (4 M KCl), nitrite reagents I (10 g sulfanilamid and 52 ml 37% HCl in 1 l deionized water), and nitrite reagens II (0.5 g N-(1)-naphthylethylenediamine dihydrochloride in 0.5 l deionized water) as well as stock-solution of standard NO_2^--N were all suction filtered through a 0.8 µm Millipore filter (Merck Millipore, Darmstadt, Germany) using a deaeration-equipment before use in order to reduce the content of particles and air bubbles. Carrier solution 2 M KCl was diluted to 4.76% using deionized water to obtain concentration equal to the diluted samples in Step 2 and 3. The NO_2^- in the samples was measured at 540 nm using a FIAStar 5000 instrument equipped with a digital dual-wavelength detector for cancelling measuring errors.

3.4. Step 1: Spectrophotometric scan of sewage sludge

SS and Soil I were spread to cover the bottom of an aluminum form and thoroughly mixed. Samples were taken diagonally from the bottom of the form, in two strikes, to get representative samples. Three different sample amounts, 6.5, 12.5 and 25 g of fresh SS, stored SS and Soil I with three replicates of each weight, respectively, were weighted into 250 ml Duran flasks containing 100 ml deionized water. The flasks were shaken for 1.5 hours at 175 rpm in a room with constant temperature of +25°C after which the rpm was lowered to 80 rpm to allow sampling. From the SS and Soil I slurries of 2 ml sample from each flask were collected and added to 10 ml Falcon test tubes prefilled with 2 ml of 4 M KCl. The tubes were mixed and centrifuged at 4100 rpm using a swing-out rotor (Jouan CR322, France) for 10 min after which the supernatant was filtered through a Munktell No. 4 filter paper (Munktell Filter AB, Falun, Sweden) into a 10 ml test tube. The filtrates were then transferred to cuvettes and absorbance spectra (200-950 nm) scanned using a Lightwave II spectrophotometer (Biochrom Ltd, Cambridge, UK) with 2 M KCl as reference. A sample of NO_2^- mixed with nitrite reagents I and II producing a pink color was scanned to determine the wavelength peak of the NO_2^- color complex. In the evaluation and comparison of absorbance between SS and Soil I the full spectrum was used, with focus on 540 nm, i.e. the wavelength used by FIA in the NO_2^- analysis (Moorcroft et al., 2001).

3.5. Step 2: Nitrite recovery analysis

SS and Soil I were sampled and incubated using the same technique as in Step 1, above. After incubation, samples of 3 ml from slurry flasks were dispensed into 10 ml Falcon test tubes prefilled with 3 ml 4 M KCl, resulting in a total volume of 6 ml which was mixed (Fig. 3). The tubes were then centrifuged and filtered as described in Step 1, above. From each tube filtrates of 2 ml were collected and added to each of two FIA test tubes. To one of the tubes 0.1 ml of 10 mg NO_2-N l^{-1} NO_2-N stock solution, equal to 1000 ng, was added and to the other 0.1 ml of deionized water acting as control. All extracts were analyzed for NO_2^- by FIAstar 5000. By subtracting the amount NO_2^- in control (without NO_2-N addition) from that measured NO_2^- the recovery was calculated and expressed as difference in amounts (Δ NO_2–N) and as difference in calculated amounts in relation to added amount (Recovery %).

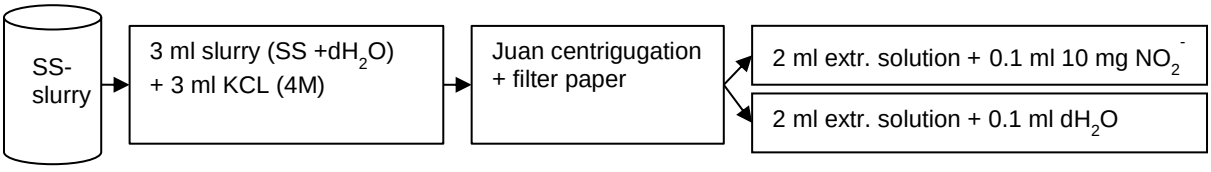

Figure 3. Experimental design in Step 2 for NO_2^- recovery analysis of sewages sludge.

3.6. Step 3: Effects of extraction and centrifugation refinements on nitrite recovery

Five replicates each of 25 g stored SS and Soil I was sampled using the same technique as in Step 1, above, and weighted into 250 ml Duran flasks. To each flask 100 ml of deionized water were added and then placed on a shaking table at 175 rpm for 1.5 h in a room with constant temperature of +25°C. At sampling the shaker rate was lowered to 80 rpm and 2 ml slurry extracted. The following three (A-C) experimental treatments were applied (Fig. 4):

- Treatment A. The 2-ml slurry samples were added to 2 ml 4 M KCl in 10 ml Falcon test tubes. The tubes were shaken and centrifuged at 4100 rpm for 10 min in a centrifuge with swing-out rotor (Jouan CR322) after which the supernatant was filtered through Munktell No. 4 filter paper. Two samples of each 1.5 ml were transferred to 10 ml FIA test tubes. To one tube 0.075 ml stock solution of 10 mg NO_2-N l^{-1} was transferred, equal to 750 ng NO_2-N, and to the other tube 0.075 ml deionized water was added.
- Treatment B. The 2-ml slurry samples were added to 2 ml 4 M KCl in 10 ml Falcon test tubes. The samples were shaken after which 2 x 2 ml were dispended into Eppendorf test tubes and centrifuged at 15000 rpm for 10 min in Eppendorf centrifuge (Microcentrifuge 5424 R, Eppendorf AG, Hamburg, Germany). From the supernatant two samples of each 1.5 ml were transferred to 10 ml FIA test tubes. To one tube 0.075 ml stock solution of 10 mg NO_2-N l^{-1}, equal to 750 ng NO_2-N, and to the other tube 0.075 ml deionized water was added.
- Treatment C. The 2-ml slurry samples were centrifuged in Eppendorf centrifuge (Microcentrifuge 5424 R) at 15 000 rpm for 10 min. One ml of the supernatant was transferred to each of two test tubes, after which 1 ml 4 M KCl was added to each tube. To one tube 0.1 ml stock solution of 10 mg NO_2-N l^{-1}, equal with 1000ng NO_2-N was added and to the other tube 0.1 ml deionized water was added.

All samples from the three treatments were analysed for NO_2^- by FIAstar 5000. NO_2 -N values of Soil I being below detection limit was adjusted to 0 mg NO_2-N l^{-1}. Recovery was calculated as in Step 2, above.

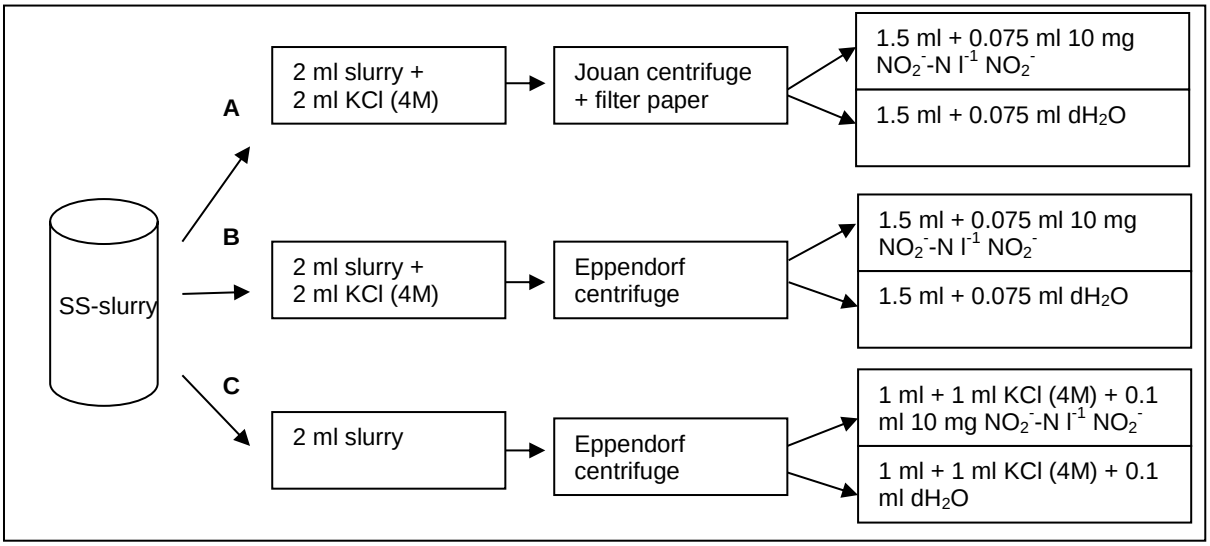

Figure 4. Schematic figure over the three experimental treatments (A - C) in Step 3.

3.7. Step 4: AOB activity tests of sewage sludge and optimization of sample size

Sewage sludge and soil were sampled using same technique as in Step 1, above. Three replicates each of 6.25, 12.5 and 25 g thawed (frozen at -20°) stored SS, 25 g refrigerated (stored at +2°C) stored SS and 25g thawed Soil I (frozen at -20°), were weighted into 250 ml Duran flasks. To each flask 100 ml PAO substrate tempered to +25°C was added. The substrate contained potassium phosphate buffer (100 mM, pH 7.24), ammonium sulphate (0.4 mM) and sodium chlorate (15 mM). Slurry samples of 2 ml were taken after 2 h and then at every hour to receive a total of 5 samples. The slurry samples were added to 10 ml Falcon test tubes prefilled with 2 ml 4 M KCl. After shaking the tubes, 2 + 2 ml were dispensed into 2 separate Eppendorf test tubes which were centrifuged at 15000 rpm for 10 min in Eppendorf centrifuge (Microcentrifuge 5424 R). The supernatant were transferred to 10 ml FIA test tubes and analysed for NO_2^- by FIAstar 5000 and the ammonium oxidation rates PAO (ng NO_2^- g dw^{-1} h^{-1}) were calculated as mean values from linear regression of NO_2^- formation over time from each flask.

3.8. Step 5: Application of PAO assay on sewage sludge

Three replicate each of 12.5 g thawed and room tempered samples of mixed (start and end) and top and bottom (end) SS from the different treatments of the pilot storage study (M, MC, TC and MAC) and 25 g thawed Soil I were weighted into 250 ml Duran flasks. At start of the assay 100 ml PAO substrate tempered to +25°C was added. The flasks were placed on a shaking table at 175 rpm in a room with constant temperature of +25°C and slurry samples of 2 ml were taken after 2 h and then at every hour to receive a total of 5 samples. The 2-ml slurry samples were added to 2 ml 4 M KCl in 10 ml Falcon test tubes. The samples 2 + 2 ml were shaken, dispended into 2 separate Eppendorf test tubes and centrifuged at 15 000 rpm in 10 min in Eppendorf centrifuge (Microcentrifuge 5424 R). From the supernatant samples were transferred to 10 ml FIA test tubes using an automate pipette. NO_2^- was analyzed with FIAstar 5000 and the ammonium oxidation rates PAO (ng NO_2 -N g^{-1} dw h^{-1}) were calculated as mean values from linear regression of NO_2^- formation over time from the triplicate.

The samples were after the assay added to cuvettes and absorbance spectra 200-950 nm of each sample were scanned using a Lightwave II spectrophotometer (Biochrom Ltd, Cambridge, UK) with 2 M KCl as reference. PAO assay of five extra replicates from mixed (end) SS from storage treatment MC of the pilot study was additionally performed to determine repeatability and consistence of the PAO assay.

3.9. Step 6: PAO assay of soil with applied sewage sludge

Portions of 25 g thawed and sieved Soil II were weighted into 250 ml Duran flasks. Then, three replicates each of thawed and mixed (end) SS from each of the pilot study storage treatments MC (0.556 g), M (0.591 g), TC (0.434 g) and MAC (0.482 g) were added to the flasks and mixed with the soil corresponding to 13 700 kg fresh weight ha^{-1}. Three flasks with soil but without sludge application were used as control. All flasks were then pre-incubated at +25°C for 12 h prior to assay of PAO. At start of the assay 100 ml PAO substrate was added and PAO assayed as described in Step 4 and ISO15685:2012. NO_2^- was analyzed with FIAstar 5000 and the ammonium-oxidation rates (PAO; ng NO_2 -N g^{-1} dw h^{-1}) was calculated as mean values of the triplicates from linear regression of NO_2^- formation over time from each flask.

3.10. Data treatment and statistical analysis

The data were statistically analysed in Excel using the add-in software XLSTAT (ver. 2015.1.03.15485, AddinSoft). One-way ANOVA followed by the Tukey (HSD) multiple

comparison test was used for repeated tests of paired differences between treatments and weights regarding absorbance, nitrite content, NO_2^- recovery and PAO. Student t-test was used in comparing absorbance, NO_2^- recovery and PAO-rates in the method development steps. Differences between the treatment were deemed statistically significant at $p<0.05$.

4. Results

4.1. PAO assay development (Step 1 - 4)

4.1.1. Step 1

Result

In the spectrophotometric scanning of SS and Soil I sample extracts, high amplitude of noise was displayed at 200 - 225 nm after which a minimum was reached. After this the absorbance increased until around 290 nm where a maximum was reached (Fig. 5). After this peaking, the absorbance gradually leveled off displaying minimum absorbance at 900 nm. The absorbance increased with increasing amounts of SS or soil (Table 1). At 540 nm the absorbance values for refrigerated fresh SS (FMS), stored SS (SMS) and frozen Soil I, all differed significantly from each other ($p<0.05$). The weight 25 g gave significantly higher absorbance than both 6.25 and 12.5 g for SS and Soil I ($p<0.05$). The absorbance of stored SS at 12.5 g did not significantly differ from the reference, i.e. Soil I at 25 g (Student, T-test, $p>0.05$). The absorbance was higher for fresh and stored SS than Soil I at equivalent weight. The extracts from fresh SS were visibly more colored than stored.

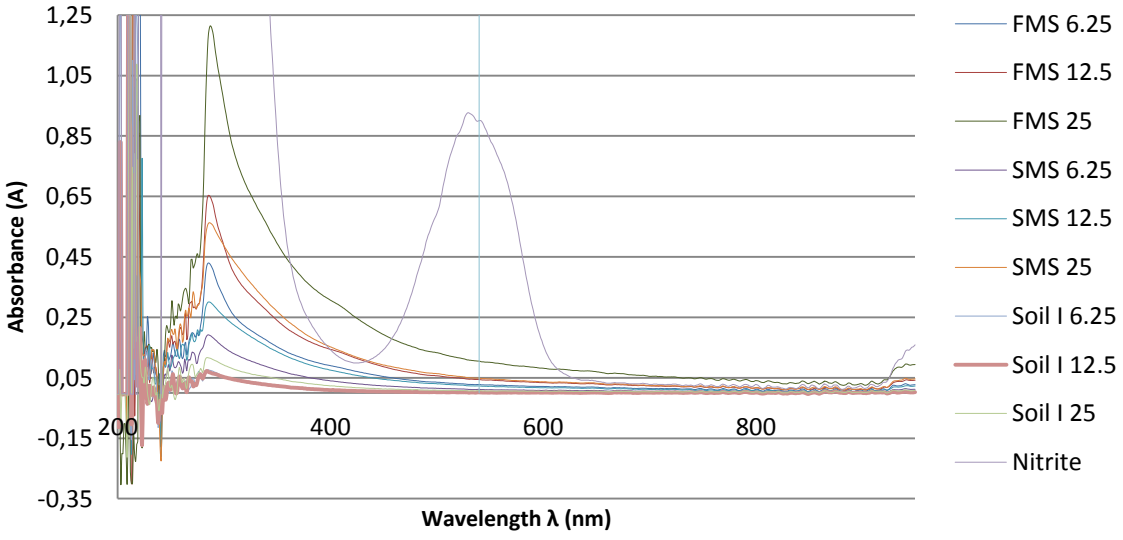

Figure 5. Absorbance spectrum of fresh (FMS) and stored (SMS) sewage sludge extracts and soil extract (Soil I) using different sample sizes (mean, n=3). The azo-dye complex, formed in spectrophotometric detection of NO_2^- (Nitrite) was also scanned. Vertical line indicates 540 nm, i.e. the wavelength used for spectrophotometric analysis of NO_2^-.

Table 1. Absorbance at 540 nm of fresh (FMS) and stored (SMS) sewage sludge extracts, and soil extract (Soil I) (mean ± standard deviation, s.d.; n=3)

Sample	Absorbance		
	6.25 g	12.5 g	25 g
FMS	0.028 ±0.0049^{Ac}	0.044 ±0.0020^{Ac}	0.105 ±0.0160^{Bc}
SMS	0.012 ±0.0020^{Ad}	0.023 ±0.0026^{Ad}*	0.049 ±0.0053^{Bd}
Soil I	0.001 ±0.0007^{Af}†	0.002 ±0.0015^{Af}	0.007 ±0.0084^{Bf}

†n=2
Same capital letter in same row indicate no significant difference ($p<0.05$; ANOVA, Tukey's HSD)
Same lower-case letter in same column indicate no significant difference ($p<0.05$; ANOVA, Tukey's HSD)
*No significant difference from 25 g reference Soil I ($p<0.05$); Student t-test)

Conclusions
During incubation under standard PAO conditions:

- Fresh dewatered mesophilically digested SS (FMS) has stronger color than the same SS stored (SMS).
- Absorbance of 12.5 g FMS and SMS at 540 nm did not differ from that of 25 g soil (Soil I).
- Extracts of SS has relatively high turbidity and coloration, implying that the centrifugation step has to be further improved to eliminate this problem.

4.1.2. Step 2

Result
The NO_2^--N content increased proportionally with increasing amount of SS, however, the amounts 6.25 and 12.5 g of Soil I had NO_2^--N content below the detection limit 0.01 mg NO_2^--N l^{-1} and, hence, did not display the same correlation (Table 2).

The recovery of NO_2^--N, i.e. the difference between background NO_2^--N and that analyzed after adding 1000 ng NO_2^--N tended to increase, though not statistically proved ($p>0.05$), with increasing sample amount of both SS and Soil I (Table 2). The lowest recovery was achieved from 6.25 g FMS (76.51%) and the highest from 25 g Soil I (99.47%) (Fig 6). The recovery of NO_2^- were significantly higher for the control Soil I than for FMS ($p<0.05$) while no statistical difference was seen between the different amounts of FMS and SMS or Soil I ($p>0.05$).

SMS had a dry weight of 48% and FMS 30%. SMS had significantly higher content of NO_2^- per unit dry weight compared with fresh FMS and frozen Soil I (Student,T-test, $p<0.05$) (not shown in table). The NO_2^--N content of FMS was 560 ±70.4 ng NO_2^--N g^{-1} dw (n=9), around 14 times lower than that of stored SS (7970 ±1190 ng NO_2^--N g^{-1} dw; n=9). Soil I had a NO_2^--N content of 25 ±34 ng NO_2^--N g^{-1} dw (n=9).

Table 2. Nitrite in extracts of fresh (FMS) and stored (SMS) sewage sludge, and soil extract (Soil I), before and after addition of 1000 ng NO_2^--N and the calculated difference (After – Before = Δ NO_2-N; mean ± s.d; n=3)

Sample		Nitrite (ng NO_2-N)		
		6.25 g	12.5 g	25 g
FMS	Before	11.9 ±2.4	22.4 ±1.2	42.7 ±3.2
	After	777.0 ±176.9	885.5 ±34.5	914.9 ±44.1
	Δ NO_2-N	765.1 ±175.6	863.1 ±33.3	872.2 ±41.0
SMS	Before	207.9 ±3.6	521.5 ±57.6	1109.9 ±48.0[†]
	After	1027.6 ±190.0	1348.9 ±305.2	1972.6 ±119.8
	Δ NO_2-N	819.7 ±186.5	827.4 ±269.5	914.8 ±48.0
Soil I	Before	0.0 ±0.0[1]	0.7 ±1.2[1]	14.0 ±1.21
	After	961.1 ±47.2	973.7 ±29.8	1008.7 ±34.2
	Δ NO_2-N	961.1 ±47.2	973.0 ±30.7	994.7 ±33.5

[†]n=2

[1]NO_2^--N resulting in negative values has been corrected to 0 ng NO_2^--N

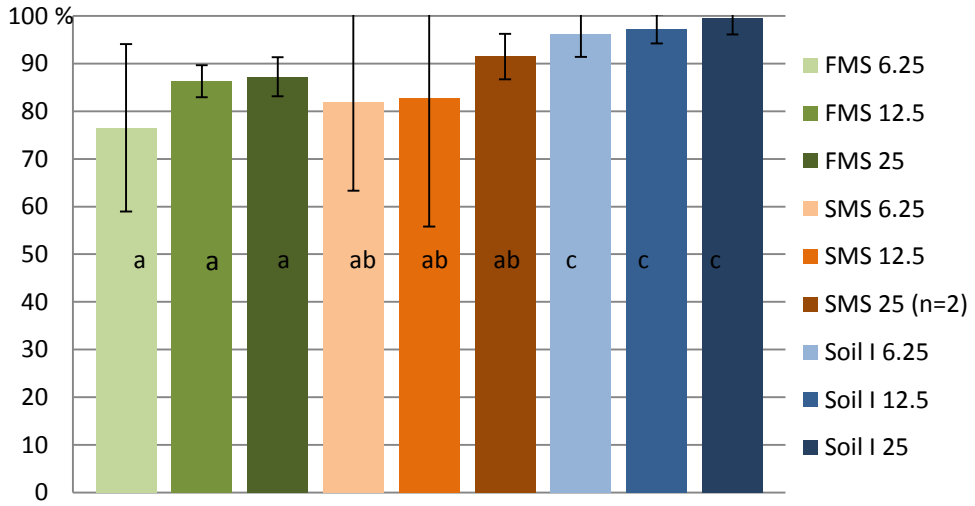

Figure 6. Nitrite recovery analysis of fresh (FMS) and stored (SMS) sewage sludge extracts, and soil extract (Soil I) of different amounts sample amounts: 6.25, 12.5 and 25 g. Error bars represent mean ± s.d.; n =3 (SMS 25, n=2). Different letters at the bars indicate statistical difference ($p<0.05$; ANOVA, Tukey's HSD).

Conclusions

During assay under standard PAO conditions:

- Stored mesophillically digested sewage sludge (SMS) displayed higher NO_2^- content than fresh sewages sludge (FMS) of equivalent type and arable soil.
- The accuracy of spectrophotometeric detection of NO_2^- was higher for SMS than for FMS.
- The centrifugation step of SS extracts must be improved further to achieve more reliable NO_2^- analysis.

4.1.3. Step 3

Result

The SMS extracts B and C, centrifuged in Microcentrifuge for 10 min gave a lower visible supernatant coloration than when centrifuged with Jouan CR322 provided with swing-out rotor. The precipitate of SS-particles in the bottom of the Eppendorf-tube was well pelleted and the supernatant could easily be pipetted without contaminants of any visible particles. The three centrifugation methods (treatments A-C; Fig. 4) seemed to influence the detection of NO_2^- as well as the recovery of added NO_2^- in SMS and Soil I (Table 3 and Fig. 7). The NO_2^- content analyzed in stored SMS was significantly higher than that of Soil I. However, the NO_2^- content of SMS exceeded the upper standard and was calculated by linear regressing line, whereas soil had NO_2^- content under the detection limit. SMS (dry weight 48%) had a NO_2^--N content of 30 700 ±750 ng NO_2 -N g^{-1} dw (n=12) which was approximately 4 times higher than that observed for SMS in Step 2, above (results not shown in table).

Treatment C resulted in higher NO_2^- content for SMS and Soil I than treatment A and B ($p<0.05$) (Table 4). The recovery of NO_2^- based on the difference between the background NO_2^- content and added NO_2^- was higher for the Soil I than for SMS (Fig. 7). Treatment A resulted in significantly higher NO_2^- recovery than treatment C ($p<0.05$). However, treatment B tended to yield higher NO_2^- recovery than treatment A and C for both SMS and Soil I.

Table 3. Nitrite detection in three different sample treatments (A-C) of extracts from stored (SMS) sewage sludge and soil (Soil I) before and after addition of known amount NO_2^--N (Added NO_2-N) and calculated difference (After – Before = Δ NO_2^-) (mean ± s.d; n = 5)

Type	Sample	Nitrite (ng NO_2^--N)		
		Treatment A	Treatment B	Treatment C
SMS[1]	Before	3021.6 ±52.3 ^{Aa†}	3131.4 ±27.5 ^{Aa}	4272.2 ±20.7 ^{Ab}
	Added NO_2 -N	750.0	750.0	1000.0
	After	3608.3 ±149.2	3793.5 ±117.5	4932.4 ±96.1
	Δ NO_2^-	586.7 ±96.9	662.1 ±110.6	660.2 ±95.0
Soil I[2]	Before	0.0 ±0.0 ^{Ba}	0.0 ±0.0 ^{Ba}	0.0 ±0.0 ^{Bb}
	Added NO_2 -N	750.0	750.0	1000.0
	After	700.6 ±34.0	718.8 ±50.9	896.7 ±108.3
	Δ NO_2^-	700.6 ±34.0	718.8 ±50.9	896.7 ±108.3

†n=2

[1]NO_2^- values exceeded the detection limit.

[2]NO_2^- values under the detection limit.

Same capital letter in same row indicate no significant difference ($p<0.05$; ANOVA, Tukey's HSD)

Same lower-case letter in same column indicate no significant difference ($p<0.05$; ANOVA, Tukey's HSD)

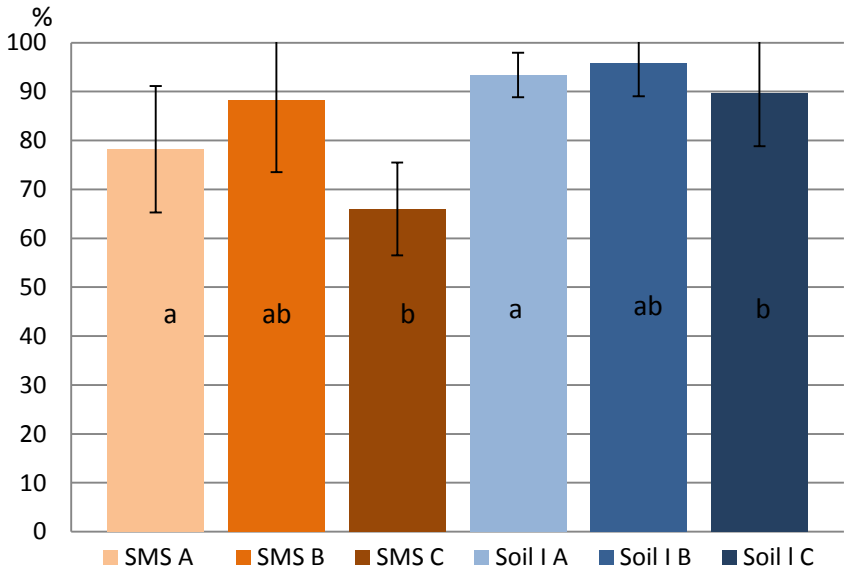

Figure 7. Nitrite recovery analysis of stored (SMS) sewage sludge extracts and soil extract (Soil I) using three centrifugation methods (A, B and C). Bars represent mean ± s.d.; n = 5 (SMS A, n=2). Different letters at the bars indicate statistical difference ($p<0.05$; ANOVA, Tukey's HSD).

Conclusions

During incubation under standard PAO conditions:

- The recovery of added known amounts of NO_2^- two extracts was lower in stored mesophillically digested sewages sludge (SMS) than in soil (Soil I) in all three treatment methods tested.
- Treating the SS extract by centrifugation in micro centrifuge at 15 000 rpm for 10 min (Treatment B) indicated the most reliable NO_2^- recovery for SS.

4.1.4. Step 4

Result

In SMS stored frozen at -20°C until analysis the NO_2^- accumulated linearly over time in the PAO assay in all three sample amounts tested, 6.25, 12.5 and 25 g (r^2 >0.9) (Table 4, Fig. 8). After 2 h incubation, i.e. at the first slurry sampling in the PAO assay, the NO_2^- concentration in the reference (25 g Soil I) was 577.1 ±21.6 ng NO_2^--N g^{-1} dw, which was significantly ($p<0.05$) lower than those of 6.25, 12.5 and 25 g SMS (10 040 ±1 839, 6 720 ±1 328 and 8 403 ±2 770 ng NO_2^--N g^{-1} dw), respectively (cf. Fig. 6). The PAO rate of SMS, irrespective of sample amount, was significantly higher than that of 25 g reference soil (Soil I; $p<0.05$). The PAO rate of 6.25 g SMS was 1.7 and 2 times higher than those in 12.5 and 25 g, respectively ($p<0.05$) (Fig. 9). SMS, refrigerated at +2°C during four weeks, did not show any NO_2^- accumulation over time (results not shown).

Table 4. Potential ammonium oxidation (PAO) rates at three different sample amounts of stored sewage sludge (SMS) and soil (Soil I) in the assay (mean ± s.d; n=3)

Sample	Dry weight (%)	PAO-rate (ng NO_2 -N g^{-1} dw h^{-1})		
		6.25 g	12.5 g	25 g
SMS	45.8	1220.6 ±105.8^{Aa}	719.8 ±46.2^{Ab}	610.9 ±164.3^{Ab}
Soil I	77.2	-	-	241.9 ±6.3^{Bb}

- No value for Soil I
Same capital letter in same row indicates no significant difference ($p<0.05$; ANOVA, Tukey's HSD)
Same lower-case letter in same column indicates no significant difference ($p<0.05$; ANOVA, Tukey's HSD)

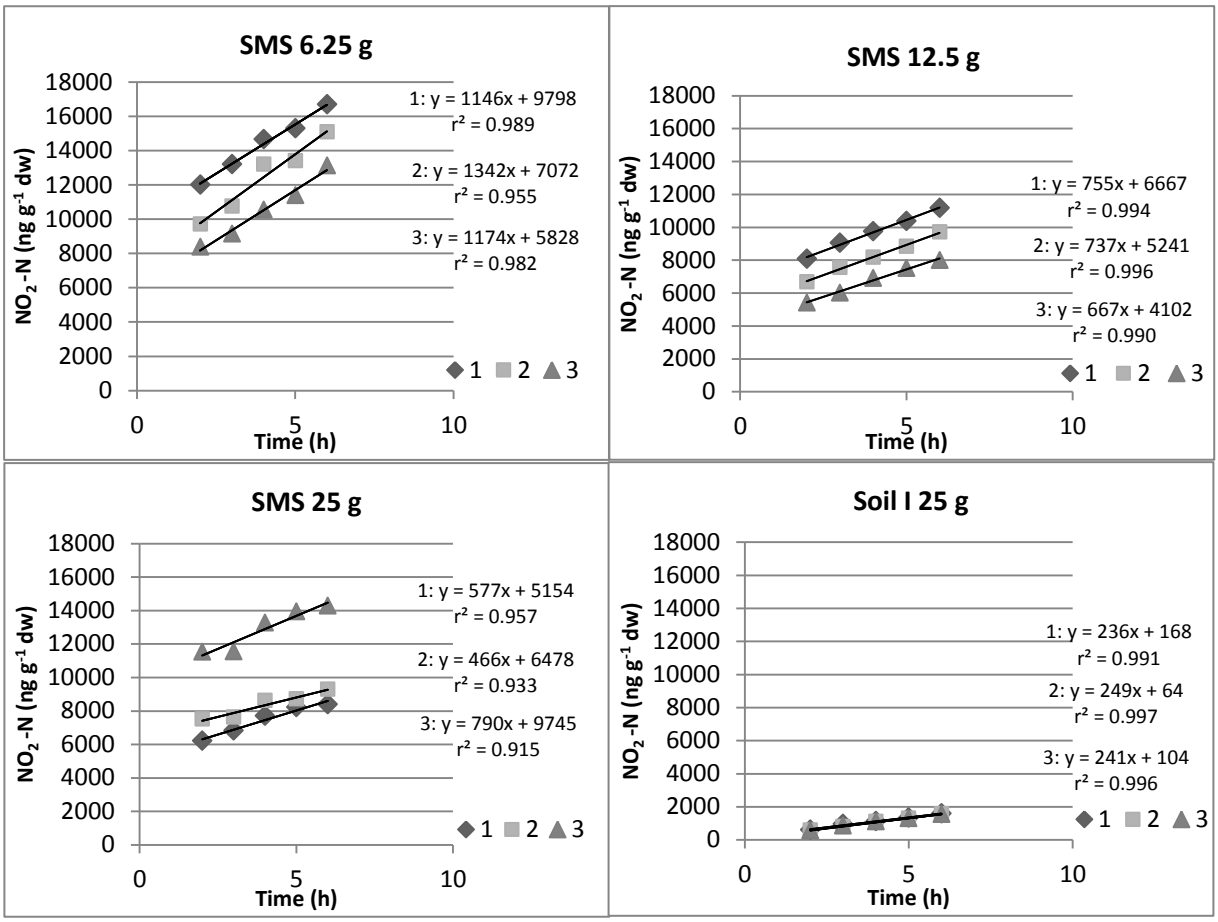

Figure 8. Accumulation of NO_2^- over time in PAO assay of three different sample amounts (6.25, 12.5 and 25 g) of stored sewage sludge (SMS) and 25 g soil (Soil I). Equations represents linear regression equations (y = kx + m) of three replicates (1-3) where the slope (k) is the PAO rate in ng NO_2 -N g^{-1} dw h^{-1}.

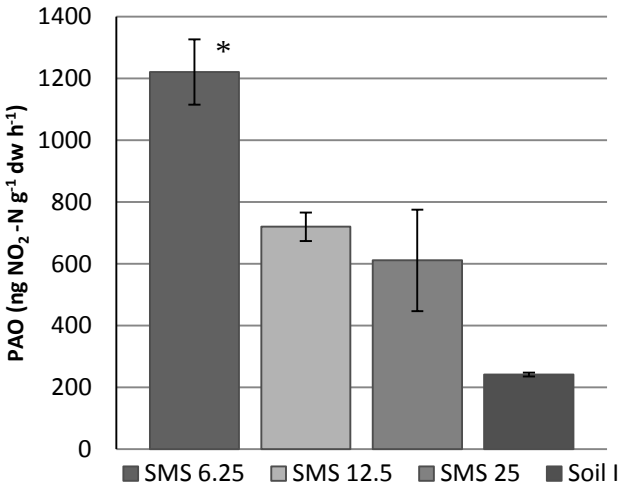

Figure 9. Potential ammonium oxidation rate (PAO) in three different amounts of stored (SMS) sewages sludge and 25 g soil (Soil I). Bars represent mean ± s.d; n=3, and asterisk (*) represents significant different value ($p < 0.05$, ANOVA, Tukey's HSD).

Conclusions

Stored mesophillically digested sewage sludge (SMS) display NO_2^- accumulation over time, i.e. PAO activity, and it is possible to measure its rates with the new method developed.

- The specific PAO rates in SMS tended to decrease with increasing sample amount in the assay.

4.2. PAO rates in fresh and stored SS (Step 5)

4.2.1. Mixed samples

The visual impression of the SS texture differed between the storage treatment applied in the pilot study where mesophilically digested SS (M, MC and MAC) appeared more compact whereas thermophilically digested SS (TC) had a more porous and cloggy texture before and after storage period.

The NO_2^- concentration in SS subjected to different sanitation treatments (M, MC, MAC and TC) were before storage lower than the detection limit of the FIA (0.01 mg NO_2 -N l^{-1}) which resulted in fluctuating NO_2^- concentration detected over time in the PAO assay. SS of treatment M, MC and TC, before storage, displayed only tendencies of PAO activity while that in MAC was significantly higher ($p<0.05$) (Table 5, Fig. 10). Non-stored sludge of all treatments had significantly lower PAO rate than the control Soil I ($p<0.05$).

The PAO rate in SS of M, MC, MAC and TC stored for one year was generally higher than that in corresponding non-stored SS. Stored MC tended to have higher PAO rates than that of Soil I, but TC was the only SS displaying significant higher value ($p<0.05$). Moreover, TC was the only SS displaying higher PAO rate than corresponding non-stored SS, and the rate was higher than in any other of the stored SS ($p<0.05$). In contrast, MAC did not show any large difference in PAO rate before and after storage.

Table 5. Potential ammonium oxidation (PAO) rate of different types of sewage sludge subjected to different sanitation treatments before and after storage for one year, and soil as reference (mean ± s.d; n=3)

Treatment	Sampling point	Dry weight (%)	PAO rate (ng NO_2-N g^{-1} dw h^{-1})	r^2-value
M	Start	28.8	28.4 ±97.7	0.426 ±0.281
	End	20.1	119.1 ±88.3	0.768 ±0.165
MC	Start	28.8	28.5 ±97.7	0.426 ±0.281
	End	22.0	351.5 ±31.7	0.986 ±0.009
MAC	Start	27.6	127.6 ±21.6	0.966 ±0.035
	End	25.4	142.2 ±32.2	0.946 ±0.011
TC	Start	27.7	15.3 ±6.3	0.302 ±0.188
	End	28.2	2420.2 ±1657.4	0.975 ±0.026
Soil I	-	76.3	251.4 ±3.4	0.999 ±0.001

M; Mesophilically digested sewage sludge stored without a cover, i.e. no cover
MC; Mesophilically digested sewage sludge stored with a cover
MAC; Ammonia treated mesophilically digested sewage sludge (addition of 1.5% urea by weight) stored with a cover
TC; Thermophilically digested sewage sludge stored with a cover
-Not relevant.

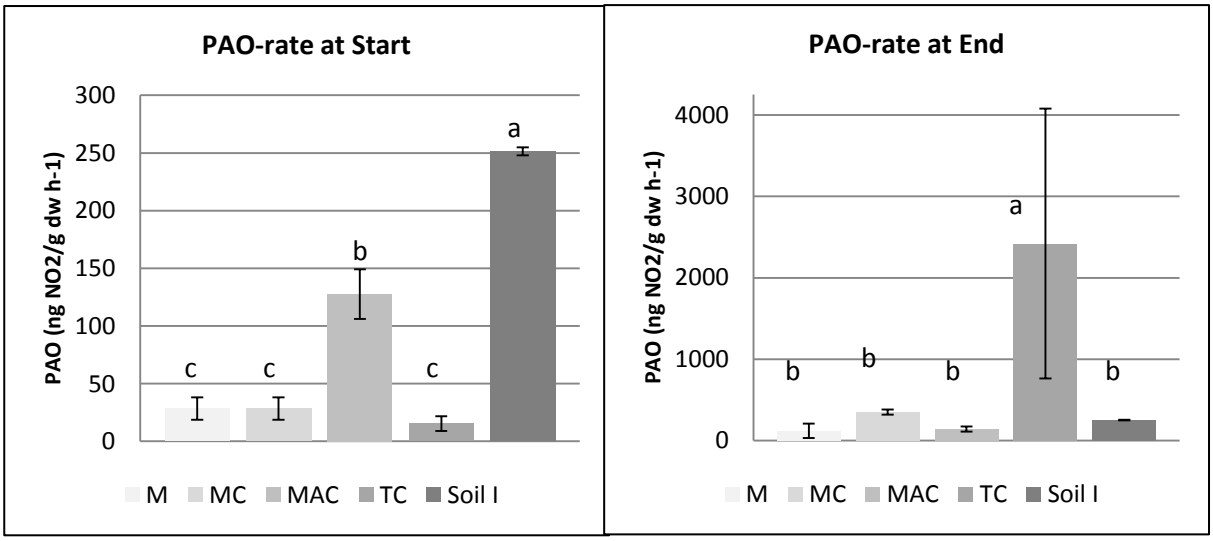

Figure 10. Potential ammonium oxidation (PAO) rate, before and after storage for one year of, of sewage sludge subjected to different sanitation treatments. Bars represent mean ± s.d; n=3. Different letters above bars indicate statistical difference ($p<0.05$, ANOVA, Tukey's HSD). For treatment abbreviations see Table 7. Note the difference in scaling of y axes of start (left) and end (right) panel.

4.2.2. Top and bottom layer

The PAO rate in all SS subjected to the different sanitation treatments after one year of storage was generally higher in the top-layer of the storage cylinders compared with the bottom-layer. The PAO rate ranged between 141 and 2390 ng NO_2^--N g^{-1} dw h^{-1} in the top-layer and 48.0 and 244 ng NO_2^--N g^{-1} dw h^{-1} in the bottom-layer (Table 6). The differences in PAO rates between the top and bottom for MAC were small, whereas large differences were observed in treatment M, MC and TC. Significant differences in PAO rates between top and bottom were shown for stored MC and TC ($p<0.05$), whereas differences were not significant for M and MAC.

The PAO rate in the top-layer indicated differences between sanitation treatments, where MAC stood out with 8 to 17 times lower PAO rate than those in the other treatments. MC and TC displayed significantly higher PAO rates than MAC and Soil I ($p<0.05$), whereas M did not differ from the other treatments. In the bottom-layer the treatments did not display any significant differences of PAO rates ($p>0.05$), however there were an indication of higher PAO rates in TC and MAC compared with M and MC (Table 8).

Table 6. Potential ammonium oxidation (PAO) rate of sewage sludge at top and bottom layer of storage units with different types of sludge and sanitation after one year of storage, and soil as reference (mean ± s.d; n=3). For treatment abbreviations see Table 5.

Treatment	Sampling position	Dry weight (%)	PAO rate (ng NO_2-N g^{-1} dw h^{-1})	r^2-value
M	Top	17.6	1173.2 ±200.5^{ab}	0.995 ±0.005
	Bottom	26.6	48.0 ±36.1^{b}	0.605 ±0.519
MC	Top	20.2	1817.9 ±1140^{a}	0.992 ±0.002
	Bottom	26.7	78.5 ±13.2^{b}	0.966 ±0.038
MAC	Top	20.1	141.2 ±29.3^{b}	0.998 ±0.015
	Bottom	26.8	119.5 ±20.1^{b}	0.921 ±0.038
TC	Top	33.1	2390.7 ±792.2^{a}	0.995 ±0.002
	Bottom	27.4	243.7 ±253.5^{b}	0.875 ±0.082
Soil I	-	76.5	175.1 ±6.3^{b}	0.940 ±0.019

Same lower-case letter in same column indicates no significant difference ($p<0.05$; ANOVA, Tukey's HSD)
-Not relevant

4.2.3. Absorbance

The spectrophotometric absorbance values at 540 nm differed generally between the slurry extracts (without added NO_2^- reagents) collected from PAO assay of SS treated differently (Fig. 11). The extracts of non-stored (start) and stored (end) MAC were visible more colored than the other treatments and had high absorbance values that differed significantly from the other treatments and the Soil I ($p<0.05$). The absorbance values of TC, before (start) and after storage (end), did not differ from 25 g Soil I neither from 12.5 g fresh (FMS) or stored (SMS) SS used in development Step 1 ($p>0.05$) (Fig. 11).

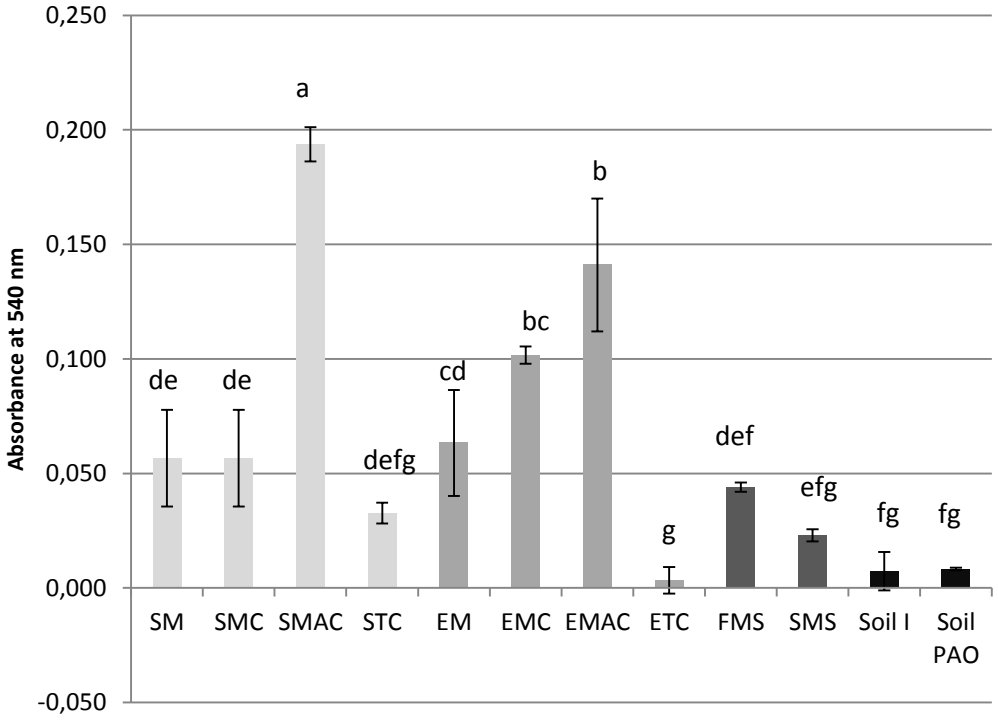

Figure 11. The absorbance of PAO extracts at 540 nm for sewage sludge (M, MC, MAC and TC) at start (S) and end (E) of pilot storage period (blue bars) and for fresh (FMS) and stored (SMS) sewage sludge (red bars). Extracts from PAO of soils used in in development Step 1 (Soil I) and Step 5 (Soil PAO) are displayed as references (black bars). Bars represent mean ± s.d; n=3. Different letters above bars indicate statistical differences ($p<0.05$) ANOVA, Tukey's HSD. For treatment abbreviations M, MC, MAC and TC see Table 5.

4.2.4. Repeated PAO assay

The repeated PAO assays of stored mesophillic SS (MC) and Soil I resulted in PAO rates of 254 ±76 (n=5) and 214 ±4 (n=3), ng NO_2^--N g^{-1} dw h^{-1} respectively, which were somewhat lower values than those previously reported in Step 5 (352 ±32 (n=3) and 251 ±3 (n=3) ng NO_2^--N g^{-1} dw h^{-1}, respectively (Table 5, Fig. 12). However, the values from the two different times of analysis did not significantly differ ($p>0.05$).

Overall some temporal variation seemed to occur, as indicated by the repeated measurements of Soil I in the different development steps. PAO rates of Soil I 242 ±6.3 (Step 4), 251 ±3 (Step 5), 235 ±16.5 (Step 5) ng NO_2-N g^{-1} dw h^{-1}, respectively were not significantly different (Student T-test, $p>0.05$) . However, PAO rate 214 ±4 (Step 5) were significant different from 242 ±6.3 (Step 4) and 251 ±3 (Step 5) (Student T-test, $p<0.05$).

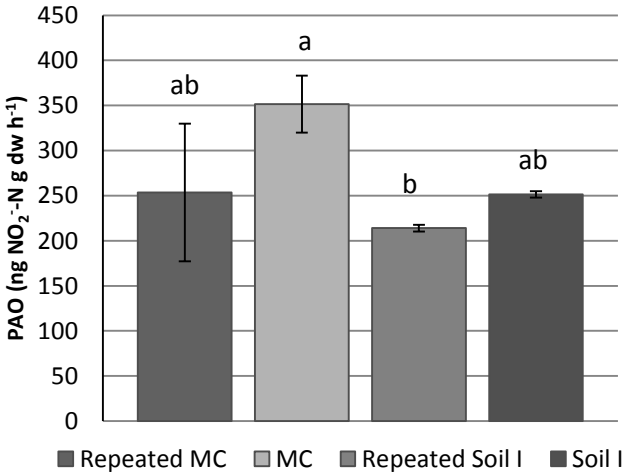

Figure 12. Potential ammonium oxidation (PAO) rates of stored mesophilic sludge (MC; n=3) and soil (Soil I) (n=3) from Step 5 and MC (n=5) and Soil I (n=3) in Step 5 (repeated analyses). Bars represent mean ± s.d., and bars with different letters are statistical different ($p<0.05$) ANOVA, Tukey's HSD.

Conclusions

- Stored and fresh SS display PAO activity, however with considerably higher rates in the former.
- Stored thermophillically digested sewages sludge (TC) displayed significantly higher PAO rates than the other treatments.
- Ammonia treatment of SS (MAC) displayed higher NO_2^- concentration at first sampling time of fresh SS in the PAO assay and consequently higher PAO rates than fresh non-ammonia treated SS.
- At the end of the storage period the top-layer of all the storage cylinders for SS displayed higher PAO rates than the bottom layer.
- The new PAO assay for sewage sludge was repeatable yielding consistent result with only low variation.

4.3. PAO assay of sewage sludge applied to soil (Step 6)

The PAO rate of Soil II without SS addition was 798 ±270 ng NO_2^--N g^{-1} dw h^{-1} (n=3; Table 7), which was around three times higher than that in Soil I. Though the addition of MAC, M and MC tended to lower the PAO activity in Soil II, none of the added SS significantly affected the intrinsic soil PAO rate ($p>0.05$) (Table 7).

Table 7. Potential ammonium oxidation (PAO) rate in soil before (Soil II) and after addition of mesophilically and thermophilically digested sewage sludge subjected to different sanitation methods (mean ± s.d; n=3). For treatment abbreviations see Table 5.

Treatment	Dry weight (%)	Added SS (g, wet weight)	PAO-rate (ng NO_2-N g^{-1} dw h^{-1}	r^2-value
M	22.0	0.591	734 ±130.8[a]	0.960 ±0.021
MC	20.1	0.556	767 ±123.0[a]	0.975 ±0.019
MAC	25.4	0.482	708 ±95.0[a]	0.974 ±0.013
TC	28.2	0.434	796 ±328.9[a]	0.971 ±0.021
Soil II	77.9	-	798 ±269.5[a]	0.994 ±0.002

Same lower-case letter in same column indicates no significant difference ($p<0.05$; ANOVA, Tukey's HSD)

Conclusions

- Application of SS to Soil II of different sanitations treatments (M, MC, TC and MAC) did not change the PAO activity in the soil; however some inhibitory effect was indicated with strongest effect displayed by MAC.

5. Discussion

5.1. PAO assay development (Step 1 - 4)

The basic assumption of the PAO assay is that it estimates the total amount of ammonia oxidizing enzymes in a system. This is achieved by creating optimal environment for the enzymes in the assay vessel and then determine the rate of NO_2^- formation. Optimization means creating the environment yielding the highest specific enzyme activity at a given temperature. Thus in the optimization process the individual test parameters, evaluated one at a time, yielding the highest and most accurate result should be chosen and included in the PAO protocol. In developing the PAO assay for SS, treatment of the slurry samples collected in the time series of the PAO assay is critical. This emphasizes the importance of choosing the correct amount of SS for the assay as well as the best extraction and centrifugation methods of the slurry samples collected during the assay, i.e. choosing methods yielding the clearest sample extract for reliable spectrophotometric analysis of NO_2^- by FIA.

To be able to detect NO_2^- accurately in SS extracts by spectrophotometric methods such as FIA the first step in the development of the PAO assay (development Step 1, PAO assay section 1 and 6 in Fig. 2) involved trying to reduce interference of color and particles. Background absorbance of SS extract similar to that of 25 g control Soil I was desirable as this previously has been shown to produce extracts clear enough (ISO, 2012). The spectrometric scan of SS extracts in development Step 1 showed low absorbance in the critical wavelength for the FIA NO_2^- analysis using 12.5 g stored SS. The absorbance did not significantly differ from that of 25 g control Soil I ($p>0.05$). Thus, 12.5 g SS should not interfere with the spectrophotometric analysis of NO_2, though some coloration and particles of the SS extracts were still visible.

Determination of NO_2^-, using spectrophotometric methods after adding Griess reagent in an acidic environment, is based on the diazotization reaction yielding a pink color that can be measured colorimetrically at 540 nm (Moorcroft et al., 2001; Ahmed et al., 1996; Ivanov, 2004; Karlberg, 1989). Colored samples with absorbance spectrum within the photometric range 500-600 nm could therefore disturb the detection of NO_2^- (Karlberg, 1989). Hence, high coloration of fresh SS could cause problems of the spectrophotometer analysis. Absorbance of the SS extracts before addition of Griess reagent was approximately 20 times higher than extracts of soil at corresponding sample amount (Step 1, Table 1). Furthermore, the NO_2^- content in fresh SS was 14 times lower than that in stored SS (Step 2). High background of NO_2^- in stored SS, resulting in a stronger coloration of the Griess reagent, could possibly override the absorbance from intrinsic SS coloration, while measurements of the low NO_2^- content in fresh sludge could be problematic in the PAO assay.

For reliable determinations of NO_2^- concentrations in SS, further improvement of the extraction method was required (PAO assay, section 1, 5 and 6, Fig. 2). This was done in a NO_2^- recovery test. Following the soil protocol for PAO the recovery of added NO_2^- to fresh and stored SS extracts in Step 2 was 77 - 91% for all sample weights tested while it was considerably higher for soil (96 - 99%) (Table 2). The NO_2^- recovery efficiency surprisingly increased with increasing SS and soil amount which could possibly be explained by a higher sensitivity of the FIA with increasing total NO_2^- concentrations. The difference in NO_2^- recovery between SS and soil indicated that either coloration or particles, or other substances in the SS extract disturbed the spectrophotometric detection of NO_2^-. The SS contained metals that might have interfered with Griess reaction. Ivanov (2004) and Karlberg and Pacey (1989) reported increasing disturbance of metals in the order Hg < Ag < Bi < Pb < Fe < V < Cu of which Fe concentration is the highest in the SS used in this study (Appendix 1, Table 1 and 2)

(Ivanov, 2004; Karlberg and Pacey, 1989). It is also possible that other SS components such as cationic polyelectrolytes could have bound NO_2^-. Thus, the absolute NO_2^- concentration in SS might not be detectable by spectrophotometric analysis using Griess reagents (Ivanov, 2004; Davidson et al., 2008), but comparing amount of accumulated NO_2^- after different storage treatments using the same sludge type and amount would be possible.

As reduction of coloration and particles of SS are more easily accomplished than trying to elude interfering substances, 25 g of stored SS was chosen in Step 3 for further refinement by various centrifugation and extraction methods in an attempt to increase the NO_2^- recovery (PAO assay, section 4 - 6, Fig. 2). Only stored SS was tested as future analysis would mainly cover stored SS and the amount of 25 g was thought to give better detection of NO_2^- despite some coloration and presence of particles could be expected in these extracts. The refinement of centrifugation and extraction of SS resulted in centrifugation of 2 ml extract followed by analysis of 1.5 ml supernatant (treatment B in Step 3) giving the highest NO_2^- recovery for both SS and soil. However, obtaining NO_2^- recover rates of SS lower than 100% and also lower recovery than that of soil in Step 2 indicated that the sample processing was still restrained by coloration and possible interfering substances. In spite of this, treatment B was chosen as the best method it the subsequent PAO assay of SS. Treatment C (Step 3) resulted in a low NO_2^- recovery but higher NO_2^- content for both SS and soil compared with treatment A and B which indicated a delayed ammonium oxidation inhibiting effect of KCl when stopping the reaction in the sampling procedure. The activity in treatment A and B proceeded possibly during the centrifuge process prior to the KCl addition which was made 10-20 min after sampling. This emphasizes the importance of adding KCl immediately after sample extraction from the PAO assay. Treatment A in Step 3, following the soil protocol for PAO, ISO15685:2012 (ISO, 2012) and identical to that used in Step 2, showed no difference (Student t-test, $p>0.05$) in NO_2^- recovery of 25 g stored SS when comparing the two steps (Step 2 and 3). However, soil did differ (Student t-test, $p<0.05$) with 99% NO_2^- recovered in Step 2 as compared to 93% in Step 3 (Fig. 6 and 7), the latter possibly being explained by measurement biases.

In evaluating the AOB activity of SS while optimizing sample size in Step 4 (PAO assay, section 1 and 7, Fig. 2), PAO activity in stored SS could be demonstrated, where sample amount of 6.25 g showed the highest rate followed by 12.5 and then 25 g. The decreasing PAO with increasing sample amount of SS could possibly be due to increasing concentrations of contaminants inhibiting the PAO activity. The AOB are sensitive to environmental changes and is a feature that has been exploited in PAO assays for testing toxicity of pollutants in soils (Pell et al., 1998; Pell and Torstensson, 2003). To avoid risks for disturbance of the AOB amounts of SS in the PAO assay should probably be kept low, meaning 6.25 g. However, small sample amounts also means difficulties in assaying the activity if rates are low such as that in fresh, not stored SS

5.1.1. The new PAO assay and protocol

The decision of choosing 12.5 g SS in the final assay of PAO was based on the results of a more stable PAO assay with high r^2-value in the linear regression as well as lower variation between the replicates compared with the other SS amounts (Fig. 8). This resulted in stable accumulation of NO_2^- over time yielding solid PAO estimates. It could be argued that using the 6.25 g SS in the PAO assay showing the highest specific PAO rate of the three SS amounts tested in experiment Step 4 would fulfil the aim of choosing the amount yielding the optimal PAO rate. However, as both fresh and stored SS should be assayed in the final experiment (Step 5), and as previous experiment (Step 2) indicated very low NO_2^-

concentrations in fresh SS (FMS 6.25 g) there is an obvious risk of getting NO_2^- in concentrations under the FIA detection limit of 0.01 mg NO_2 -N l^{-1}. The higher amount of 12.5 g would therefore most likely increase the ability of assaying PAO in both fresh and stored SS. Choosing 12.5 g there is a risk of achieving PAO rates being somewhat lower than the actual PAO rates *in vivo*. It could be speculated that the lower rates using larger sample amounts could be due to possible inhibiting contaminants in the SS. To overcome this further development of the PAO for SS is needed. Nevertheless, if the small error in rate determination is systematic the method would still provide a valuable tool in comparing different methods of storing sludge for sanitization purpose like that in Step 5.

From the above work in developing of a new PAO assay for SS the following protocol is suggested. Triplicates of 12.5 g SS is weighted into separate 250 ml Duran flasks. To each flask 100 ml PAO substrate (100 mM K2HPO4/ KH_2PO_4 buffer at pH 7.24, 0.4 mM $NH_4)_2SO_4$ and 15 mM $NaClO_3$) tempered to +25°C is added. Flasks are then placed on a shaking table at 175 rpm in a room with constant temperature of +25°C. After slowing down the shaking speed to 85 rpm a slurry sample of 2 ml is taken after 2 h after which the shaking speed is reset to 175 rpm. After this new samples are taken every hour following the same procedure to receive a total of 5 samples. The 2-ml slurry samples are added to 10 ml Falcon test tubes prefilled with 2 ml 4 M KCl to stop the reaction. The samples are mixed thoroughly using a vortex mixer and dispensed to 2 x 2 ml Eppendorf test tubes and centrifuged at 15 000 rpm in 10 min using a micro centrifuge. The supernatant are transferred to 10 ml FIA test tubes using an automate pipette. All extracts are analyzed for NO_2^- by e.g. FIA (FIA, Tecator, Höganäs, Sweden; application note ASN 5200). The potential ammonium oxidation rate is calculated by linear regression of NO_2^- formation over time and PAO is the mean value of the rates achieved in the three flasks expressed in ng NO_2^--N g dw^{-1} h^{-1}.

5.2. PAO activity of processed sewage sludge (Step 5)

The results from development Step 4 gave clear indications of PAO activity in SS. Moreover, the activity was higher in SS that had been stored for seven months in open piles compared to that of fresh SS. Development of PAO activity in SS during storage was also evident from the observed increasing NO_2^- concentration of SS kept refrigerated at +2°C for 20 days in Step 3. This led to concentrations exceeded the FIA upper detection limit of 1 mg NO_2 -N l^{-1}. The same SS analyzed 10 days earlier (in Step 2) showed 4 times lower NO_2^- concentration, well in the range of FIA detection, which indicated active ammonium oxidation bacteria (AOB) even at low temperatures. The SS showed no PAO activity four weeks later which indicated that supporting factors like NH_4^+ or O_2 had been depleted. The NO_2^- concentration of refrigerated SS increased over time between experimental Step 2 and 3, but then leveled off displaying the same concentration eight days later in Step 4. AOB activity in temperatures of 2°C has previously been demonstrated by (Avrahami and Conrad, 2005; Avrahami et al., 2003).

5.2.1. Start (mixed samples)

The AOB activity in the pilot storage study was low in fresh (non-stored) mesophilically digested (M, MC, and MAC) and thermophilically (TC) digested SS. As the AOB are sensitive to environmental changes their activity ought to be low in fresh dewatered SS newly originating from post-treatment in an anaerobic digester, especially when digested at theromophilic temperatures (Jiang and Bakken, 1999). Also ammonia (NH_3) treatment by adding urea should have an inhibiting effect on the microbial population (Anthonisen et al., 1976). The slightly higher PAO rate of NH_3 treated fresh SS could however be explained by increased aeration as SS were spread out on the ground for incorporation of urea by hand

prior to setting up the pilot-study. Degradation of urea to NH_3 and then further to NH_4^+ was possible as treated SS was uncovered and aerated for some time before covered which could have decreased the direct sanitation effect of NH_3. The NH_3 treatment is effective as long as the NH_3 concentration is contained by covering the treated material (Vinnerås, 2013). The NO_2^- concentration in the non-stored M, MC and TC was lower than the detection limit of the FIA meaning that low activity causing accumulation of NO_2^- during the PAO incubation could not be detected in the present study. PAO activity of non-stored SS was lower than that of Soil I, indicating that the microbial activity was lowered by anaerobic digestion. However, the the non-stored SS was kept frozen just over 2 years which might have affected the microbial characteristics when unfrozen. Even though the microbial characteristics in soil should not fluctuate over time when kept frozen at -20°C (Stenberg et al., 1998a), this has not been tested on SS.

5.2.2. End (mixed samples)

The AOB activity in stored SS was for each treatment generally higher than corresponding non-stored. Storage most likely increased the NH_4^+ content in the sludge due to N mineralization and also access of O_2, both being essential factors for AOB activity (Robertson and Groffman, 2007; Tolli and King, 2005). Increased NH_4^+-N levels after storage were seen in M, MC and MAC treated sludge (Appendix 2, Table 1), whereas decreased levels were found in TC. The decreased levels of NH_4^+ and also accumulated NO_2^- and NO_3^- after storage support the result of high PAO activity in TC compared with the other treatments (Table 5). Increasing contents of organic N of TC indicated microbial growth and immobilization of N. However, the total N had decreased after storage which indicated gaseous N losses with possible emissions of N_2O through nitrification, nitrifying-denitrification or denitrification activity, with N_2O production being favored by limited oxygen supply (Poth and Focht, 1985) and presence of high concentrations of NO_2^- (Firestone and Davidson, 1989; Stein, 2011). The NH_4^+-N content was at start the same for the mesophilically and thermophilically digested SS, and hence, the difference in PAO activity at the end of the storage period could be an effect of the observed differences in SS texture. The porous and aggregated texture of the thermophilically digested SS (TC) probably increased the O_2 availability for AOB, initiating higher PAO activity, whereas the structure of mesophillically digested SS (M, MC and MAC) were more compact. Difference in O_2 availability inside and outside SS aggregates in the TC sludge is also possible which could explain the high variation in PAO activity among the TC replicates.

The AOB activity seemed not to be affected by the thermophilic temperatures in the digestors producing the sludge used in the TC treatment, which has been suggested by (Jiang and Bakken, 1999). Nyberg et al.(2006) showed that anaerobic digestes generated in thermophilic temperatures inhibited ammonium oxidizing bacteria more than digestates from mesophilic temperatures and argued that organic pollutants degrade more efficient in low temperatures (Leven and Schnurer, 2005). However, these suggested inhibitory effects seams not have affected the AOB activity in TC. The observations of PAO activity in the TC sludge are supported by Kowalchuk et al. (1999) who demonstrated presence AOB in thermophilic environment and Jarvis et al. (2009) who demonstrated both presence and activity of AOB in thermophilic composts.

The ammonia (NH_3) treatment (MAC) showed a long-term decline in AOB growth as this SS displayed 40% lower PAO activity than the same sludge stored without NH_3 (MC). The low N_2O emissions observed from MAC treatment in the long-term storage trial (Jönsson et al., 2014) strengthen the findings of inhibited nitrification and possible also denitrification,

probably caused by NH_3 formation from added urea. At the end of the storage period the NH_4^+- N content of the MAC treated sludge had increased the from 7 to 9 kg ton^{-1} wet weight SS (Appendix 2, Table 1). In addition, the low C/N ratio (<5) of the MAC treated sludge reflects the value of ammonia treated SS as N-fertilizer product in agricultural as suggested by Vinnerås (2013).

At the end of the storage period there were no significant differences in PAO activity between MC and M, even though the former tended to be somewhat higher. The dry weight of MC was higher than that of M (Table 5) which should have positively affected the O_2 availability for AOB leading to higher PAO activity. However, the aerated top-layer of M most likely favored AOB activity during storage. The total N in the M treatment was lower than that of MC reflecting the volatization of N as N_2, N_2O and NH_3 (Appendix 2, Table 1).

5.2.3. Top and bottom layer

The importance of O_2 availability for ammonium oxidation of stored SS was seen from the results of top and bottom layer samples. The PAO activity was generally higher in the top-layer than in the bottom layer, the latter being O_2 restricted. However, such a difference in PAO activity between top and bottom layer was not seen in the MAC treated sludge. The MAC sludge might be more homogeneous in O_2 as the SS were mixed and aerated when urea was incorporated.

5.3. PAO assay of SS applied to soil (Step 6)

Application of SS to Soil II did not change the PAO activity in the soil. However, some inhibitory effect was indicated with strongest effect displayed by MAC. The NH_3 inhibiting effect by urea on PAO activity (Table 5) could have affected also the soil AOB as the SS application probably resulted in NH_4^+ concentrations higher than that in the PAO substrate. SS from all storage treatments displayed lower PAO activity than the soil itself. Inhibition of PAO by contaminants in the SS such as heavy metals, organic pollutants etc. cannot be excluded. Inhibition of AOB activity has been seen in other studies where similar organic products such as biogas residues and pig-slurry were applied to soil (Abubaker et al., 2015; Levén et al., 2006). The impact of SS on the PAO activity in soil in the present study was probably limited as only small amounts of SS, 2% of the assayed soil amount, were added. Nevertheless, the initial NO_2^- concentration of each treatment added to soil were higher than that in the soil itself, which indicate that the SS application should have affected the soil N content.

The PAO rate of Soil II without SS addition was 798 ±270 ng NO_2^--N g^{-1} dw h^{-1} (n=3) which could be considered moderate to high activity for a Swedish arable soil (Stenberg et al., 1998b), and around three times as high as than that in Soil I. High intrinsic PAO indicates the ability of a soil to mineralize and convert N. The control Soil I showed slightly lower PAO rate in the present experiments (240 ng NO_2^--N g^{-1} dw h^{-1}, n=4) than reported previously for the same soil (288 ng NO_2^- -N g^{-1} dw h^{-1}) (Abubaker et al., 2015). The microbial characteristics should not fluctuate over time when soil is frozen at -20°C (Stenberg et al., 1998a), meaning the PAO rates from the same soil should not differ. However, in the present study the assay was different using centrifugation of 2 ml extracts followed by analysis of 1.5 ml supernatant compared to the PAO protocol for soil. In addition, even though the soil was collected in the same field spatial variation in AOB activity are likely to have influenced the results.

The SS of MC, M and MAC were nearly water saturated whereas TC were dry and aggregated, which implied difficulties in getting equal samples. The soil had higher moisture when sieved which probably resulted in aggregate patterns differing to those when sieving a dried soil which may have introduced variation among the samples. The NO_2^- detection by FIA were interfered, possibly by contaminated NO_2^- standard solutions which was indicated by an approximately 13% higher reference standard curve than those when assaying PAO in Steps 4 and 5. However, this error did not affect the possibility to compare the treatments within the same batch of assays. In addition, to be able to compare with previous PAO-assays data were adjusted by lowering with 13% (mg NO_2^--N l^{-1}).

5.4. Implications of PAO in SS and SS amended soil

The results show that nitrification occurs in SS and that AOB activity is high especially in treatments where NH_4^+ and O_2 resources are good and not disturbed by inhibiting compounds. The PAO activity decreased when O_2 seemed to become limited and when urea was added such as in the MAC treatment (Table 5), whereas the opposite were seen in TC treatment.

A lower PAO activity in the SS decreases the transformation of NH_4^+ to leachabel NO_2^- and NO_3^- which retains the NH_4^+ pool and increases its SS value as N-fertilizer. In addition, supplementing SS with urea increased its content of NH_4^+ which should further enriche the fertilizer value the SS. Application of SS to soil in the present study showed a small tendency of inhibiting the ammonium oxidation (Table 7). This suggests that the N_2O emissions may decrease as less NO_2^- and NO_3^- indicates not only low AOB activity but also will retard the activity of denitrifying bacteria in the soil matrix. As ammonia-oxidizing bacteia are senistive to disturbance PAO has been used in evaluating toxic effects of chemicals (Pell et al., 1998; Pell and Torstensson, 2003; Jezierska-Tys and Frac, 2008). Thus a low observed PAO activity could possibly be a result of AOB inhibition by chemical contaminants. Such an effect was indicated in development Step 4 (Fig. 9) where increasing amount of SS resulted in decreased PAO-rate, which if true should contradicts the value of SS spread on arable land.

Today, SS that are not approved for soil application are mainly stored uncovered at landfills in Sweden, which means that SS are exposed to shifting weather conditions similar to that in the top-layer of the M treatment. Uncovered storage implicate higher availability of O_2 and increased exchange of volatilized N (NH_3, N_2O, N_2) with the atmosphere. In addition, infiltration of precipitated water will transport mineralized N (NH_4^+) and NO_3^- to the bottom-layer sustaining nitrification and denitrification. Emissions of N_2O were two times higher from M than MC (Jönsson et al, 2014) as both nitrification and denitrification are supported in environment of fluctuating O_2 (Clemens et al., 1997). The emissions of N_2O from storage of SS have been reported in several studies (Jönsson et al. 2014; Flodman, 2002), and the measured PAO activity in SS shown in the present study indicates that nitrification is one likely candidate of the observed emissions.

5.5. Further improvement of the PAO assay

A major challenge in developing a PAO protocol for SS was to achieve a sample preparation method yielding true NO_2^- concentrations in the FIA analysis. This was due to problems in getting pure extracts. Despite efforts to centrifuge and filtrate the extracts some interference at the used wavelength of 540 nm still seemed to remain. This problem seemed to be more severe for the MAC treated sludge. For better accuracy in the spectrometric detection of NO_2^-, discoloration chemicals could be used in the sample preparation. Furthermore, presence of inhibitory chemicals like heavy metals and other contaminants interfering with the diazotization reactions forming the nitrite color complex to be analyzed in the

spectrophotometer can not be excluded which might have lowered the efficiency of the analysis. Also, the microbial characteristics in SS might fluctuate over time when kept frozen at -20°C. These analyses on SS has not been done before as done for soil (Stenberg et al., 1998a), and has to be considered in further development of the PAO assay.

Variation among the SS replicates was seen for all storage treatments, whereas soil replicates displayed only low variation. This was however expected as the variation within SS replicates reflects the spatial variation between three true replicates (containers) while the soil originated from samples taken from a small spot. Lowering the variation of the SS could be accomplished by increasing the numbers of replicates from each container. Such a procedure would however be time consuming. The low variation among soil samples could also be explained by soil being slightly dried, sieved and frozen before withdrawing samples, whereas SS samples were frozen fresh and only mixed thoroughly before extracting samples for the various steps of the PAO development. The different texture of the SS in the different treatments also made it difficult to take accurate samples. The TC treated sludge was more aggregated and the M treated sludge had higher water content than the other SS. The variation in fresh weight was though compensated for, as the dry weight of each sample was recorded and used in the calculations of the PAO rate.

Further improvement of the PAO assay could possibly be a tool among others to classify SS by microbial activity and evaluate the contaminants effect in different batches of SS. Moreover, the assay can also be used as a tool, as in this study, to evaluate different storage methods of SS regarding nitrification contribution to N_2O emissions and leakage of NO_2^- /NO_3^-.

6. Conclusion

This study show that ammonia oxidation occurs in fresh and stored digested and dewatered sewage sludge (SS). The new developed PAO assay enables determination of sanitation treatment effects in SS of which ammonia treatment seemed to have better reducing effect on nitrification and possibly denitrification, than thermophilic temperature.

More specific:

- During the development of the PAO assay, SS extracts where centrifuged in a micro centrifuge at 15 000 rpm for 10 min (Treatment B) which resulted in the most reliable NO_2^- recovery for SS and was incorporated in the new PAO assay for SS.
- The specific PAO rates decreased with increasing sample amount in the assay, which indicated content of AOB inhibiting substances in the SS. Therefore, using the new PAO assay there is a risk of achieving PAO rates being somewhat lower than the actual PAO rates *in vivo.*
- The assay proved to be repeatable and yielded consistent result with only low variation.
- Termophillically treated SS (TC) had a porous structure which supplied O_2 and kept the ammonia-oxidizing bacteria (AOB) active during the one-year storage despite coverage and digestion in thermophile temperatures.
- Ammonia treated SS (MAC) inhibited the AOB displaying a long-term effect as the PAO rate were kept low and at a constant level throughout the storage period of one year.

- Application of SS to soil did not change the PAO activity in soil, however some inhibitory effects was indicated with strongest effect displayed by MAC.
- Inhibition of the AOB activity leads to reduction of nitrate, also it is important to control factors supporting nitrification during storage of SS to reduce substrate availability for further transformation to N_2O.

To overcome the observed biases in the new assay further development of the PAO for SS is needed. However, the observed deviation in rates was low and systematic which should still provide a valuable tool in assessing PAO rates and comparing different treatment methods for storing SS for sanitization purpose.

7. Acknowledgement

I am grateful to my supervisor Mickael Pell at the Department of Microbiology of Swedish University of Agricultural Sciences that have been supportive and encouraging during the development work of the assay and for critical reading of the essay.

Agnes Widén for providing sewages sludge samples to my study and information about the pilot study of different storage treatments.

Elisabet Börjesson, Maria Hellman, Harald Cederlund and Johnny Ascue Contreras for assistance in the laboratory and introduction to the detection method FIA.

Eric Cato at the wastewater treatment plant, Kungsängsverket in Uppsala for providing samples of sewages sludge for the development of the PAO assay.

Kersti Linderholm for support, critical reading of the essay and being encouraging.

Tina Putz and Lea Wittorf, Anton Pallin, Simon Isaksson and Tong Liu for great companionship, support and making me look forward to come the office and laboratory every day.

Last but not least I thank my family and all friends for believing in me and always being supportive and encouraging.

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Appendix 1 – Sewage sludge used in the development work

Characteristics of fresh and stored SS used in the developing Steps 1- 4.

Table 1. Chemical characteristics of fresh dewatered mesophilically digested sewage sludge (FMS) from Kungsängen WWTP, Uppsala (January 2014).

Parameter	Result	Unit
pH	7.4	
Dry weight	27.9	%
Al	8.44	g kg^{-1} DW
Ca	27.7	g kg^{-1} DW
Fe	91.7	g kg^{-1} DW
K	1.47	g kg^{-1} DW
Mg	2.16	g kg^{-1} DW
Mn	0.166	g kg^{-1} DW
N	49.7	g kg^{-1} DW
P	29.4	g kg^{-1} DW
Ag	1.66	mg kg-1 DW
As	2.56	mg kg-1 DW
Au	0.248	mg kg-1 DW
B	22.8	mg kg-1 DW
Bi	3.95	mg kg-1 DW
Cd	0.499	mg kg-1 DW
Co	2.89	mg kg-1 DW
Cr	17.6	mg kg-1 DW
Cu	279	mg kg-1 DW
Hg	0.636	mg kg-1 DW
Mo	7.39	mg kg-1 DW
Ni	12.7	mg kg-1 DW
Pb	10.2	mg kg-1 DW
Se	1.51	mg kg-1 DW
Sn	13.5	mg kg-1 DW
U	59.5	mg kg-1 DW
V	23.7	mg kg-1 DW
W	2.63	mg kg-1 DW
Zn	283	mg kg-1 DW

DW; dry weight

Table 2. Chemical characteristics of mesophilically digested sludge (SMS) from Kungsängen WWTP (July 2013), dewatered and stored for seven months at Hovgården, Uppsala.

Parameter	Result	Unit
pH	7.2	
DW	28.9	%
Al	11.0	g kg^{-1} DW
Ca	25.8	g kg^{-1} DW
Fe	87.4	g kg^{-1} DW
K	0.853	g kg^{-1} DW
Mg	2.46	g kg^{-1} DW
Mn	0.198	g kg^{-1} DW
N	45.1	g kg^{-1} DW
P	30.3	g kg^{-1} DW
Ag	2.11	mg kg-1 DW
As	2.25	mg kg-1 DW
Au	0.328	mg kg-1 DW
B	17.7	mg kg-1 DW
Bi	5.43	mg kg-1 DW
Cd	0.480	mg kg-1 DW
Co	2.86	mg kg-1 DW
Cr	17.0	mg kg-1 DW
Cu	453.0	mg kg-1 DW
Hg	0.748	mg kg-1 DW
Mo	7.19	mg kg-1 DW
Ni	14.0	mg kg-1 DW
Pb	13.1	mg kg-1 DW
Se	2.17	mg kg-1 DW
Sn	<10	mg kg-1 DW
U	61.8	mg kg-1 DW
V	24.7	mg kg-1 DW
W	2.36	mg kg-1 DW
Zn	489.0	mg kg-1 DW

DW; dry weight

Appendix 2 – Sewage sludge from storage trial

Sewage sludge samples collected from a storage trial with four different storage treatments set up in fully randomized design with three replicates.

Table 1. Characteristics of digested sewage sludge at start and end of a one-year storage period (mean, n=3) (from Jönsson et al., 2014).

					(kg ton^{-1} fresh weight)					
Treatment	Sampling point	Dry weight, DW (%)	Volatile solids, VS (% of DW)	pH	Tot-N	Org-N	NH_4^+-N	NO_3^--N/ NO_2^--N	Tot -C	Tot -C/ Tot- N
M	Start	29.4	61.2	7.7	11.7	8.9	2.9	0.001	101.8	8.7
	End	22.5	58.3	8.2	11.4	8.1	3.4	0.001	70.9	6.2
MC	Start	29.4	61.2	7.7	11.7	8.9	2.9	0.001	101.8	8.7
	End	24.2	58.1	8.2	12.8	8.9	4.0	0.001	76.5	6.0
MAC	Start	29.1	61.6	8.6	16.8	10.2	6.6	0.005	100.2	6.0
	End	25.1	59.6	8.6	18.9	9.7	9.2	0.001	82.0	4.3
TC	Start	29.5	51.4	7.7	8.1	5.6	2.6	0.001	81.8	10.1
	End	28.8	49.1	6.5	7.6	7.0	0.6	0.154	72.3	9.5

M; Mesophilically digested sewage sludge stored without a cover, i.e. no cover
MC; Mesophilically digested sewage sludge stored with a cover
MAC; Ammonia treated mesophilically digested sewage sludge (addition of 1.5% urea by weight) stored with a cover
TC; Thermophilically digested sewage sludge stored with a cover

Table 2. Characteristics of digested sewage sludge at 0.15 m from top and 0.15 m bottom in storage cylinders at end of a one-year storage period (mean, n=3) (from Jönsson et al., 2014). See Table 1 for abbreviations.

					(kg ton^{-1} fresh weight)					
Treatment	Sampling position	Dry weight, DW (%)	Volatile solids, VS (% of DW)	pH	Tot-N	Org-N	NH_4^+-N	NO_3^--N/ NO_2^--N	Tot -C	Tot-C/ Tot- N
M	Top	17.7	58.7	7.7	8.1	6.8	1.3	0.0	56.2	6.9
	Bottom	27.0	59.4	8.3	13.6	9.6	3.9	0.0	84.8	6.2
MC	Top	20.8	58.0	8.0	10.4	7.8	2.6	0.0	64.7	6.2
	Bottom	27.2	59.4	8.4	41.3	9.7	4.7	0.0	85.4	6.0
MAC	Top	20.6	59.2	8.5	16.2	8.5	7.8	0.0	63.7	3.9
	Bottom	27.3	60.5	8.6	22.3	9.8	12.5	0.0	89.6	4.0
TC	Top	33.2	49.0	6.4	8.6	8.5	0.0	0.5	78.9	9.2
	Bottom	27.9	53.2	8.0	8.9	6.1	2.7	0.0	75.2	8.5

Appendix 3 – Soils used in the development work

Soil used as reference in developing Steps 1 – 4, PAO assays of SS from storage trial and PAO assay of SS applied to soil.

Table 1. Chemical, physical and microbial characteristics of Soil I used in development Steps 1 - 5 (from (Abubaker et al., 2015).

Characteristics	
Chemical	
pH	5.6
Tot-C (%)	1.3
Tot-N (%)	0.1
Tot-P (g kg^{-1} DW)	0.7
Tot-S (g kg^{-1} DW)	0.2
P-AL (mg 100 g^{-1} DW)	4.8
K-AL (mg 100 g^{-1} DW)	18.5
Physical	
Sand (%)	14-20
Silt (%)	36-44
Clay (%)	37-49
WHC (g H_2O g^{-1} soil DW)	0.511
Moisture content (% of WHC)	34
Microbial	
PAO (ng NO2-N/g^{-1} DW min^{-1})	4.8
PDA (ng N2O-N/ g^{-1} DW min^{-1})	7.8
Basal respiraiton (µg CO_2 g^{-1} dw h)	0.782

DW; dry weight

Table 2. Parameters of Soil II used in PAO assay with SS application (from (Jönsson et al., 2014).

Characteristics	
Chemical	
pH	6.9
Dry weight (%)	83.5
Tot.-C (%)	19.7
Tot.-N (%)	2.4
NH_4^+-N (mg kg^{-1} DW)	6.0
NO_2^--N+NO_3^--N (mg kg^{-1} DW)	4.8
Tot.-P (g kg^{-1} DW)	6.2
Tot.-S (g kg^{-1} DW)	0.21
K (g kg^{-1} DW)	0.78

DW; dry weight

Appendix 4 – Populärvetenskaplig sammanfattning

Kan man man mäta nitrifikationsaktiviteten i lagrat slam och hur påverkas den av olika lagringsmetoder?

Avloppsslam innehåller många värdefulla växtnäringsämnen som i ett resursuthålligt samhälle måste kunna återföras till jordbruksmark. Det är dock vanligt att slammet innehåller föroreningar som t.ex. sjukdomsalstrande bakterier, tungmetaller och kemiska miljögifter. En metod att minska bakteriehalten i slammet innan såsom föreslagits är att lagra slammet under ett år innan spridning. En annan metod är att behandla slammet med urea. Under lagringstiden riskerar man dock att förlora kväve (N) genom emissioner av lustgas (N_2O), en potent växthusgas, och läckage av nitrat (NO_3^-) till grundvattnet vilket kan störa vattenmiljön genom bl.a. övergödning. Det är därför viktigt att hitta lagringsmetoder för slammet som minskar förlusterna av dessa kväveföreningar. Den mikrobiella processen nitrifikation påverkar förekomsten av båda dessa kväveföreningar och det är därför intressant att undersöka nitrifikationsaktiviteten i slam. Dock har inga mätningar gjorts i lagrat avloppsslam tidigare så det är svårt att veta hur stor nitrifikationsaktiviteten är. I denna studie efterforskades därför huruvida nitrifikationsaktivitet finns i lagrat avloppsslam och om nitrifikationen kan bidra till kväveläckage i form av N_2O emissioner. Vidare var det intressant att undersöka om nitrifikationen påverkas av olika hygieniseringsmetoder. Målet för studien var att utveckla en metod för att mäta nitrifikationsaktiviteten i avloppsslam. I metoden bestäms den potentiella ammonium-oxidationshastigheten (PAO) i provet, d.v.s. den högsta möjliga hastigheten av det första steget i nitrifikationen där ammonium omvandlas till nitrit. Denna metod finns redan beskriven för mätningar i jord och denna användes som underlag i utvecklingen av metoden för slam. Ett ytterligare mål har varit att utvärdera huruvida nitrifikationen skulle kunna förklara avgången av N_2O från lagrat slam.

PAO bestäms genom att sätta en liten mängd prov (slam eller jord) till en flaska. Till flaskan sätts och ett substrat bestående av en ammoniumlösning buffrad till pH 7,24 och säkra god syretillgång genom konstant omrörning. Genom att blockera det andra steget i nitrifikationen, d.v.s. ombildningen av nitrit till nitrat kan man genom spektrofotometrisk analys mäta hastigheten varmed nitrit ackumuleras. Detta görs genom att ta prover från försöksflaskan först efter två timmar och sedan en gång i timmen under totalt 6 timmar. Linjär regressionsanalys används sedan för att bestämma lutningen på kurvan (PAO) som beskriver ökningen av mängden nitrit i flaskan över tiden. PAO värdet kan bl.a. användas för att visa att nitrifikationsbakterier är aktiva eller för att tolka om aktiviteten i ett jordprov påverkas av slamtillsats.

I utvecklingsarbetet med att anpassa mätmetoden till slamprover användes avvattnat färskt och lagrat avloppsslam. Arbetet genomfördes i följande fyra steg: (1) Spektrofotomerisk skanning av slamextrakt, (2) analys av känd mängd nitrit som satts till slamextrakt, (3) förfining av extraktions- och centrifugeringsmetoder och (4) test av optimal provmängd för AOB aktivitet i slammet. Utvecklingsarbetet resulterade i ett modifierat protokoll för PAO jämfört med det som används för jord. Mängd slamprov i testet minskades från 25 till 12,5 g vilket signifikant reducerade bakgrundsfärgen på slamextraktet. Mängden extrakt vid provtagning minskas från 5 till 2 ml för att kunna använda en högre centrifugeringshastighet och ta bort ytterligare slampartiklar och bakgrundsfärg innan nitritanalys. Den nya metoden resulterade i ackumulerande nitrathalter under 6 timmars perioden och en linjär regression visade på en stabil kurva med konstant hastighet. Förutom att resultaten visade att den nya metoden kan användas för att mäta PAO i avloppsslam visade den också att lagrat avloppsslam kan ha en betydande PAO aktivitet, vilket inte har rapporterats tidigare.

Genom att använda den nya PAO metoden utvecklad för slam, var det möjligt att testa nitrifikationen i slam från ett lagringsförsök med fyra olika lagrings- och hygieniseringsbehandlingar, med och utan täckning. Resultaten visade att långtidslagrat termofilt rötat avloppsam hade signifikant högre PAO än mesofilt rötat slam. Skillnaden berodde troligen på slammens struktur där det mer porösa termofila slammet möjliggjorde nitrifikation genom en ökad syresättning under lagringen. Mesofilt rötat slam som behandlats med ammoniak uppvisade låga PAO värden, vilket påvisade att ammoniak haft en negativ inverkan på nitrifikationsbakteriernas aktivitet. Lustgasmätningar som gjordes på samma slam i en tidigare studie gick i linje med PAO värden i denna studie. Lustgasemissionerna från det termofilt rötade slammet var höga medan de var låga för det ammoniak behandlade slammet. Detta visar på att ammoniak inte bara inhiberade nitrifikationen utan möjligen även denitrifikationen, och som båda bidrar till lustgasavgång.

Sammantaget visar denna studie på att nitrifikationsaktivitet pågår i lagrat avloppsslam och är olika stor beroende på vilken hygieniseringsmetod som används. Nitrifikationen är en viktig del i kvävets kretslopp och styr tillgången på nitrat som är substrat till lustgasproducerande dentirifikationsbakterier. I en miljö med skiftande syretillgång, som i en slamhög vilken utsätts för regn och torka, kan en ökad tillgång på nitrat även öka produktionen av lustgas vilket bör undvikas. Genom att behandla slam med ammoniak kan man under lagringsperioden nå en minskad nitrifikation vilket visats i denna studie. Problemet med föroreningar i slammet kvarstår dock för att kunna sluta kretsloppet av växtnäringsämnen fullt ut, och dessa föroreningar verkade även påverka nitrifikationsbakterierna. I utvecklingsskedet av metoden tenderade PAO värden att minska med ökade provmängder av slam vilket troligen är en inverkan av ökande koncentrationer av föroreningar i slammet. För att säkerställa metodens tillförlitlighet krävs ytterligare utveckling. Trots detta öppnar den nya PAO metoden möjligheter för att kunna avgöra skillnader i mellan olika hygieniseringsmetoder i slam och möjligen avgöra föroreningars inverkan på mikroorganismerna i jorden innan spridning på jordbruksmark.