Effects of Fertilisation with Biogas Residues on Crop Yield, Soil Microbiology and Greenhouse Gas Emissions

Recycling of Plant Nutrients from Bioenergy Production

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2012

Acta Universitatis agriculturae Sueciae
2012:46

Cover shows the different perspectives studied in the thesis
(Photo: Created by thesis author)

ISSN 1652-6880
ISBN 978-91-576-7682-5

Print: SLU Service/Repro, Uppsala 2012

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Abstract

The amount of residues generated by biogas production has increased dramatically due to worldwide interest in using renewable energy. Biogas residues (BRs) originate from anaerobic degradation of different types of rural and urban organic wastes and have been proposed as organic fertilisers because of their high content of ammonium and other valuable macro- and micro-nutrients. However, application of BRs to agricultural soils may be accompanied by environmental risks, since they may contain heavy metals and organic pollutants. Therefore the effects of BRs on crop production and on the soil ecosystem and environment urgently need to be investigated before their wider use. This thesis evaluated and compared different types of BRs against cattle slurry, pig slurry, compost and mineral fertiliser with respect to their (1) ability to provide plants with necessary nutrients, (2) impact on the soil microbial ecosystem and (3) effects on emissions of the greenhouse gas nitrous oxide (N_2O). The results from short-term laboratory experiments and a long-term field trial showed that BRs increased crop yield to the same extent or more than conventional mineral fertiliser and compost, but less than pig slurry. BRs generated from source-separated organic household waste had a tendency to give higher crop yield and soil microbial activities than other BRs. BRs had no general negative effect on soil respiration, but substrate-induced respiration decreased significantly in organic soil on addition of BRs. Although all BRs initially inhibited potential ammonium oxidation and potential denitrification activity, no long-term negative effects were detected. BRs stimulated ammonium assimilation, which can temporarily decrease nitrogen availability to the plant. Furthermore, the bacterial community structure in the sandy soil was altered by BRs and cattle slurry, but no significant change was seen in the community structure of clay and organic soil. Application of BRs and animal slurry increased N_2O emissions, but the total losses and flux patterns were affected by fertiliser type and soil type. In conclusion, the fertiliser value of BRs should be regarded as high and they apparently have no long-term adverse effects on soil microbial functions and structures. Thus the problematic amounts of residues associated with expansion of biogas production could be turned to advantage, as these residues seem to be safe and competitive fertilisers.

Keywords: animal manure, bacterial community structure, biogas residues, crop yield, microbial activity, nitrous oxide emission, soil type

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Dedication

To my parents, brothers and sisters

The important thing in science is not so much to obtain new facts as to discover new ways of thinking about them.

William Lawrence Bragg, Nobel Prize for Physics, 1915

Contents

List of Publications

This thesis is based on the work reported in the following papers, which are referred to by their Roman numerals in the text:

I **Abubaker, J.**, Risberg, K. & Pell, M. (2012). Biogas residues as fertilisers - effects on wheat growth and soil microbial activities. *Applied Energy* 99, 126-134.

II Odlare, M., Arthurson, V., Pell, M., Svensson, K., Nehrenheim, E. & **Abubaker, J.** (2011). Land application of organic waste - Effects on the soil ecosystem. *Applied Energy* 88, 2210-2218.

III **Abubaker, J.**, Risberg, K., Jönsson, E., Pell, M., Dahlin, S. & Cederlund, H. Short-term effects of biogas residue and pig slurry application on soil microbial activity (Submitted).

IV **Abubaker, J.**, Cederlund, H., Pell, M. & Arthurson, V. Bacterial community structures and microbial activities of different soils amended with biogas residues and cattle slurry (Submitted).

V **Abubaker, J.**, Odlare, M. & Pell, M. Nitrous oxide emissions from different soil types amended with biogas residues and cattle slurry (Submitted).

VI Odlare, M., **Abubaker, J.**, Lindmark, J., Pell, M., Thorin, E. & Nehrenheim, E. (2012). Emissions of N_2O and CH_4 from agricultural soils amended with two types of biogas residues. *Biomass and Bioenergy* 44, 112-116.

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The contribution of the author to the papers included in this thesis was as follows:

I Participated in planning and setting up the experiment, performed part of the laboratory work and data evaluation, and wrote most of the manuscript.

II Participated in measuring the nitrogen mineralisation capacity and potential ammonium oxidation in soil, and made a minor contribution to writing.

III Participated in the planning, performed part of the laboratory work and data evaluation, and wrote most of the manuscript.

IV Planned the whole experiment, performed all laboratory work and the majority of the data evaluation and wrote most of the manuscript.

V Carried out the majority of the planning, performed all the laboratory work and did most of the data evaluation and writing.

VI Participated in the planning, performed part of the laboratory work and data evaluation, and contributed in writing the manuscript.

In addition to Papers I-VI, the author contributed to the following papers and manuscripts within the timeframe of the project:

- Rodhe, L., **Abubaker, J.**, Ascue, J., Nordberg, Å. & Pell, M. Greenhouse gas emissions from pig slurry during storage and after field application in northern European conditions (Submitted).

- Odlare, M., Pell, M., Arthurson, V., **Abubaker, J.** & Nehrenheim, E. Combined mineral N and organic waste fertilization - effects on crop growth and soil properties (Submitted).

Abbreviations

AOA	ammonia oxidising Achaea
AOB	ammonia-oxidising bacteria
BCS	bacterial community structure
BR	biogas residue
BR-A	biogas residue type A
BR-B	biogas residue type B
BR-C	biogas residue type C (called BR-A in Papers IV and V)
BR-D	biogas residue type D (called BR-B in Papers IV and V)
BR-E	biogas residue type E
B-resp	basal respiration
BR-F	biogas residue type F
BR-G	biogas residue type G (solid fraction)
C/N	carbon to nitrogen ratio
CH_4	methane
CO_2	carbon dioxide
CS	cattle slurry
GHG	greenhouse gas
N_2	Denitrogen
N_2O	nitrous oxide
NH_2OH	Hydroxylamine
NH_3	Ammonia
NH_4^+	Ammonium
NMC	nitrogen mineralisation capacity
NMS	non-metric multidimensional scaling
NO	nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOB	nitrite-oxidising bacteria

NPK	mineral fertiliser (nitrogen, phosphorus, potassium)
NPS	mineral fertiliser (nitrogen, phosphorus, sulphur)
PAO	potential ammonium oxidation
PDA	potential denitrification activity
PS	pig slurry
qCO_2	metabolic quotient
SIR	substrate-induced respiration
TRF	terminal restriction fragment
T-RFLP	terminal restriction fragment length polymorphism
WHC	water-holding capacity
μ_{SIR}	microbial specific growth rate

1 Introduction

1.1 Background

Worldwide, in urban and rural areas, large amounts of organic wastes are produced every day from different sources such as livestock, industries, agriculture and households. These wastes need to be managed and utilised in an appropriate way. One way to make waste management sustainable is to use the organic wastes as sources in renewable energy production, to produce *e.g.* biogas. However, the use of organic wastes in bioenergy production also leads to generation of 'secondary' residues, because not all organic material fed into a bioprocess can be utilised by the microbes. Such residues are known to be rich in plant nutrients and can be used as fertilisers, thereby recycling the nutrients back to agricultural soils (Odlare *et al.*, 2011; Svensson *et al.*, 2004). The application of organic residues to agricultural soils has become an important approach to increase soil fertility (Pérez-Piqueres *et al.*, 2006; Ayuso *et al.*, 1996), but also presents challenges, as the beneficial effects of these materials may be accompanied by risks to both the soil and the environment due to contaminants (Bationo *et al.*, 2007; Albihn, 2002). Most types of organic fertilisers such as conventional animal manures and crop residues have been extensively studied from different perspectives, *e.g.* crop yield, soil microbiology and environmental effect (Zhong *et al.*, 2010; Möller *et al.*, 2008; Goyal *et al.*, 2006; Grandy *et al.*, 2006; Ghosh *et al.*, 2004; Goyal *et al.*, 1992), whereas organic residues generated from renewable energy sources, such as biogas residues (BRs), are poorly documented.

The number of biogas plant reactors is increasing and will continue to increase rapidly in many countries over the coming years to meet the demand for energy (Rutz, 2010). Within the European Union, biogas production increased six-fold from 1990 to 2005 (EUROSTAT, 2007). In early 2010,

about 5,900 biogas plants were installed in Eastern Europe (Zuber, 2010). In Germany, the biogas market is booming and currently there are about 4,500 biogas plants (Rutz, 2010). In 2009, biogas was produced at 230 digestion plants in Sweden and there are about 50 ongoing biogas projects to be finished in 2012 (Biogasportalen, 2011). Because of this great increase in number of anaerobic digestion plants, large amounts of BRs are expected to be produced (Angelidaki *et al.*, 2003). For instance, Swedish Waste Management reported that in 2011 more than 555,000 tons biodegradable wastes were digested in biogas processes, resulting in 594,000 tons of slurry BRs (Swedish Waste Management, 2012).

The biogas process, also called anaerobic digestion, consists of the stages hydrolysis, fermentation, acetogenesis and methanogenesis (Angelidaki *et al.*, 2011), all of which are performed by diverse groups of microorganisms, leading to the overall degradation of complex organic compounds. In the initial hydrolysis stage, polymers such as carbohydrates, fats and proteins in the raw material are hydrolysed by extracellular enzymes to monomeric fatty acids, simple sugars and amino acids. In the fermentation and acetogenesis stages, the monomers are further degraded by fermenting and acetogenic bacteria to hydrogen gas (H_2), carbon dioxide (CO_2), alcohols, organic acids (including acetate), ammonia (NH_3) and hydrogen sulphide (H_2S). In the final stage (methanogenesis), strictly anaerobic methanogenic archaeans form methane mainly from CO_2 and H_2 (hydrogenotrophic methanogens) or acetate (acetotrophic methanogens), but also form small amounts of dinitrogen (N_2), NH_3 and H_2S (Deublein & Steinhauser, 2008). During anaerobic digestion, a large proportion of the energy contained in the organic waste is retained in the methane molecules (Neves *et al.*, 2006). At the same time, the nitrogen (N) and most other nutrients are preserved in the residues (Massé *et al.*, 2007). For example, organically bound N in manure and crop residues is mineralised to ammonium (NH_4^+), which is a soluble form of N that plant roots can easily absorb (Möller & Stinner, 2009). Overall, anaerobic digestion in a biogas plant results in a residue that differs profoundly from the 'raw materials' fed to the process in having *e.g.* a higher pH, lower contents of dry matter and total carbon (C), a higher proportion of NH_4^+-N to total N and a lower carbon to nitrogen (C/N) ratio. However there is generally no alteration in total N, potassium (K) and phosphorus (P) (Field *et al.*, 1984; Kirchmann & Witter, 1992). Therefore, application of BRs can be expected to lead to different effects in arable soil compared with the use of regular organic fertilisers (Levén & Schnurer, 2005; Engwall & Schnürer, 2002; Angelidaki *et al.*, 2000; Grossi *et al.*, 1998).

A wide range of organic wastes can be used as ingoing substrate in biogas production, *e.g*. animal manure, slaughterhouse waste, source-sorted municipal household waste, restaurant waste and waste from the food industry, sludge from wastewater treatment, crop residues, energy crops and by-products from the biofuel industry (Börjesson & Mattiasson, 2008; Deublein & Steinhauser, 2008; Kvasauskas & Baltrėnas, 2008; Edström *et al.*, 2003). Due to this wide range of organic materials used in biogas production, the residues generated can vary in their properties (Amani *et al.*, 2010), especially in their content of heavy metals, organic pollutants, pesticides and pathogens (Govasmark *et al.*, 2011). Furthermore, there are several factors related to the operating parameters of biogas processes that can affect the composition of BRs, *e.g*. retention time and operating temperature (Gallert & Winter, 1997). The type of pollutants present in BRs mainly depends on the substrate fed to the biogas reactor (Levén, 2006) and on the process operating temperature (Weiland, 2010). For instance, it has been shown that biogas plant reactors operated at mesophilic temperature may have increased degradation efficiency of organic pollutants compared with thermophilic reactors (Levén & Schnurer, 2005). Thus, BRs can be generated using different operating parameters and therefore the recycling of residues to arable soils might lead to different effects on the soil ecosystem and the environment.

1.2 Organic fertiliser and soil fertility

The use of organic residues as fertilisers increases soil fertility and sustains crop production (Diacono & Montemurro, 2010; Acton & Gregorich, 1995). In general, soil fertility can be directly or indirectly related to the physical, chemical and biological properties of that particular soil (Bulluck *et al.*, 2002; Knoepp *et al.*, 2000). Many studies have shown that altering one of these types of properties through *e.g*. climate variation or an agricultural practice such as application of fertilisers will automatically result in a change in the other two types of properties (Doran, 2002; Knoepp *et al.*, 2000). Recycling of organic residues to arable soils has been proven to be an efficient way of improving the physical, chemical and biological properties of the soil (Odlare *et al.*, 2008).

The physical soil properties mainly constitute texture, structure, porosity, bulk density and water-holding capacity (WHC), which in turn control the oxygen availability and soil drainage capacity. For instance, a soil with poor structure will have restricted aeration, and thus the oxygen supply to the plant roots will be limited (Currie, 1962). The benefits of organic fertilisers, *e.g*.

farmyard manure, on soil physical properties have been studied extensively and it has been observed that these organic fertilisers decrease soil bulk density and increase WHC, porosity, aggregation and biological activity (Hati *et al.*, 2006; Marinari *et al.*, 2000; Simek *et al.*, 1999; Ritz *et al.*, 1997; Schjønning *et al.*, 1994). Furthermore, organic fertilisers build up soil organic matter, which is a key attribute of soil fertility as it acts as a nutrient sink, enhances soil structure and promotes biological activity (Masciandaro & Ceccanti, 1999). Consistent long-term application of a balanced rate of mineral fertilisers in combination with organic manure has been shown to improve soil physical health and increase crop productivity (Hati *et al.*, 2007).

The most common chemical soil properties used to describe a soil are pH, salinity (electric conductivity), cation exchange capacity (CEC), organic matter and C/N ratio (Darilek *et al.*, 2009). Of these, organic matter, CEC and soil pH probably have the most profound influence on soil microbiological function and plant nutrient availability (Bagayoko *et al.*, 2000; Modaihsh *et al.*, 1989). For instance, pH influences the solubility of many nutrients, with most nutrients being more available at a pH between 5.5 and 7.5 (Skinner *et al.*, 1992). The effects of applying organic fertiliser vary depending on the type of organic residue used. Short-term application of cattle manure increases the pH and thereby the quantity of plant-available P and K (Whalen *et al.*, 2000). Application of organic fertiliser improves the CEC of the soil and, hence, results in increased nutrient availability (Lehmann *et al.*, 2003). Furthermore, N fertilisation can cause acidification by release of hydrogen ions (H^+) from nitrification when NH_3 is oxidised to NO_3^- (Hansen & Mullins, 2006). The C/N ratio of the organic matter is an important indicator of N turnover in the soil and N availability to the plant and microorganisms. In soils amended with organic material with C/N ratios below 20, net N mineralisation dominates, while at higher C/N ratios net assimilation is the dominating process (Myrold, 1999).

Biological properties play important roles in soil fertility and nutrient cycling. Microorganisms, *i.e.* bacteria, archaea and eukarya (*e.g.* fungi), can have direct or indirect effects on soil fertility through decomposition of organic matter and nutrient transformation processes (Diacono & Montemurro, 2010). Application of organic fertilisers promotes growth of beneficial organisms that are responsible for nutrient cycling (Liu *et al.*, 2009). In addition, soil microorganisms have positive effects on the physical properties of the soil by creating stable aggregate structures through their exudation of extracellular polysaccharides, which tend to improve soil aeration and drainage of water,

and facilitate root penetration (Bronick & Lal, 2005). It is well known that N, P and K are important elements for crops and it has been shown in several studies that the majority of the nutrients released by microbial decomposition are available to plants (Kalburtji *et al.*, 1997; Lee & Butler, 1977). Nitrogen may be the most important element, as it is needed in large quantities by soil microorganisms and plants to build up their biomass. Nitrogen is usually added to the soil either by fertilisation with inorganic or organic fertilisers or through stimulation of bacterial N fixation of atmospheric N_2 (Babiker *et al.*, 1995). Furthermore, not the entire N in organic fertilisers is present in a form available to the roots, as some of it is organically bound, and therefore has to be converted to mineral form such as NH_4^+ or nitrate (NO_3^-) by soil microorganisms before it is ready to be taken up by the crop. Moreover, microbial activity can lead to losses of gaseous N to the atmosphere, mainly nitric oxide (NO), nitrous oxide (N_2O) and N_2. Soil microbial activities are regulated by soil properties such as the C/N ratio of organic matter, oxygen status, soil pH, soil moisture, temperature and aeration (Ciarlo *et al.*, 2007; Yanai *et al.*, 2003; Zheng *et al.*, 2000; Maag & Vinther, 1999; Goodroad & Keeney, 1984). Therefore, any change in these properties can cause a direct or indirect effect on the soil microorganisms. Some microbial activities, such as nitrification, are particularly sensitive to changes in soil properties, which make them suitable as indicators of disturbance in the soil system (Dick, 1992).

2 Aims and objectives of the thesis

The overall aim of this thesis was to evaluate the effect of fertilising arable soil with biogas residues. Specific objectives were to study some selected new types of BRs and compare them with conventional animal manure with respect to their:

1. Potential to provide crops with the necessary nutrients, mainly N, which was studied under laboratory conditions to observe short-term effects and under field conditions to reveal long-term effects.

2. Influence on soil microbial activities and bacterial community structure in several projects under both laboratory and field conditions in order to evaluate short- and long-term effects.

3. Influence on N_2O emissions in different soil types, which was studied in short laboratory incubations under controlled conditions.

The above perspectives are discussed in the thesis based on the results reported in **Papers I-VI,** structured into three chapters: Crop yield (**Papers I** and **II**), soil microbiology (**Papers I**, **II**, **III**, **IV** and **V**) and nitrous oxide emissions (**Papers V** and **VI**).

3 Crop yield

Like other organic fertilisers, BRs contain N and various macro- and micro-nutrients needed by plants for growth. The level of nutrients may differ from one type of BR to another (**Paper I**). In fact, the different types of organic material used to feed biogas processes are the most likely reason why the BRs vary considerably in nutrient content. Therefore, the performance of BRs as fertilisers can be expected to vary. Most studies to date have focused on investigating one single type of BR (Bougnom *et al.*, 2012; Odlare *et al.*, 2011; Svensson *et al.*, 2004), which is not enough to draw general conclusions about their general fertilisation value. One obvious way of reporting the fertiliser value of BRs is to compare them with well-known fertilisers such as animal manures and mineral fertilisers. Animal manures have probably been used since the Neolithic era began, with domestication of cattle and pigs, and have proven to be valuable and trusted fertilisers on most agricultural soils (Materechera, 2010; Möller *et al.*, 2008; Ghosh *et al.*, 2004; Chadwick *et al.*, 2000; Jackson & Smith, 1997; Schjønning *et al.*, 1994). Mineral fertilisers are recognised as the most convenient and perhaps the best fertiliser if added properly, because they usually contain the three essential macro-nutrients nitrogen, phosphorus and potassium (NPK) in the proportions needed by the crop and in a form ready for immediate uptake by the plant (Martin-Ortiz *et al.*, 2009). Several studies have shown that fertilisation with anaerobically digested cattle slurry and pig slurry results in higher yields than application of commercial fertilisers such as compost and mineral fertilisers (Chantigny *et al.*, 2008; Kocar, 2008). However, these results may not be applicable to all types of anaerobically digested residues generated from a wide range of organic wastes. Therefore, there is a need for broader studies to evaluate the residues originating from different sources. Ideally, evaluation of a fertiliser should be carried out under field conditions. However, field studies can be expensive and difficult to perform but also difficult to interpret due to the fluctuating weather

factors. Therefore, performing initial experiments under laboratory conditions can be helpful as a first step in the evaluation process by providing detailed knowledge that may be difficult to obtain under field conditions (Falk & Heckman, 2009). This chapter discusses the effect of BRs on crop yield based on the results of the short-term laboratory experiment reported in **Paper I** and the long-term field trial reported in **Paper II**.

3.1 Short-term effect on spring wheat yield

The performance of four types of BRs (BR-A, BR-B, BR-C and BR-D) on spring wheat yield was studied in a pot experiment under controlled conditions and compared with the performances of pig slurry (PS), as a conventional organic fertiliser, and NPK (30:11:24), as a typical mineral fertiliser (**Paper I**). The BRs were produced from a wide range of substrates: slaughterhouse waste and source-separated organic household waste (BR-A and BR-C); distiller's waste from ethanol production and cereals (BR-B); and silage from ley, source-separated organic household waste and sludge from grease traps (BR-D). Furthermore, the operation temperature for the biogas reactors was mesophilic for BR-A and BR-D, and thermophilic for BR-B and BR-C. The retention times varied between 40-50 days for BR-A, BR-B and BR-C, and it was 20 days for BR-D. Spring wheat was cultivated in pots and incubated in a growth chamber set to Swedish summer conditions for a growth period of 77 days. The fertilisers in the study were applied at three rates corresponding to field rates of 35, 70 and 140 kg NH_4^+-N ha^{-1}.

PS application resulted in higher total crop biomass yield than the other treatments at all three fertilisation rates. We attributed the good performance of the PS to its high content of P and Zn, which were present in only low concentrations in all the BRs. The P is essential for plant growth, as all cells need this element for synthesis of membranes and nucleic acids. An increase in P level will not only result in higher wheat grain yield (Hussain *et al.*, 2008), but also an increase in the shoot to root biomass ratio (Blackshaw *et al.*, 2004). The importance of P for crop yield is discussed by Svensson *et al.* (2004), who concluded that BRs should be complemented with P to become a full fertiliser. Another explanation for the lower yield generated from BRs compared with PS could be zinc (Zn) deficiency. Zn is an important component for plant growth and development as it is a constituent of the enzymes responsible for driving many biochemical functions in the plant cell (Clemens, 2010; Rehm & Schmitt, 1997). It has been shown that Zn can affect crop yield (Sawan *et al.*, 2008) and that fertiliser should supply 22.5 kg Zn ha^{-1} for the highest economic

return in wheat production (Abbas *et al.*, 2010). Furthermore, it has been reported that Zn deficiency can occur in crops grown on sandy soil with low organic matter content (Rehm & Schmitt, 1997), which may have been the case in our study, since the sandy soil used in the pot experiment had low organic matter content. In fact the amount of Zn that was added in our experiments at fertilisation corresponding to 35, 70 and 140 kg NH_4^+-N ha^{-1} ranged between 3 and 16 kg Zn ha^{-1} for the BRs and 12 and 47 kg Zn ha^{-1} for PS.

Moreover, the lower total biomass yield in BR-fertilised pots could be related to low N availability, resulting from competition from NH_4^+ assimilation by soil microorganisms, which was seen in the dose-response test experiment (**Paper III**). The BRs stimulated net NH_4^+ assimilation by microorganisms at amendment rates ranging between 17.5 and 280 kg NH_4^+-N ha^{-1}. Furthermore, in the same experiment, PS addition resulted in net mineralisation at all used amendment rates (17.5 -1120 kg NH_4^+-N ha^{-1}). This means that all the NH_4^+ applied with the PS was available to the plant at least during the 10 first days after application. These results suggested that microbial assimilation should be taken into account when calculating crop N requirements before fertilisation with BRs.

The yield of ear biomass did not differ between the different BRs at a fertilisation rate of 35 kg NH_4^+-N ha^{-1}, while at 70 kg NH_4^+-N ha^{-1} the yield was higher in the BR-D treatment and at 140 kg NH_4^+-N ha^{-1} it was significantly higher in the BR-A treatment. At fertilisation rates of 35 and 140 kg NH_4^+-N ha^{-1}, straw yield was not significantly different among the BRs, but at 70 kg NH_4^+-N ha^{-1}, BR-D application resulted in higher straw yield compared with BR-A, BR-B and BR-C, but was not different compared with PS. The root biomass was significantly higher only at BR-D application at all fertilisation rates compared with the other BRs, but it was low compared with PS. Based on the above results, it seemed that BR-D, which was generated from source-separated organic household waste, had a tendency to produce higher crop biomass yield than the other BRs. High crop yield after fertilisation with BR derived from organic household waste compared with compost and mineral fertiliser has been reported in several previous studies (Haraldsen *et al.*, 2011; Svensson *et al.*, 2004; Båth & Rämert, 1999). However, it is difficult to obtain consistent conclusions on the fertiliser value of BRs from source-separated organic household waste, since their composition varies depending on various local factors. For instance, factors influencing the quality include the sorting criteria applied, such as the efficiency of residents in sorting properly, the design of local storage bins (containers or paper sacks) and the

system used for treatment prior to the biological step, *e.g*. use of disc screen, screw separator and magnetic separator (la Cour Jansen *et al.*, 2004). In addition, the fertilisation value of BRs can differ depending on several other factors related to the operating parameters of the biogas process such as temperature and retention time. For example, BR-D was originated from a biogas plant operated at mesophilic temperature with short retention time (20 days), which probably reduced the degradation efficiency of organic components. However, knowledge about the effect of these parameters on the fertiliser value of BRs in crop production is still scarce.

3.2 Long-term effect on barley yield

Long-term field studies are particularly valuable in determining the sustainability of agricultural practices and providing information on their environmental effects. Therefore, a field trial was established in autumn 1998 at a site located in east-central Sweden (59°37'N, 16°33'E), where the performance of BR-E applied to a crop rotation consisting of barley was evaluated and compared with compost and a mineral fertiliser (NPS) (**Paper II**). The BR-E used during the first years of the trial was obtained from a biogas plant in Stockholm, where source-separated household waste was co-digested with restaurant waste. From 2005 the BR-E was collected from the municipal biogas plant in Västerås, close to the study site, where the biogas was produced from source-separated household waste and silage from a ley crop. The compost used for comparisons was produced at the municipal composting plant in Västerås, where source-separated household waste (70%) was mixed with chopped park and garden litter (30%) before composting. The N in the NPS mineral fertiliser was made up of 50% NH_4^+-N and 50% NO_3^--N. In the field trial, all fertilisers were applied at a rate corresponding to application of total N (mineral plus organic) at 100 kg ha^{-1}.

In 2006 the yield of barley did not differ significantly between the plots fertilised with BR-E and compost and the unfertilised plots, although there was a trend for higher yield in the BR-E treatment (**Paper II**). NPS gave the highest yield, which was probably due to its high content of ready available mineral N. However, in terms of mean values for all four years when barley was grown, *i.e.* 1999, 2001, 2004 and 2006, then BR-E gave significantly higher yield than compost and the unfertilised control (**Paper II**). One reason for the better performance of BR-E is that this material contains higher amounts of mineral N in the form of NH_4^+ (Massé *et al.*, 2007), whereas compost contains only small amounts. The N application rate in the field trial was relatively low, and

therefore a pronounced yield response to easily available N could be expected. However, the strategy of this experiment was to supply rather low amounts of N, thereby allowing the capacity of the organic wastes to deliver plant-available N to be assessed. If excess N had been supplied from the start, these effects could have been obscured.

3.3 Comparison of short-term and long-term effects

In the field trial (**Paper II**), the BR-E was applied at a rate corresponding to 100 kg total N ha^{-1}, which was equivalent to 61 kg NH_4^+-N ha^{-1}. The closest addition rate in the pot experiment (**Paper I**) was 70 kg NH_4^+-N ha^{-1}, but by constructing a calibration curve, including the fertilisation rates 35, 70 and 140 kg NH_4^+-N ha^{-1}, the ear yield at a fertilisation rate of 61 kg NH_4^+-N ha^{-1} in the pot experiment could be estimated. This allowed comparison of yield patterns between corresponding fertiliser types used in the field trial and pot experiment. In the field trial, BR-E application gave lower barley grain yield than the mineral fertiliser (**Paper II**), whereas in the pot experiment the different BRs tested gave higher wheat ear yield than the mineral fertiliser (**Paper I**). One reason for the high barley grain yield in the field mineral fertiliser treatment could be that the high intrinsic N pool of clay soil, which had been fertilised once a year during the eight-year experiment. Therefore, the combination of soil organic N pool and the mineral fertiliser, easy to be taken up, was clearly more efficient in delivering N than when BR-E and compost were added in the field trial. It has been shown that long-term application of organic residues may increase the soil organic N pool by up to 90%, acting as a resource for mineralising microorganisms in future cropping seasons by giving a slow, continuous release of N (Diacono & Montemurro, 2011). Indeed, the sandy soil used in the pot experiment had not been fertilised for several years before being collected and used, and therefore it had a generally low N pool and low nutrient status, although the P status was high. Furthermore, it displayed low intrinsic microbiological activity in terms of nitrogen mineralisation capacity (NMC) and potential ammonium oxidation (PAO) compared with 52 Swedish arable soils of various origins (Stenberg *et al.*, 1998b). Moreover, the clay soil in the field trial had higher fertility than the sandy soil used in the pot experiment, as can be seen in the unfertilised treatment (control) where barley yield in the field was not statistically different compared with BR-E and compost treatments (**Paper II**). Therefore, the efficiency of the mineral fertiliser (NPK) in the nutrient poor sandy soil used in the pot experiment was low compared with the efficiency of organic fertilisers containing not only N, P and K but also a variety of other nutrients. The effect

of soil type on crop yield should be considered when evaluating fertiliser efficiency. Thus, while soil with poor nutrient status more distinctly reveals restraints, it should also display the potential performance of a fertiliser. Hence, poor soils are probably preferable when the quality and effect of a new fertiliser are to be evaluated, but the results obtained do not reflect the real situation when using more fertile soils.

3.4 Soil microorganisms as a nitrogen source

Soil microorganisms decompose organic matter to access C and N and to extract energy for growth of new biomass. During the decomposition process, macro- and micro-nutrients are released in simple forms that can be assimilated by plants. Organic residues contain complex forms of nutrients such as proteins, which must be mineralised through enzymatic processes commonly called ammonification or N mineralisation, leading to the formation of NH_4^+ before plant roots can take it up. However, it has also been shown that plants in some cases can use proteins or amino acids directly as an N source for growth without assistance from other organisms (Paungfoo-Lonhienne *et al.*, 2008). The importance of mineralisation as an N source was studied in the pot experiment (**Paper I**). At the end of the experiment, the NMC in all treatments was assessed anaerobically according to Waring *et al*. (1964) and Stenberg *et al*. (1998a). The total N mineralised per pot was calculated assuming a constant rate of N mineralisation throughout the experimental period of 77 days. A factor of 0.5 (*i.e*. assuming a Q_{10} temperature coefficient of 2) was applied to the data from the NMC assay performed at 37 °C to adjust the rates to the lower temperature (18 °C) set in the growth chamber for the pot experiment (Stanford *et al.*, 1973). It is known that the rate of chemical reactions and biological processes generally doubles for every 10 °C increase in temperature; this is often referred to as Q_{10} for biological systems (Hartel, 1997). The Q_{10} for N mineralisation at optimum moisture level is reported to range between 1.5 and 3 (Kladivko & Keeney, 1987; Campbell *et al.*, 1984).

Table 1. *Amount of nitrogen applied and estimated amount of nitrogen mineralised at a fertilisation rate corresponding to 70 kg NH_4^+-N ha^{-1} during 77 days of a pot experiment in wheat with no fertiliser (C1), soil without plants (C2), pig slurry (PS), four different biogas residues (BR-A, BR-B, BR-C and BR-D) and mineral fertiliser (NPK)) (**Paper I**).*

Fertiliser type	Amount of ammonium added (mg NH_4^+-N pot^{-1})	Org-N added (mg pot^{-1})	Mineralised N (mg NH_4^+-N 77 $days^{-1}$ pot^{-1})	Mineralised N (kg NH_4^+-N 77 $days^{-1}$ ha^{-1})
C1	0	0	52	22
C2	0	0	45	26
PS	141	165	150	74
BR-A	141	69	69	34
BR-B	141	83	82	41
BR-C	141	42	70	35
BR-D	141	84	101	50
NPK	141	0	72	37

The calculated gross N mineralisation in the C1 control soil (with wheat plants but without fertilisation) was 52 mg NH_4^+-N pot^{-1} (Table 1). The corresponding mineralisation rates at fertilisation corresponding to 70 kg NH_4^+-N ha^{-1} ranged from 69 to 101 mg NH_4^+-N pot^{-1} in the pots fertilised with BRs, which corresponded to 34 to 50 kg NH_4^+-N ha^{-1}. The mineralisation rate of the pots fertilised with PS was 150 mg NH_4^+-N pot^{-1}, which corresponded to 74 kg NH_4^+-N ha^{-1}. The total estimated N released was within the range (14 - 155 mg N kg^{-1} in 84 days) reported by Bregliani *et al*. (2010). The results emphasise the importance of soil microorganisms as N suppliers on application of organic fertilisers. As the most easily degradable material is mineralised first the rate will gradually slow down, but will probably be enough to contribute to the mineral N supply during the following cropping seasons (Sadej & Przekwas, 2008). Our results indicate that NMC was positively correlated with the amount of organic N added to the soil. Fertilisation with different types of BRs resulted in different amounts of mineralised N. The amount of mineralised N was higher in the soil amended with PS and BR-D, which could be the explanation for the higher total biomass yield generated from these two residues (**Paper I**).

3.5 Summary

The residues generated from biogas processes have a higher concentration of NH_4^+ compared with conventional animal manure and compost, which makes their potential fertilisation value high. Most plant nutrients such as P, K, Mg and a number of other essential trace elements from the raw material fed to the

biogas process remain in the BR. All the BRs tested here had positive effects on crop yield, but these effects were of different magnitude. The BRs used in the short-term study (BR-A, BR-B, BR-C and BR-D) gave higher total crop biomass yield than mineral fertiliser but lower yield than PS (**Paper I**). The long-term study showed that BR-E gave significantly higher yield than compost and the unfertilised control, but lower yield than mineral fertiliser (**Paper II**). In the short-term pot experiment, BR-D (generated from source-separated household waste) showed a tendency to give higher crop yield than the other BRs (BR-A, BR-B and BR-C) and NPK (**Paper I**), which is in agreement with results reported in a recent study (Haraldsen *et al.*, 2011). The results from the NMC tests emphasise the importance of soil microorganisms as N suppliers on application of organic fertilisers. This is evident from the calculated high amounts of mineralised N in the soil amended with PS and BR-D, which correlated with high total plant biomass yields (**Paper I**).

4 Soil microorganisms

The soil system consists of five major components: organic material, minerals, water, air and organisms. The only living component is the organisms, consisting of microorganisms, fauna and plant roots. Different types of indigenous microorganisms exist in the soil, including bacteria, archaeans, fungi, algae, protozoa and viruses (Fierer *et al.*, 2007). Bacteria are known to be the most numerous organisms (Zwart *et al.*, 1994). The bacterial population in soil typically ranges from 10^5 to 10^9 g^{-1} soil and it has been estimated that 1 g unpolluted soil typically contains 10^6 bacterial species (Gans *et al.*, 2005). The next most numerous microorganisms in soil is the actinomycetes, numbering 10^6 to 10^7 g^{-1} soil (Wollum, 1999). However, in terms of biomass fungi are the most dominant soil microorganism, with 500 to 5,000 kg ha^{-1} (Metting, 1993) and generally, the fungal biomass is greater than the bacterial biomass in agricultural soils (Ananyeva *et al.*, 2006). The relative abundance of archaeans, a diverse and widespread group found in terrestrial and aquatic ecosystems (Schleper *et al.*, 2005), is also high, with its biomass ranging from 1 to 9% of soil microorganisms (Eilers *et al.*, 2012). About 95-99% of the bacteria in the soil are not culturable by common laboratory methods (Torsvik *et al.*, 1998; Pace, 1997; Borneman *et al.*, 1996; Amann *et al.*, 1995), but it is still possible to study them by using molecular approaches or measuring their functions (Heribert, 2001). Soil microorganisms play a major role in the decomposition of organic matter and recycling nutrients. However, soil microorganisms, like all other organisms, are affected by a variety of factors such as climate, fertiliser regime, soil moisture, temperature and various soil properties. It has been shown that application of organic fertiliser stimulates the soil microbial function (Peacock *et al.*, 2001; Pascual *et al.*, 2000; Hu *et al.*, 1999). However, it has also been shown that application of sewage sludge to soil may have adverse effects on microorganisms due to an associated increase in the concentration of heavy metals (Witter *et al.*, 2000; Dahlin *et al.*, 1997;

Chander & Brookes, 1993). Hence, the impact of fertilisation on the soil microbial system is in most cases pronounced but depends on the type of fertiliser used. Crucial questions examined in this thesis were whether the application of BRs stimulates the soil microorganisms or suppresses them, and whether all types of BRs have the same effect on the soil microorganisms. This chapter discusses these questions based on the results reported in **Papers I**, **II**, **III**, **IV** and **V**.

4.1 Bacterial community structure

Microbial communities are defined as multi-species assemblages, in which organisms live together in a contiguous environment and interact with each other (Konopka, 2009). Knowledge on microbial community structure and activity is useful for understanding changes in soil fertility and broad-scale ecosystem functioning under different fertilisation regimes (Zhong *et al.*, 2010; Ge *et al.*, 2008; Mele & Crowley, 2008; Liu *et al.*, 2006). The soil microbial community can be studied by one of several molecular biological methods based on extraction of universal nucleic acids (DNA and RNA). The general strategy for studying the genetic structure of bacterial communities is by amplifying 16S rRNA genes or functional genes using the polymerase chain reaction (PCR), where after the product can be analysed with a number of different techniques. For instance, the amplified fragment can be profiled by denaturing and temperature gradient gel electrophoresis analysis (Muyzer, 1999) or terminal restriction fragment length polymorphism (T-RFLP) analysis, or cloned and sequenced for further analysis of phylogenetic diversity (McCaig *et al.*, 1999a).

T-RFLP is one of the most frequently used so-called fingerprinting methods (Liu *et al.*, 1997) because of its relative simplicity and high reproducibility (Osborn *et al.*, 2000). The method has been used for comparative microbial community analyses in a wide range of environments such as soil, water, marine environments and plant tissues (Thies, 2007; Pérez-Piqueres *et al.*, 2006; Schmidt *et al.*, 2006; Katsivela *et al.*, 2005; Noll *et al.*, 2005; Marsh, 1999). T-RFLP is a semi-quantitative PCR-based method in which the PCR product is cleaved with a specific restriction enzyme, generating fragments of the same gene but with different lengths. By separating the PCR fragments, molecular fingerprints of the dominant members of complex microbial communities can be obtained. These fingerprint profiles can then be visualised in electropherograms and compared for different treatments and/or environments. In this thesis, the effect of BR application on soil bacterial

community structure (BCS) was studied in a short-term incubation experiment (**Paper IV**) and a long-term field trial (**Paper II**), using the T-RFLP method in both cases. The individual terminal restriction fragments (TRFs) discovered in the short-term incubation experiment was compared to DNA sequences of known identity within the database library using the software Microbial Community Anal. 3 (MiCA; http://mica.ibest.uidaho.edu/trflp.php) (Shyu *et al.*, 2007) (**Paper IV**). TRF identification by use of a database library has been proven to be consistent with results from clone libraries (Hackl *et al.*, 2004; Dunbar *et al.*, 2000). TRFs generated from the long-term field trial were also sequenced and identified by comparison with a clone library (**Paper II**).

4.1.1 Bacterial community after biogas residue application

It has been shown that application of organic fertilisers such as animal manure and compost to soil increases the accumulation of organic C, which in turn stimulates the microbial biomass and induces changes in the microbial community structure (Peacock *et al.*, 2001; Frostegard *et al.*, 1997). BR contains organic C and N, but as the raw materials have been treated anaerobically, different types of chemical changes to the organic molecules in the residue have probably occurred compared with aerobically treated materials (Field *et al.*, 1984). Consequently, adding these types of residues to soil may alter the BCS in different ways. In order to detect the effect of BRs on soil BCS, a short-term incubation experiment was set up in which sandy, clay and organic soils were incubated with two types of BRs (BR-C and BR-D) and cattle slurry (CS) for 120 days (**Paper IV**). The short-term incubation with all three residues resulted in a significantly altered BCS in the sandy soil, possibly due to growth of some bacterial species induced by the organic C and nutrient-rich BRs and CS. The observed shifts in BCS were probably also amplified by the low indigenous nutrient content and low microbiological activity of this soil at the start of the experiment compared with the clay and organic soils. Furthermore, compared with 52 Swedish arable soils of various origins, the sandy soil displayed low intrinsic activity in terms of basal respiration (B-resp), substrate-induced respiration (SIR) and NMC (Stenberg *et al.*, 1998b), which clearly indicates that nutrient levels in this soil before amendment were too low to sustain the growth and activity of microorganisms.

In contrast to the sandy soil, the BCS in the clay soil showed only small change, irrespective of the fertiliser type added. Resistance of the BCS to change was also seen in the long-term field trial, in which BR-E, compost and mineral fertiliser were applied to clay soil (**Paper II**). The inertness of the bacterial population in clay soils to change could be accounted for by its

greater indigenous bacterial diversity causing greater community stability *per se*. Theoretical and empirical work in soil ecology has postulated that higher diversity within a community also increases ecosystem stability (Tilman *et al.*, 2006; Loreau, 2000). However, T-RFLP does not reflect the richness and diversity of the whole soil bacterial community, but rather that of its dominant members. Other methods such as pyrosequencing-based analysis of DNA can provide substantially more sequence information about diversity (Roesch *et al.*, 2007; Sogin *et al.*, 2006). Based on the SIR results (**Paper II**), we assumed that the clay soil had higher microbial biomass than the sandy soil, which inferred higher bacterial diversity. The association between bacterial diversity and microbial biomass has been discussed by Cenciani *et al.* (2009). Positive and significant correlations between bacterial diversity and soil microbial biomass were also observed by Murphy *et al.* (2011) and Ndaw *et al.* (2009). Moreover, fine-textured soils such as clays host higher microbial diversity than coarse-textured soils, as shown by Sessitsch *et al.* (2001).

Identification of dominant species

The MiCA software was applied to data from **Paper IV**, where alteration in soil BCS was observed, to establish the identities of the most dominant TRFs. The T-RFLP profiles of 16S rRNA genes from bacterial communities in the amended soils revealed significant species variations between the different treatments and soil types, particularly in the sandy soil treatments (**Paper IV**). The TRF 307 (*i.e*. 307 basepair long) in the study, representing the very dominant peak in the sandy soil amended with CS, was not one of the dominant peaks in the control treatment. Furthermore, TRF 620 and TRF 622 were distinct in the sandy soil amended with BR-C and CS, but not in soil amended with BR-D and the control. The organic soil amended with BR-D had TRF 226 as its most dominant TRF, whereas this was only present as a very minor TRF in the other treatments. It should be noted that bacteria added with the BRs (BR-C and BR-D) and CS may also have contributed to the observed change in BCS. In the sandy soil amended with CS, the most dominant peak was identified as *Bacillus, Streptococcus* or an uncultured bacterium. *Bacillus* spp. is a common member of the soil microflora and its existence in CS has been shown by Bagge *et al.* (2010), whereas certain types of *Streptococcus* (*e.g*. *Enterococcus*) are frequently found in the faeces of both humans and animals. Consequently, the dominant bacterial group detected in the sandy soil amended with CS may have originated from the gut flora of cattle. The growth of these species in the soil during incubation was probably induced by the content of dissolved organic C in CS (Cleveland *et al.*, 2007). Furthermore, the

most dominant peak in the organic soil amended with BR-D was classified as a member of the *Rhodopseudomonas* group, previously shown to have strong relationships to species of *Nitrobacter* (Seewaldt *et al.*, 1982). *Rhodopseudomonas* (Burke *et al.*, 1974) but also *Nitrobacter* (Hankinson & Schmidt, 1988) have been isolated from soil with low pH, indicating that the organic soil in the present study may be a suitable environment for these bacterial groups. It is also well known that *Rhodopseudomonas* is able to degrade organic pollutants, including aromatic compounds that are usually found in organic soils (Oda *et al.*, 2001). Furthermore, some species belonging to *Rhodopseudomonas*, *i.e*. *Rhodopseudomonas faecalis*, have been isolated from an anaerobic biogas plant reactor (Zhang *et al.*, 2002). This indicates the ability of this group of bacteria to grow in biogas plant reactors under anaerobic conditions, which may explain why they dominated in the organic soil after incubation with BR-D.

In the long-term field trial, the T-RFLP profiles of 16S rRNA genes from soil bacterial communities revealed only small variations between the different treatments (**Paper II**). In order to establish the types of bacteria present in soil samples from the different treatments, a clone library was constructed from the soil treated with BR-E. In general, most cloned sequences were assigned identities as uncultured and undefined bacteria. The bacterial identities assigned to the different TRFs belonged to various genera and classes, as evident from maximum likelihood trees. For example, the sequence coupled to the dominant TRF 18 appeared to be closely related to the nitrite-oxidising genus *Nitrospira* (Attard *et al.*, 2010), whereas TRF 14 was grouped together with *Microlunatus ginsengisoli*, a chemoorganotrophic bacterium that grows on a variety of simple organic molecules (Cui *et al.*, 2007). In addition, *Bradyrhizobium*, a nitrogen-fixing bacterium (Lodwig *et al.*, 2003), *Nitrosomonas*, an ammonia-oxidising bacterium (Engel & Alexander, 1958), and *Gemmatimonas* were also found in the treated soils. In fact, *Gemmatimonas* has been isolated from an anaerobic–aerobic sequential batch reactor operated under enhanced biological phosphorus removal conditions for wastewater treatment (Zhang *et al.*, 2003). This means that this species also can grow in anaerobic biogas plant reactors and therefore it was detected in the long-term field trial as a result of fertilisation with BR.

4.2 Microbial activities

Microbial activities comprise enzymatic reactions that occur in the soil during the life cycle of microorganisms as a result of produced intracellular or

secreted extracellular enzymes (Yamasaki & Hayashi, 1982). Soil microbial enzymes are important in the degradation of organic compounds and recycling of nutrients such as N, P, sulphur (S) and other essential metals and elements to plant-available forms. Therefore, soil microbial activities play critical roles in determining the efficiency of nutrient cycling in natural ecosystems (Van Der Heijden *et al.*, 2008). It has also been shown in several studies that soil microbial activities can be used as indicators to probe changes in soil fertility (Lopes *et al.*, 2010; Araújo *et al.*, 2008; Svensson, 2002; Brendecke *et al.*, 1993). Moreover, measuring enzymatic activity is a useful tool for assessing the functional diversity of soil microbial communities or soil organic mass turnover (Kandeler *et al.*, 1999). Several different methods have been used to assess soil microbial activities related to N or C turnover (Fig. 1). However, most of these methods do not reflect the actual microbial activity but rather the potential microbial activity under optimal conditions. This section discusses the impact of BRs on soil microbial activities based on the short-term effects reported in **Papers I**, **III**, **IV** and **V**, as well as the long-term effects based on the results in **Paper II**.

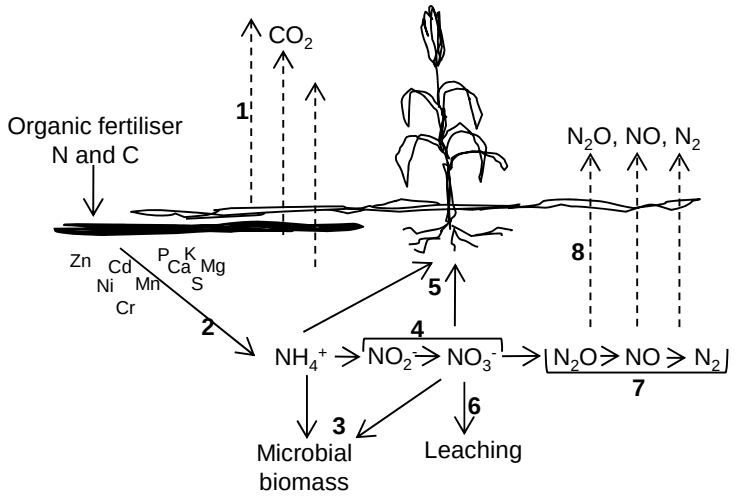

Figure 1. Schematic diagram of organic fertiliser decomposition and nutrients released by soil microbial activities. (1) Soil respiration, (2) nitrogen mineralisation or ammonification, (3) microbial ammonium and nitrate assimilation, (4) nitrification, (5) plant ammonium and nitrate assimilation, (6) nitrate leaching, (7) denitrification and (8) gaseous nitrogen losses to the atmosphere.

4.2.1 Respiration

Measuring soil respiration is probably the most common way to provide information on soil microbial activity (Michelsen *et al.*, 2004; Stenström *et al.*, 2001). B-resp, SIR, microbial specific growth rate (μ_{SIR}) and metabolic quotient (qCO_2) have proven to be useful biological parameters that can be obtained from measuring soil respiration (Stenström *et al.*, 2001). B-resp is defined as the background activity of a soil measured as production of CO_2, usually monitored over a period of a few hours up to a week after the soil has equilibrated to minimise disturbances from preceding soil sampling and handling (Martens, 1995). SIR is a measure of CO_2 emitted from the soil after saturating the microorganisms with glucose and the response can be used to derive the total soil microbial biomass. The principle of SIR is that most soil microorganisms instantaneously respond to glucose and immediately increase their respiration. The response is linearly related to the biomass C, which has been shown to be generally well correlated to the biomass index determined by the chloroform fumigation incubation method (Anderson & Domsch, 1978). The μ_{SIR} value can be obtained from the SIR response curve after fitting by non-linear regression using the formula proposed by Stenström *et al.* (2001). Furthermore, the respiration response can be used to divide the biomass into active and dormant microorganisms. The qCO_2, defined as B-resp divided by SIR, *i.e*. basal soil activity per unit of biomass, can be used as an indicator of disturbance, where a high qCO_2 value indicates a stressed soil microbial ecosystem (Wardle & Ghani, 1995).

The effect of BRs on B-resp, SIR, μ_{SIR} and qCO_2 was studied in a short-term incubation experiment (**Paper IV**). The experiment was performed using three soil types, a sandy, clay and organic soil, incubated at +20 ^{o}C for 120 days after amendment with two types of BRs (BR-C and BR-D) and CS. Non-amended soil was used as the control. An additional experiment was performed to study the immediate effect on B-resp of application of four BRs (BR-A, BR-B, BR-C and BR-D) and PS to clay soil (**Paper III**). The results from both experiments showed no negative effect on B-resp (**Papers III** and **IV**). However, this was expected since soil respiration is performed by most soil organisms and therefore, it is generally not very sensitive to metals (van Beelen & Doelman, 1997) or to organic pollutants (Walton *et al.*, 1989). On the contrary, addition of some specific toxic substances can stimulate basal soil respiration but inhibit other enzymatic reactions, as described by Tu (1992). In the incubation experiment (**Paper IV**), CS increased B-resp significantly in most treatments compared with the BRs (BR-C and BR-D), an effect that we attributed to the high amount of organic C added with CS. The CS had not

been treated anaerobically in the same way as the BRs, a process during which most of the easily available C is probably consumed into methane (CH_4) and CO_2. The added C content has been reported to be positively correlated with basal soil respiration (Enwall *et al.*, 2005). However, in the clay soil used in our incubation experiment, BRs (BR-C and BR-D) did not increase B-resp significantly compared with the control (**Paper IV**). This effect could possibly be explained by low C availability in BRs or by physical protection of the organic C against microbial attack (Balesdent *et al.*, 2000; Dexter *et al.*, 2000). In the long-term field (**Paper II**), none of the treatments produced a clear effect on B-resp.

Incubating sandy and clay soil with BR-C, BR-D and CS did not alter SIR significantly compared with the control (**Paper IV**). However, adding residues to the organic soil significantly reduced SIR compared with the control, indicating a decreased ability of soil microorganisms to utilise the organic C and assimilate it into their biomass in this particular soil. The explanation for this effect may lie in the low pH of the organic soil (pH 4.3). Inhibited mineralisation of glucose and reduced microbial biomass in acidic conditions has been reported previously by Sleutel *et al.* (2012) and Sawada *et al.* (2009). Another explanation might be that at low pH the solubility of some heavy metals increases (*e.g.* Cd, Cu, Pb and Zn) (Yobouet *et al.*, 2010) and become toxic to soil microorganisms. This has been observed to restrict the growth of microbial biomass (Aciego Pietri & Brookes, 2008; Flis *et al.*, 1993). Although Anderson *et al*. (1997) found that both the fumigation-extraction method and SIR should be used to estimate microbial biomass in soils over a wide range of pH. Heribert (2001) showed that the SIR method is less appropriate for determination of biomass in the lower pH range. In addition, acid conditions have been shown to inhibit growth of most organisms and slow down many important activities, *e.g*. N fixation, nitrification and organic matter decay (Hansen & Mullins, 2006). However, in the long-term field trial (**Paper II**), adding BR-E and compost to clay soil both significantly increased SIR.

The μ_{SIR} in the clay and organic soils showed no significant change after incubation with BR-C, BR-D and CS (**Paper IV**). In the long-trial too (**Paper II**), BR-E and compost did not produce a clear effect on μ_{SIR}. In the sandy soil, BR-C and CS treatments increased the μ_{SIR} significantly compared with BR-D and the control, indicating a shift towards more rapidly growing microorganisms (**Paper IV**). In the dose-response test experiment (**Paper III**), amendment with different BRs (BR-A, BR-B, BR-C and BR-D) resulted in different μ_{SIR}, with higher rates in soil amended with PS and lower rates in the

BR-A treatment. We speculated that PS contained more growth-stimulating materials than BRs, since it had not been treated anaerobically in the way that BRs had. Moreover, μ_{SIR} was higher in the BR-C treatment compared with the other BRs (BR-A, BR-B and BR-D), which may be related to the wide range of substrate fed to this biogas reactor, yielding a residue with a wider variety of nutrients (**Paper III**). However, the effect of application of BRs generated from different combinations of organic wastes on the soil microbial system is poorly documented.

Elevated qCO_2 was observed in the sandy and organic soils, but no change was seen in the clay soil (**Paper IV**). The high qCO_2 indicated that the soil microorganisms might have been stressed at incubation with BR-C, BR-D and CS. However, it is likely that the stress detected in the organic soil was caused by the low pH in this soil, in combination with the heavy metal content of the residues. This is in agreement with Blagodatskaya & Anderson (1998), who found that qCO_2 was strongly affected by soil pH and that the stress was higher in soil with low pH.

4.2.2 Nitrogen mineralisation

Nitrogen mineralisation is simply defined as the biological transformation of organic N to NH_4^+ (Fig. 1) and is a vital part of the N cycle (Nahm, 2005). During mineralisation, organic N compounds such as proteins and nucleic acids in crop residues, animal manure and dead microorganisms are degraded by enzymes produced by most microorganisms and soil animals under both aerobic and anaerobic conditions. Two types of enzymes are involved in N mineralisation: (1) extracellular enzymes, which degrade organic N polymers, and (2) intracellular enzymes, which degrade organic components within the cell (Myrold, 1999). Nitrogen mineralisation is an important feature due to its contribution to soil fertility by supplying microorganisms and plants with mineral N, but also for being an indirect regulator of N leaching and cause of gaseous losses (Akkal-Corfini *et al.*, 2010; Yanan *et al.*, 1997). Therefore, N mineralisation has been employed to detect general changes in soil fertility (van Beelen & Doelman, 1997). Using an anaerobic incubation method originally described by Waring & Bremner (1964) and modified by Stenberg *et al.* (1998a) the NMC was assessed in different experiments to investigate the effect of BRs on the soil ecosystem (**Papers I**, **II**, **III** and **IV**).

In a pot experiment (**Paper I**), NMC was determined in the soil 77 days after application of four types of BRs (BR-A, BR-B, BR-C and BR-D), and PS and NPK at rates corresponding to 35, 70 and 140 kg NH_4^+-N ha^{-1}. The results

showed that PS and BRs enhanced NMC in the soil at all fertilisation rates compared with unfertilised soil. Among the four BRs, BR-D increased NMC significantly compared with the other BRs. As the fertilisation rates in the experiment were based on the NH_4^+ content of the residues, large but varying amounts of organic N were added, which probably explained the different responses in soil NMC. BR-D contained a higher proportion of organic N than the other BRs (**Paper I**). This was probably due to this residue having originated from a biogas plant operated with short retention time and at mesophilic temperature, which reduced the degradation efficiency of the organic components. Furthermore, some studies have reported that BR generated from source-separated organic household waste can contain high total N (Haraldsen *et al.*, 2011; Svensson *et al.*, 2004; Båth & Rämert, 1999). NMC was also measured in different soil types after incubation with two types of BRs (BR-C and BR-D) and CS for 120 days (**Paper IV**). The results showed that the NMC increased in all soils after incubation with the organic residues. Moreover, BR-D, which was generated from source-separated organic household waste at mesophilic temperature with short retention time, also increased NMC significantly compared with BR-C in this experiment (**Paper IV**).

The long-term field study showed that both BR-E and the compost significantly increased the NMC in the soil compared with the mineral fertiliser and unfertilised control (**Paper II**). These results are in agreement with previous observations in the same long-term field study, where the BR-E, sewage sludge and pig manure were reported to increase the NMC already four years from the start of the trial (Odlare *et al.*, 2008). The increase in NMC after application of organic residues can most likely be explained by the addition of organic N and organic C, which act as energy sources for many soil microorganisms (Goyal *et al.*, 2006). Wang *et al.* (2001) and Weintraub & Schimel (2003) have shown that the NMC is correlated with organic N content, rather than with the microbial activity itself. At low organic C/N ratios, soil microorganisms in search of C and energy break down and release the N components in the organic material, resulting in net N mineralisation.

4.2.3 Nitrogen assimilation

Nitrogen assimilation is the general process by which plants and microbes take up NH_4^+ or NO_3^- for further incorporation into amino acids and nucleotides to form proteins and nucleic acids, and also to synthesise chlorophyll (Fig. 1). In the short-term incubation study on clay soil amended with BR-A, BR-B, BR-C and BR-D (**Paper III**), the NMC test displayed net assimilation at application

rates ranging between 35 and 280 kg NH_4^+-N ha^{-1} and net mineralisation at higher application rates (560 and 1120 kg N ha^{-1}). The C/N ratio in the organic fertiliser is thought to be the main regulator of these two processes. Decades of research have shown that when organic amendments with C/N ratios below 20 are added to a soil, net mineralisation will dominate, while at higher C/N ratios net assimilation should dominate (Myrold, 1999). In the present study the C/N ratio in the BRs (BR-A, BR-B BR-C and BR-D) ranged between 4.2 and 12.1, while it was 13.6 in the PS, and thus theoretically all should have stimulated mineralisation, leading to a net release of NH_4^+. However, net release was only observed at the higher doses and it therefore seems that it is not only the C/N ratio that determines which process dominates. Thus, efforts must be made to identify specific compounds in BRs that play critical roles in N turnover. It has been suggested in several studies that decomposition and mineralisation of N in organic residues can be regulated by a number of compounds such as proteins, polyphenols and soluble carbohydrates, but also cellulose-like, hemicellulose-like and lignin-like components (Thuriès *et al.*, 2002; Rowell *et al.*, 2001; Constantinides & Fownes, 1994; Lerch *et al.*, 1992; Palm & Sanchez, 1991). Furthermore, some studies have reported significant correlations between potential N mineralisation and a water-soluble fraction of the organic residue. Qafoku *et al.* (2001) observed that potential N mineralisation was strongly correlated with water-soluble organic N, but poorly correlated with the C/N ratio. In addition, De Neve & Hofman (1996) reported that N mineralisation had a higher correlation with the water-soluble fraction of crop residues than with the C/N ratio. Therefore, it seems that the C/N ratio is only a rough index of the fate of organic N in soil and that other composition variables play important roles in determining the balance between N mineralisation and assimilation processes. In addition, **Paper III** showed the importance of dose for N mineralisation. At high doses of organic residue there seemed to be a deficiency of easily available C in the soil, which limited the ability of the soil microorganisms to assimilate NH_4^+, and thus net mineralisation could be observed. This hypothesis was supported by the observations made in a supplementary glucose experiment. After adding three levels of glucose to the soil without amendment and also to soil amended with high doses of BR-D or $(NH_4^+)_2SO_4$, net assimilation dominated and increased with increasing glucose concentration (Fig. 2), which is in agreement with observations by Azam *et al.* (1993).

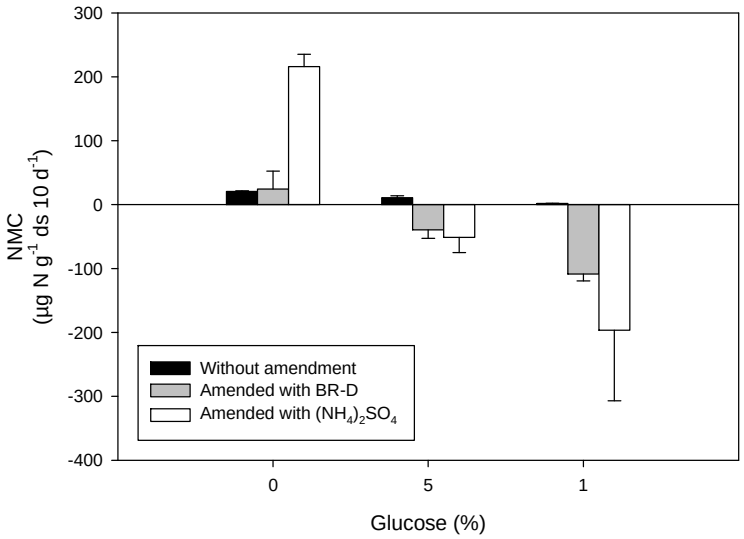

Figure 2. Nitrogen mineralisation capacity (NMC) of unamended soil and soil amended with biogas residue type D (BR-D) or $(NH_4)_2SO_4$ at a rate corresponding to 1120 kg NH_4^+-N ha^{-1} for different concentrations of glucose (0, 0.5 and 1%). Error bars represent mean ± standard deviation (s.d.); n = 3.

Adding glucose along with the highest dose of BR-D and $(NH_4)_2SO_4$ resulted in a transition from mineralisation to assimilation. The results from the supplementary experiment clearly indicated that available C was a limiting factor for the soil microorganisms. A correlation between the content of easily available C in organic residues and the amounts of N immobilised was also demonstrated by Kirchmann & Lundvall (1993). They concluded that fatty acids acted as an easily decomposable C source for soil microorganisms, stimulating assimilation of N. Another scenario could be that at high doses the amount of N present in the organic residues is larger than that required by the microbial biomass, resulting in net N mineralisation with release of NH_4^+ (**Paper III**).

4.2.4 Ammonia oxidation

Ammonia oxidation is the first step in the nitrification where NH_3 is oxidised to nitrite (NO_2^-) by ammonia-oxidising bacteria (AOB), and then in the second step NO_2^- is oxidised to NO_3^- by nitrite-oxidising bacteria (NOB) (Fig. 1) (Prosser & Martin Embley, 2002; Kowalchuk & Stephen, 2001; Zumft, 1997). Nitrification is conducted by a specialised group of chemolithotrophic and autotrophic bacteria that derive their energy from the oxidation process and C from assimilation of CO_2 (Tolli & King, 2005). Heterotrophic filamentous

fungi have also long been known as important nitrifiers, especially in forest soils (Stams *et al.*, 1990). More recently, ammonia-oxidising achaeans (AOA) have been found in various marine, limnic and terrestrial ecosystems (Leininger *et al.*, 2006). The importance of AOA as NH_3 oxidisers in soil is higher than previously thought (Kelly *et al.*, 2011), but the full extent of their contribution has yet to be revealed. It has been shown that microbial NH_3 oxidation has high sensitivity to changes in soil management practices, probably due to the complex biochemical cell machinery needed for the lithoautotrophic way of life (Kowalchuk & Stephen, 2001; McCaig *et al.*, 1999b). Therefore, determining NH_3 oxidation activity has been suggested as a suitable tool to detect toxicity (Odlare & Pell, 2009; Pell *et al.*, 1998), *e.g.* from contaminants such as heavy metals and organic toxicants (Odlare & Pell, 2009; Oved *et al.*, 2001; Phillips *et al.*, 2000; Pell *et al.*, 1998). In this thesis, a PAO assay was used as a tool to investigate the effect of BRs addition to soil (**Papers I**, **II**, **III** and **V**). PAO is defined as the specific increase in NO_2^- concentration per unit of time under non-limited substrate concentration and optimal pH (ISO 15685, 2004; Pell *et al.*, 1998).

In the pot experiment reported in **Paper I**, PAO was measured 77 days after fertilisation with four types of BRs (BR-A, BR-B, BR-C and BR-D), PS and NPK. The results showed that both BRs and PS increased PAO compared with the NPK treatment and unfertilised control. Moreover, no negative effects were detected after incubation of a sandy, clay and organic soil for 11 and 24 days with BR-C, BR-D and CS (**Paper V**). Furthermore, the long-term field trial showed that PAO increased significantly after application of BR-E (**Paper II**). Such a positive effect was also seen in a long-term study by Chu *et al.* (2008), where addition of organic fertiliser increased the nitrification potential compared with mineral-fertilised soils and the control. The higher total C in the organic fertilisers might have led to higher release of CO_2, stimulating growth of autotrophic ammonium oxidisers but also increasing NH_4^+ availability due to N mineralisation.

On the other hand, the dose-response test (**Paper III**) showed an immediate inhibitory effect of BRs (BR-A, BR-B, BR-C and BR-D) and PS addition on NH_3 oxidation at amendment rates corresponding to 35, 70, 140, 280, 560 and 1120 kg NH_4^+-N ha^{-1}. This inhibitory effect can probably be attributed to the presence of heavy metals (Rother *et al.*, 1982) and other pollutants in the organic residues. It has been shown that BRs generated from reactors fed with slaughter-house waste, organic household waste, restaurant waste and food industry waste can contain organic pollutants such as plasticisers, phenols and

pesticides, which can be toxic to AOB (Levén *et al.*, 2006; Engwall & Schnürer, 2002; Nilsson *et al.*, 2000). A toxic effect was also observed by Nyberg *et al*. (2004) when anaerobic digestion residues were applied to soil at a rate of 140 kg N ha^{-1}. However, the inhibitory effects observed in **Paper III** may last for just a very short period, after which nitrifying organisms return to their normal activity. After 24 h of incubating sand, clay and organic soil with BR-C and BR-D, PAO was not negatively affected but had on the contrary increased in the clay soil compared with the activity before amendment (**Paper V**). The temporary inhibition effect might be beneficial to the environment by delaying the oxidation of the NH_4^+ in the soil and thereby reducing losses of NO_3^- by leaching to the groundwater or as N_2 and N_2O by denitrification.

4.2.5 Denitrification

Denitrification is the anaerobic respiration process by which nitrogen oxides, mainly NO_3^- and NO_2^-, are reduced to N_2O, NO and N_2 (Fig. 1). Anaerobic conditions trigger the bacteria to use the nitrogen oxides as an alternative terminal electron acceptor to oxygen in their respiration. Denitrification is a common trait in soil and is performed by many chemoorganotrophic and heterotrophic bacteria that may comprise 0.1-5% of the total bacterial population (Philippot *et al.*, 2007). Denitrification has been proven to be sensitive to disturbances and hence is ideal for detection of the presence of contaminants such as heavy metals, pesticides and organic toxicants (Odlare & Pell, 2009; Pell *et al.*, 1998). Denitrification activity was investigated here after application of different types of BRs and animal slurry in short-term (**Paper III** and **V**) and long-term (**Paper II**) experiments. In these experiments, potential denitrification activity (PDA) was used as a tool to detect the effect of BRs on denitrification activity. PDA was determined in soil samples under optimal conditions for the denitrifying enzymes by use of the short-incubation C_2H_2 inhibition method described by Pell *et al.* (1996).

The dose-response experiment (**Paper III**) showed inhibitory effects on PDA in clay soil immediately after application of BRs (BR-A, BR-B, BR-C and BR-D) corresponding to 70-1120 kg NH_4^+-N ha^{-1}. A possible explanation for this inhibitory effect could be that the content of heavy metals was high in these high doses. The concentration of the copper (Cu), cadmium (Cd) and Zn in the added BRs was higher than the range reported to decrease PDA (Holtan-Hartwig *et al.*, 2002). Moreover, several studies have shown that denitrification is inhibited by organic pollutants, for example, polyaromatic hydrocarbons (PAHs) (Roy & Greer, 2000; Richards & Knowles, 1995), which have been found in anaerobically digested waste (Christensen *et al.*, 2004). In contrast to

this, addition of PS resulted in increased PDA with increasing application rate, but heating PS at 80 ºC for 15 minutes prior to addition resulted in three times lower PDA compared with untreated slurry (**Paper III**). The inhibitory effects of BRs on PDA could probably be considered beneficial for the environment, since they, at least temporarily, reduce the gaseous losses of N. The low PDA in soil amended with heat-treated PS indicates that the slurry itself contained microorganisms or enzymes with denitrifying abilities. This phenomenon has not been reported previously, but offers a credible explanation for the generally large evolution of N_2O observed after fertilisation with PS (Arcara *et al.*, 1999). It is noteworthy that the inhibitory effects on PDA caused by the BRs, interpreted as possible effects of heavy metals, were not seen in soil amended with PS, even though PS had the highest content of heavy metals. One explanation could be that the denitrifying microorganisms present in the slurry were adapted to the higher concentration of heavy metals in that material. Moreover, no negative effects were detected on PDA measured 24 h and then 11 and 24 days after application of two types of BRs (BR-C and BR-D) and CS to three soil types (**Paper V**). These results indicate that the effect observed in the dose-response experiment only lasts for a short time (**Paper III**). Moreover, the long-term field trial experiment showed that consistent fertilisation with BR-E increased PDA (**Paper II**).

4.3 Bacterial community structure and microbial activities

Data from the study reported in **Paper IV** suggest that the impact of application of BR-C, BR-D and CS to soil on BCS and microbial activity varies depending on soil type, which is consistent with observations made by Van Diepeningen *et al.* (2006). The community composition showed clear differences between the different non-amended soil types, with distinct clustering of the bacterial communities of each soil type, based on T-RFLP profiles, as evident from the non-metric multidimensional scaling (NMS) ordination plots (see Fig. 2a in **Paper IV**). Before amendment, the separation of bacterial communities among soil types in the NMS plot suggests that this was driven by microbial activity levels. For instance, the vectors corresponding to B-resp, SIR and NMC were located in the same direction and closer to the bacterial communities of the organic and clay soils. Thus, these activities were also higher than in the sandy soil. At the same time, μ_{SIR} and qCO_2 were higher in the sandy soil and located closer to it, indicating growth and possible stress of the microorganisms in this soil during incubation. Upon amendment with the different residues, the communities were still clustered based on soil type (see Fig. 2b in **Paper IV**). These results are in agreement with those of Girvan

et al. (2003) and Larkin *et al.* (2006), who proposed that soil type is the primary determinant of the bacterial community composition in arable soils rather than different amendments or management practices. Furthermore, the NMS ordination plot in **Paper IV** disclosed that the soil pH was a determinant of the BCS in the three soil types, as also confirmed by Mantel's test (*i.e.* rejection of the null hypothesis of no relationship between the two matrices). In a recent study by Nacke *et al.* (2011), it was shown that soil pH is responsible for the discrimination of soil microbial communities. This study found that the relative abundances of bacterial groups at different taxonomic levels correlated with the soil pH, but little or no relationship was seen to management type and other soil properties. The shift in BCS of the sandy soil seen in our study appeared to be significantly correlated with μ_{SIR} (see Fig. 2b in **Paper IV**), indicating a trend towards more rapidly growing species as a consequence of the treatment with organic residues. As discussed above, the sandy soil had a low nutrient content, which was also reflected in a lower microbial biomass, indicative of growth limitation of some bacterial species. Therefore, BCS changed significantly as a consequence of adding organic substances and nutrients needed for bacterial growth, which is in agreement with observations made by Chu *et al.* (2007). In addition, after amendment the vector in the NMS ordination plot representing qCO_2 moved closer to the bacterial communities of the sandy and organic soils where this parameter had increased, indicating stressed microbial communities.

The microbial activities in all three soil types showed some changes after amendment with organic residues, while the BCS of the clay soil was not altered significantly. This finding indicates that two different types of microbial shifts occur in residue-amended soils, one faster shift directly correlated with functional properties and one slower shift that may be attributed to altered microbial community composition. Thus, a shift in microbial activity does not necessarily mean that a shift in community structure can be detected. A similar conclusion was reached by Lucas *et al.* (2007), who showed that small additions of N-containing organic compounds caused changes in the soil microbial community structures, but community changes did not necessarily have an impact on extracellular enzyme activity. This conclusion was further supported by Stark *et al.* (2008), who reported that no direct relationships exists between microbial community structure, enzyme activities and N mineralisation. However, it is possible that if we had targeted specific functional groups we would have found a link between structure and function. In the study by Enwall *et al.* (2007), only a very weak link was found

between the change in total BCS and community functions, but a rather strong link when the AOB were specifically targeted.

4.4 Summary

The effect of BRs on soil microbial activities and soil BCS varies depending on the type of organic residue added and type of soil. BRs can have different chemical composition due to the different substrates used to feed biogas plant reactors, which will affect the soil microorganisms differently when added to soil. Organic residues are usually applied based on their NH_4^+-N concentration, and therefore treatment with digested residues resulted in lower total C input compared with addition of animal slurries such as PS and CS. This may make an additional contribution to the differences in microbial activity observed when comparing the effects of fertilising with BRs with those of conventional animal slurries. No negative effects on soil respiration were observed, which shows that the microbial biomass was not affected by application of BRs, except in soil with low pH (organic soil), where SIR was reduced significantly after incubation with BR-C, BR-D and CS (**Paper IV**). The initial effect of BRs on soil microbial enzymes involved in N transformation varies, as shown in the different enzymatic tests used in **Paper III**. Based on results from the dose-response test of NMC, N availability in soil can be predicted to be limiting for plants, especially when fertilised with BRs (BR-A, BR-B, BR-C and BR-D). Shortly after applying BRs, microbial N assimilation will compete with the plant roots for N. Furthermore, nitrification and denitrification activity in soil are both inhibited immediately after soil amendment with BRs (**Paper III**). The short-term effects, *i.e*. at days 1, 11, 24 (**Paper V**), 77 (**Paper I**) and 120 (**Paper IV**) and the long-term effect, *i.e*. 8 years (**Paper II**) after BR application showed no negative effect on NMC, PAO and PDA. Amending three soil types (sandy, clay and organic) with BR-C and BR-D altered BCS significantly in sandy soil, as shown in the 120-day incubation experiment (**Paper IV**). Changes in specific BCS due to the residue type occurred primarily in sandy soil. The difference in BCS change in soil is more pronounced between BRs and CS application than between application of different types of BRs. This suggests that BRs and CS have distinctly different effects on soil BCS. In clay and organic soils where the indigenous microbial biomass is high, no significant shift in BCS is likely to occur within a short time frame (**Paper IV**), which was also the case on long-term application of BR-E to clay soil (**Paper II**).

5 Nitrous oxide emissions

5.1 Background

Recycling organic residues to arable soils can carry the risk of increased emissions of greenhouse gases (GHG) such as N_2O, CH_4 and CO_2 (Flessa *et al.*, 2002), all of which contribute to global warming. N_2O is produced in the soil by microbiological activity through nitrification and denitrification (Bremner, 1997). N_2O has a global warming potential 310 times higher than that of CO_2 due to its absorptive capacity and long persistence in the atmosphere, *i.e.* 114 years (Forster *et al.*, 2007). Furthermore, emissions of N_2O have received great attention due to the potential of this gas to destroy the ozone layer protecting the earth from ultraviolet radiation from the sun (Ravishankara *et al.*, 2009). In addition, emissions of N_2O result in losses of N from the soil, thereby withdrawing availability of this nutrient from arable land (Whalen, 2000). The agricultural sector accounts for 65-80% of the total global emissions of N_2O, which means that agriculture represents the largest anthropogenic source of this gas (IPCC, 2007). A number of factors have been identified as affecting N_2O emissions from soils either directly or indirectly, of which soil moisture (Blackmer *et al.*, 1982), oxygen concentration (Hwang & Hanaki, 2000), mineral N (Peng *et al.*, 2011), available C (Velthof *et al.*, 2003), soil texture (Maag & Vinther, 1996), pH (Šimek & Cooper, 2002) and temperature (Goodroad & Keeney, 1984) are thought to be the most important. The overall soil moisture is probably the most important factor regulating N_2O production by directly affecting the cellular activities of nitrifying and denitrifying bacteria and influencing the solubility and diffusion rate of organic C, mineral N and N_2O in the soil system. In addition, soil moisture affects the reduction of N_2O to N_2, thereby influencing the gaseous composition of the gaseous emissions (Blackmer *et al.*, 1982). Soil moisture also indirectly affects N_2O emissions through dissolution and diffusion of organic C, making it

available to aerobically respiring organisms and contributing to lowering the partial pressure of oxygen in the system (FAO, 2001). The main soil microbial processes leading to emissions of N_2O are illustrated in Fig. 3.

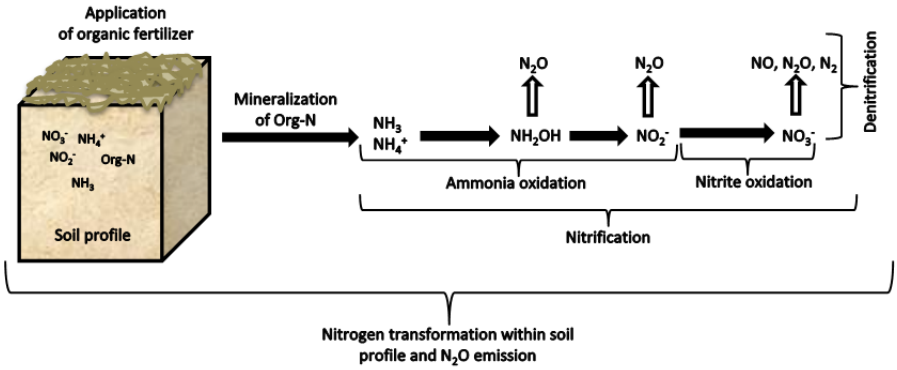

Figure 3. Nitrous oxide emissions by nitrification and denitrification during biological transformation of N.

5.1.1 Nitrification as a source of N_2O

Nitrifying bacteria are a well-known source of N_2O in soils (Davidson, 1992). Nitrifiers are mainly chemolithoautotrophic bacteria obtaining their energy from oxidation of NH_3 (Hofman & Lees, 1952). Nitrification is performed by two different groups of bacteria belonging to the β and γ subgroups of proteobacteria: AOB gaining energy from oxidation of NH_3 to NO_2^- and NOB oxidising NO_2^- to NO_3^- (Fig. 3). Common AOB in the soil belong to the genera *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus* and *Nitrosovibrio,* whereas NOB are commonly represented by *Nitrobacter* and *Nitrospira* (Myrold, 1999). AOB are widely distributed in soils, rocks, freshwater and seawater, and in wastewater treatment systems (Koops *et al.*, 2006; Teske *et al.*, 1994). Ammonia oxidation is catalysed by the enzyme ammonia monooxygenase, which oxidises NH_3 to hydroxylamine (NH_2OH). The intermediate NH_2OH is further oxidised to NO_2^- by another enzyme called NH_2OH oxidoreductase. The NO_2^- produced is further oxidised by NOB in a one-step reaction to NO_3^- using the enzyme nitrite oxidoreductase. It has been shown that N_2O can be produced through chemical decomposition of the intermediate NH_2OH (Fig. 3) (Poth & Focht, 1985; Hooper & Terry, 1979), especially under oxygen-limited conditions (Bremner, 1997). In addition, using $^{14}NH_4^+$ and $^{15}NO_2^-$ it has been shown that N_2O, at low oxygen tension, can also be produced by NO_2^- reduction (Poth & Focht, 1985). Recently, high numbers of putative ammonia monoxygenase genes in archaeans belonging to the

phylum Crenarchaeota have been detected in soil, indicating high ammonia-oxidising potential of this prokaryotic group (Kelly *et al.*, 2011), alsosuggesting contribution to N_2O emission (Cabello *et al.*, 2004). However, nitrogen metabolism is much less known in archaea than in bacteria.

5.1.2 Denitrification as a source of N_2O

Denitrification is a biological process responsible for gaseous losses of N_2O and N_2 from soil. Anaerobic conditions prompt the denitrifiers to use nitrogen oxides as an electron acceptor when oxygen is limited. The main enzymes involved in the denitrification process are NO_3^- reductase, NO_2^- reductase, NO reductase and N_2O reductase, catalysing the chain of reductions from NO_3^- to NO_2^- and then further to NO and N_2O and finally, in most cases to N_2 (Knowles, 1982). Under certain conditions, such as those in acidic soil, N_2O reductase is inhibited, so the N_2O/N_2 ratio in the gaseous product will increase (Šimek *et al.*, 2002), while in conditions of high soil moisture and low oxygen the N_2O/N_2 ratio will be low (Ciarlo *et al.*, 2007). Denitrification can lead to emissions of NO, which even though it has very short lifetime can still be emitted from the soil (Jeffrey Peirce & Aneja, 2000; Remde & Conrad, 1991). Denitrification is performed by a wide range of bacterial species (Knowles, 1982), and potent denitrifiers have been identified as belonging to the genera *Achromobacter*, *Alcaligenes, Bacillus*, *Burkholderia-Ralstonia*, *Clostridium, Moraxella*, *Pseudomonas*, *Streptomyces* and *Xanthomonas-Frateuria* (Chèneby *et al.*, 2000; Myrold, 1999; Zumft, 1997). Furthermore, N_2O can be produced by other groups of microorganisms such as archaeans (Cabello *et al.*, 2004) and fungi (Ma *et al.*, 2008). However, it has been shown that N_2O emissions are driven by bacteria rather than archaeans in N-rich grassland soils (Di *et al.*, 2010).

5.2 Fertilisation and N_2O emissions

Application of mineral fertilisers and animal manures to soils has frequently been shown to increase N_2O emissions (van Groenigen *et al.*, 2004; Akiyama & Tsuruta, 2003; Petersen, 1999; Olivier *et al.*, 1998; Beauchamp, 1997). Therefore, fertilisation with BRs can also be expected to increase N_2O emissions. However, total emissions and flux patterns of N_2O after fertilisation with BRs may differ to those from mineral fertilisers and conventional animal manures due to the fact that BRs originate from technical anaerobic processes. In a biogas reactor the raw feedstock is biochemically altered in a different way to digestion in the gastro-intestinal system of animals (Field *et al.*, 1985). Moreover, the raw material fed to the biogas process is more variable than the

feed given to animals. Anaerobic digestion increases the NH_4^+ concentration and pH in the processed material, but also decreases the organic dry matter and total organic C content. Therefore, the C/N ratio in BRs is lower than that in the raw organic substrate (Field *et al.*, 1984). During the biogas production process the most easily degradable organic C is consumed and other organic components are partly degraded, resulting in digested residues with more recalcitrant material. Such material when applied to soil might be more limiting for microbial activities than conventional organic fertilisers (Ernst *et al.*, 2008; Clemens *et al.*, 2006; Kirchmann & Witter, 1992). To get a full picture of the effects of different BR types on N_2O emissions, studies under both controlled conditions and field conditions are needed. However, in the field, climate factors are unstable, which makes the results difficult to interpret. In addition, field experiments are expensive, time-consuming and labour-intensive to conduct because of the high spatial and temporal variability of N_2O fluxes from soil (Ambus & Christensen, 1994). Nevertheless, field studies are important in order to quantify N_2O emissions on regional and global scales. For detailed understanding of how different fertiliser types affect and interact with the factors regulating N_2O emissions, experiments in controlled environments are needed.

In **Paper V**, N_2O emissions were studied in two laboratory incubation experiments performed by using a repacking soil core technique, which is a common method for studying GHG emissions under controlled conditions (Scheer *et al.*, 2009; Ball *et al.*, 2008; Dannenmann *et al.*, 2008; Jan Dick, 2006; Butterbach-Bahl *et al.*, 2002). The experiments were performed by amending three soil types (sandy, clay and organic) with two types of BRs (BR-C and BR-D) and CS. The amendments were added at a fertiliser rate corresponding to 70 kg NH_4^+-N ha^{-1}. In the first experiment N_2O fluxes were measured 12 times during 24 days, with the first gas sampling made 24 hours after amendment and thereafter on days 2, 4, 6, 8, 10, 11, 13, 15, 17, 20 and 24. The second experiment aimed to quantify the N_2O fluxes at times when peaks were expected as inferred from the first incubation experiment. Furthermore, PAO and PDA, NH_4^+ and NO_3^- as well as total C (Tot-C) and N (Tot-N) were analysed at the times when N_2O was expected to peak.

In **Paper VI**, N_2O emissions were studied in a clay and organic soil after amendment with two types of BRs: (1) a conventional biogas residue (BR-F) from a large-scale biogas plant treating ley crop silage and source-separated household waste, and (2) a solid-fraction residue (BR-G) from a pilot-scale biogas reactor treating the same material as above, but with the residues

processed in an ultra-filtration membrane unit to separate the solid fraction from the liquid fraction of the slurry. This mechanical solid-liquid separation of the digested residue was done to reduce the dry matter content of the recirculated process water and thereby increase the capacity to load and treat more material in the reactor. This allows more biogas to be produced and, hence, more electricity (Bauer *et al.*, 2009). Another benefit with solid-liquid separation is that it yields a BR with a lower water content, which makes the product easy to store and handle and lowers the costs of transportation and spreading. In the experiment the flux of N_2O was measured 24 hours and 7 days after amending the two soils with non-separated slurry and the solid fraction of the BR.

5.2.1 Effect of biogas residues on N_2O emissions

The results in **Paper V** showed that the BRs (BR-C and BR-D) and CS yielded substantially different flux patterns of N_2O and also differed in total N_2O-N losses. Amending the sandy soil with BR-D and CS led to higher total N_2O-N losses (0.32 and 0.18 mg N_2O-N m^2, respectively) than amending it with BR-C (0.02 mg N_2O-N m^2). This can probably be attributed to the higher levels of organic N and organic C in BR-D and CS than in BR-C *per se*. In fact, the actual organic N and Tot-C in the doses, corresponding to 70 kg NH_4^+-N ha^{-1}, were higher in BR-D and CS and lower in BR-C. In contrast, addition of BR-C to clay soil increased the N losses significantly compared with BR-D and CS (0.25, 0.15 and 0.02 mg N_2O-N m^2 for BR-A, BR-B and CS, respectively). The organic C content in BR-C was obviously not high enough to provide the denitrifying bacteria with the energy required to further reduce N_2O to N_2, as apparently was the case for BR-D and CS. It has been shown that applying C-rich material to fine-textured soil at relatively high soil moisture contents decrease the N_2O/N_2 ratio in the gas emitted (Weier *et al.*, 1993; Cady & Bartholomew, 1960). Therefore, we believe that the different content and quality of organic C were responsible for the higher emissions observed for the BRs. Organic soil amended with BR-C and BR-D showed lower total N_2O-N losses compared with soil amended with CS (0.09, 0.08 and 0.31 mg N_2O-N m^2 for BR-A, BR-B and CS, respectively). Furthermore, the residue type showed a clear effect on the peak size, with amendment with CS generating a larger peak than amendment with BR-C and BR-D. This may be related to both the quantity and quality of the C in CS (Florinsky *et al.*, 2004; Velthof *et al.*, 2003). The organic C in BRs may not be of the same quality as that in CS (Ernst *et al.*, 2008) because during anaerobic digestion most of the organic C will be degraded and converted to CH_4 and CO_2 (Venkata Mohan *et al.*, 2005; Angenent *et al.*, 2004).

Amending clay and organic soils with BR-G increased the N_2O flux significantly compared with the unfiltered BR-F at days 1 and 7 (**Paper VI)**. The high N_2O flux after application of BR-G could probably be explained by stimulated overall denitrification due its high dry matter content containing organic C, as described by Bauer *et al.* (2009). The filtration process resulted in a doubling of the dry matter content of the solid fraction compared with the unfiltered residue. Another interpretation could be that despite its high quantity of organic matter, the solid fraction did not contain sufficient amounts of easy available C to support the denitrifiers in completing the full denitrification pathway to N_2, thus leaving N_2O as a dominant end product. It has been reported previously that the liquid fraction of anaerobic digested residues contains most of the soluble C, such as volatile fatty acids (Lee *et al.*, 2000). Yet another factor to consider is that the pH of the residues was considerably higher in BR-G (pH 10) than in the unfiltered BR-F (pH 7.5), which should have led to increased soil pH after application and lower N_2O emissions, especially in the organic soil. However, BR-G still generated higher N_2O emissions than BR-F, and thus it seems that short-term application may not affect the soil pH and N_2O emission. Therefore, the long-term effect of fertilisation with the solid fraction of BRs needs to be investigated to determine its impact on soil pH and N_2O emissions.

5.2.2 Effect of soil properties on N_2O emissions

The physical, chemical and biological properties vary considerably among soil types, which probably affect the N_2O emissions in different ways. In this thesis, N_2O emissions were studied in sandy, clay and organic soils after application of different types of BRs (**Papers V** and **VI**). The sandy soil was coarse-textured and had low organic material content, the clay soil was fine-textured and the organic soil had high amounts of organic material and low pH. The coarse-textured sandy soil probably had good aeration compared with the fine-textured soil, which probably affected the soil microbial activities. The fine-textured clay soil had higher WHC than the sandy soil. In addition, the organic soil had low pH, which has been proven to affect the availability of soil microorganisms and nutrients (Rousk *et al.*, 2009). The differences in soil properties described above indicate that the effect of a specific BR on N_2O emission pattern could differ between the soils. However, the relationship between fertiliser type and soil properties in terms of N_2O emissions is complex due to the fact that the emissions are regulated by many factors.

Soil physical properties

The main soil physical properties are texture, structure, porosity, bulk density and soil water content, all of which can directly or indirectly regulate N_2O emissions. The three soil types used in the incubation experiments (**Papers V** and **VI**) represent a wide range of soil physical properties. The coarse-textured sandy soil was dominated by macrospores, which result in characteristic fast gas diffusion and good aeration, stimulating nitrification and lowering denitrification activities, but also lessening the likelihood of N_2O being reduced to N_2 (FAO, 2001; Maag & Vinther, 1996). Rapid gas diffusion in the sandy soil could be the reason for the higher N_2O flux observed in this particular soil compared with the clay soil (**Paper V**). In the clay soil, the N_2O and total N_2O-N losses were significantly lower after amendment with CS compared with those in the sandy and organic soils. This is in agreement with observations from the experiment reported in **Paper VI**, where the fluxes of N_2O were lower in the clay soil than in the organic soil after amendment with either BR-F or BR-G. It could be speculated that a clay soil is more prone to develop small soil aggregates with micropores in their centres containing water, thereby restricting oxygen diffusion. Such anaerobic microsites will stimulate denitrification activity and also the consumption of the intermediate N_2O from either denitrification or nitrification (Arah *et al.*, 1991). This assumption is supported by the high PDA observed in the clay soil compared with the sandy soil in **Paper V**. As a result, applying C-rich material such as CS to a clay soil will probably provide denitrifying bacteria with the C they require enhancing N_2O reductase activity thereby increasing the relative proportion of N_2 in the emission product.

Soil chemical properties

Important soil chemical properties affecting N_2O emissions are pH, cation exchange capacity (CEC), organic matter content, and content of macro- and micro-nutrients. Soil mineral N, in terms of both content and form (NH_4^+ or NO_3^-), is perhaps the most important nutrient regulating N_2O emissions (van Groenigen *et al.*, 2004). In the experiment reported in **Paper V**, we observed that amending three soil types with BR-C, BR-D and CS led to N_2O emissions peaking at different times. In the sandy soil, N_2O peaked at day 11 in all treatments and in the clay soil the peaking time varied between days 1, 6 and 13 for the different residues, whereas in the organic soil the peaks were observed at day 1 for all treatments. The results reported in **Paper VI** showed that N_2O peaked at day 1 in both the clay and organic soil. Late N_2O peaks in sandy soil were observed by Senbayram *et al.* (2009), where the N_2O peaked

one week after amendment with BR at a WHC of 65 and 85%. They attributed this delay to the low NO_3^- concentration at the start of the experiment, since they found that most of the N_2O originated from denitrification. We believe that the explanation put forward by Senbayram *et al.* (2009) could also be valid for our results, since the NO_3^- concentration was initially low in the sandy soil and high in the clay and organic soils (**Paper V**). Application of organic residues to the organic soil with low pH increased the N_2O flux significantly in both experiments (**Papers V** and **VI**) compared with sandy and clay soils, also boosted by the high NO_3^- content. It has been shown that low pH (around 4) increases the N_2O/N_2 ratio due to the inhibition of N_2O reductase (Šimek & Cooper, 2002; Šimek *et al.*, 2002). In our clay soil, the peaking time of N_2O ranged from day 1 to day 13 between the treatments with BR-C, BR-D and CS, even though the soil NO_3^- concentration was higher than in the sandy soil (**Paper V**). This could possibly be explained by the presence of hotspots making the emissions pattern unpredictable in this particular soil, as indicated by higher standard deviation compared with the other two soils (Johnson *et al.*, 2010; Von Arnold *et al.*, 2005; Velthof *et al.*, 1996). Another factor that has been identified as indirectly affecting N_2O emissions is CEC (Jarecki *et al.*, 2008). In our case, the higher CEC in the clay soil used in both studies (**Papers V** and **VI**) may have reduced the N availability through fixation of NH_4^+ to soil particles, resulting in a direct decrease in ammonia oxidation and thereby indirectly a lowering of denitrification. At day 24 the flux of N_2O had dropped to the background level in all treatments, even though the NO_3^- concentration in the three soils was high (**Paper V**). This demonstrates the importance of NH_4^+ and C for nitrification and denitrification.

Soil biological properties

Soil biological properties are defined as various activities performed by living microorganisms, soil fauna and roots. Examples of activities within the N cycle are mineralisation, nitrification and denitrification (Fig. 3). Based on the results from the incubation experiments reported in **Paper V**, PAO and PDA in the three soils at the times when N_2O generally peaked, *i.e.* day 1 and 11, could not explain the individual peaks in the soils, but were more useful in explaining the different flux patterns between soil types and total losses. PAO was low in the sandy and organic soils and higher in the clay soil, while PDA was higher in the clay and organic soils and lower in the sandy soil. This indicates that most of the N_2O flux during the first week in the sandy soil should have originated from nitrification. This was probably not the case in the clay and organic soils, where PDA was higher. However, it should be kept in mind that measuring

PAO and PDA is only indicative and in order to determine whether N_2O originates from nitrification or denitrification an alternative approach is needed, such as determining the ^{15}N-isotope abundance (Koster *et al.*, 2011; Yoshida, 1988). Using such a method, Koster *et al.* (2011) observed a rapid shift from denitrification to nitrification in soil after application of BR to soil cores. They attributed this shift to depletion of organic C, the driving force for bacterial denitrification, as indicated by decreasing CO_2 release and the presence of increasingly abundant nitrate due to ongoing nitrification. At this stage nitrification had probably become the main source of N_2O production relative to denitrification.

5.3 Summary

Application of BRs to soil will increase the N_2O emissions to different extents depending on the organic C quality in the residue added and the soil type. There is a high risk of N_2O emissions from a soil with low pH at application of either BRs or CS (**Paper V**). Application of BR solid fraction (BR-G) may increase the risk of N_2O emissions compared with the unseparated residue (BR-F) (**Paper VI**). In clay soil, the risk of N_2O emissions is higher after application of BRs compared with CS, because BRs generally contain less organic C. Thus the C supply may not be high enough to sustain denitrifying bacteria to reduce N_2O to N_2 (**Paper V**). Data based on measurements of N_2O emission and soil mineral N assays indicate that the quality of the organic C in BRs is different from that in CS, with CS perhaps containing more available organic C than BRs (**Paper V**). This is a consequence of most of the easily available organic C being consumed during digestion in the biogas process, leading to a residue containing less degradable material. However, it should be emphasised that it is still important to conduct large-scale experiments where different BRs can be tested under field conditions to validate the results presented here.

6 Conclusions and perspectives

Different types of biogas residue were studied and discussed in this thesis in terms of their fertilisation value, effect on soil microorganisms and potential to cause nitrous oxide emissions. Combining the three perspectives was useful to give a more coherent and comprehensive view of the links between the biogas plant and agricultural soil. For instance, complementary studies of crop growth and yields with analysis of microbiological mineralisation and assimilation processes were helpful in explaining the lower crop yields obtained with biogas residues compared with pig slurry. In addition, soil microorganisms are the core in the soil system, as they are responsible for beneficial cycling of plant nutrients and detrimental production of the GHG nitrous oxide.

Fertilisation value of biogas residues

The biogas residues tested had different chemical and physical properties due to the different organic substrates used to feed the biogas processes and also to variations in process parameters such as operating temperature and retention time. The high concentration of ammonium in the biogas residues makes their fertilisation value comparable to that of mineral fertilisers, which means that they can provide the crop with required nitrogen. However, in some cases the fertiliser effect may be delayed because the organic residues contain less immediate plant-available nitrogen than mineral fertilisers. This also means that an N pool of organic N will be built up in the soil and can supply the plants in following years. The residues that resulted in the lowest crop yields in our pot experiment may well display different results in other soil types due to different levels of fertility and amounts of N available from the N pool. Thus in order to fully exploit BRs in crop production, further investigations using several different soil types is needed.

Soil microorganisms

The application of biogas residues had no general short-term or long-term negative effects on soil microbial activities. However, some severe immediate inhibitory effects on the enzymes involved in nitrification and denitrification were observed on application of biogas residues. This initial effect is of concern considering soil health and provides an early warning of the presence of potential hazardous substances in the residues. However, inhibition of nitrification and denitrification also lowers N losses from the system. Our dose-response test study showed that biogas residues enhance ammonium assimilation by soil microorganisms, which might temporarily decrease nitrogen availability to the plant. However, there is a need for further studies to investigate the effect of biogas residues on nitrogen turnover in the soil, since the C/N ratio in the biogas residues could not fully explain the assimilation and mineralisation patterns observed. Biogas residues have the ability to change soil bacterial community structure depending on soil type, with sandy soil being more prone to change. This change in the bacterial community structure brought about by biogas residues is different to that caused by cattle slurry. However, in general, only small or no changes in microbial community structure could be seen after eight years of biogas residue application. Soil resistance to changes in genetic structure is beneficial, as reversion to a previous functional state is possible, for instance, if a farmer for various reasons wants to change back to the original fertiliser type after having tested a 'new' type of fertiliser.

Nitrous oxide emissions

Biogas residues, like other fertilisers containing nitrogen, can stimulate emissions of N_2O from soils. However, compared with animal slurries biogas residues have less organic carbon and high NH_4^+ concentration and therefore induce N_2O emissions from the soil in a different way. This means that the ability of biogas residues to provide denitrifying microorganisms with required carbon is restricted, which will limit the reduction of N_2O to N_2 and lead to higher N_2O emissions from some soils. Soil properties also affect the performance of biogas residues regarding N_2O emissions. Consequently, the type of organic residue and soil should be considered when trying to mitigate N_2O emissions. For instance, it could be advisable to avoid fertilisation of organic soils with C-rich material such as ordinary animal manure and instead fertilise with biogas residues. Furthermore, in clay soil the risk of N_2O emissions could perhaps be lowered by using a C-rich fertiliser at moisture

contents higher than 60% of the water-holding capacity. However, it should be emphasised that it is still important to conduct large-scale experiments where different biogas residues are tested under field conditions to validate the results presented here. Moreover, fertilisation with the solid fraction of filtered biogas residues may lead to higher N_2O emissions compared with unseparated biogas residue, especially when applied to organic soils. Further investigations are needed to identify the reason why filtered biogas residues increase N_2O fluxes. Such information can be used to guide the development of new fertilisers and devise mitigation options.

Energy perspective of biogas residues

The biogas residues tested in our study were generated from different biogas plant reactors with different operating parameters (*i.e*. substrate composition, temperature and retention time) and none of the residues originating from the processes showed negative effects on either crop yield or soil microbial activity. Interestingly, the biogas residue generated from a biogas process fed with source-separated organic household waste had a tendency to increase crop biomass yield and soil microbial activities compared with the other biogas residues. Therefore, it could be economically interesting in future to devote more effort to developing biogas processes that not only produce biogas but also high-yielding fertilisers. This also means that biogas residues should be evaluated considering the operation and management of the biogas reactor. Overall, the positive effect of using biogas residues in agriculture further enhances the importance of renewable energy as an environmentally friendly alternative, as the increasing amounts of residues associated with biogas production can be turned into an advantage as the residues should be regarded as safe and competitive fertilisers.

Acknowledgements

There is no doubt that these four years of PhD studies have taken me through different experiences that have changed me; I have learnt patience, how to solve problems and no doubt I have also become wiser. During my PhD studies I met some people who contributed to this change in me and therefore especially deserve to be mentioned and thanked.

First of all, I would like to thank my main supervisor **Mikael Pell** for guiding me through the exciting world of sciences and for his encouragement, advice, constructive criticism and all the scientific challenges given me. I am very grateful for being guided towards both scientific and personal independence. I would also like to thank you and your wife, **Hjördis**, for inviting me to your home several times and offering me wonderful dinners, thank you so much for all shared moments!

Veronica Arthurson, my co-supervisor, thanks for assisting and sharing your knowledge in molecular biology and I wish you all the best with your new science direction, a doctor setting out to become a doctor again.

My co-authors for the papers in this thesis, thank you all for the collaboration: **Harald Cederlund, Monica Odlare**, **Sigrun Dahlin**, **Kajsa Risberg**, **Lena Rodhe**, **Åke Nordberg**, **Johnny Ascue**, **Erik Jönsson**, **Emma Nehrenheim**, **Kalle Svensson**, **Johan Lindmark** and **Eva Thorin**.

I am grateful to the NL faculty programme **MicroDrivE**at SLU and its research group for having me as a member.

Also, I would like to take this opportunity to thank **Johan Schnürer** and **Bengt Guss** for all their help and advice during my PhD studies.

I would especially like to thank **Hans Jonsson** for his care about my study situation and **John Stenström** for his kindness and for giving help when it was needed.

My thanks also go to my colleagues and staff at the Department of Microbiology for creating a friendly work environment and for giving help when it was needed: **Leticia Pizzul**, **Lotta Levén**, **Sture Larsson**, **Maria Erikson**, **Volkmar Passoth**, **Sarah Hallin**, **Elisabet Börjesson**, **Maria Westerholm**, **Gunnar Börjesson**, **Karin Önneby**, **Johanna Blomqvist**, **Ievgeniia Tiukova**, **Susanne Broqvist**, **Ann-Cristine Lundquist**, **Ingemar Baselius**, **Ingemar Baselius**, **Anna Schnürer**, **Lotta Jäderlund**, **Åsa Svanström**, **Greta Hulting**, **Maria Hellman**, **Christopher Jones**, **Karin Enwall**, **Sture Larsson**, **Lars Frykberg**, **Solveig Geidnert**, **Stefan Roos**, **Bettina Müller**.

My friends **Osama Swisi**, **Mohamed Rahayem**, **Khalid Nadeem**, **Nizar Enwaji**, **Sahar Dalahmeh**, **Mohammed Al-Azzawi**, **Kheilfa El- Tarhony**, **Mohsen El-kharam**, **Mohamed Elghali**, **Omar Omar** and **Ali El-Mabsoot**, thank you all for your friendship and all shared moments. Special thanks to **Guma Abdeldaim** for helping me when I arrived in Sweden for the first time.

Many thanks also go to the Libyan Government, represented by **Ministry of Higher Education**, for financial support during the study period. Thanks also go to the **Libyan Embassy**.

I would like to thank my parents, my father **Abdullah** and my mother **Nadia**, for their continuous encouragement and support. Many thanks too to my brothers, sisters and cousins for their support. My brother **Magdi**, thanks a lot for taking care of all my stuff and matters in Libya during all these years. Above all, I thank my **Allah** (Glory to him) for helping me to successfully complete my studies.

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