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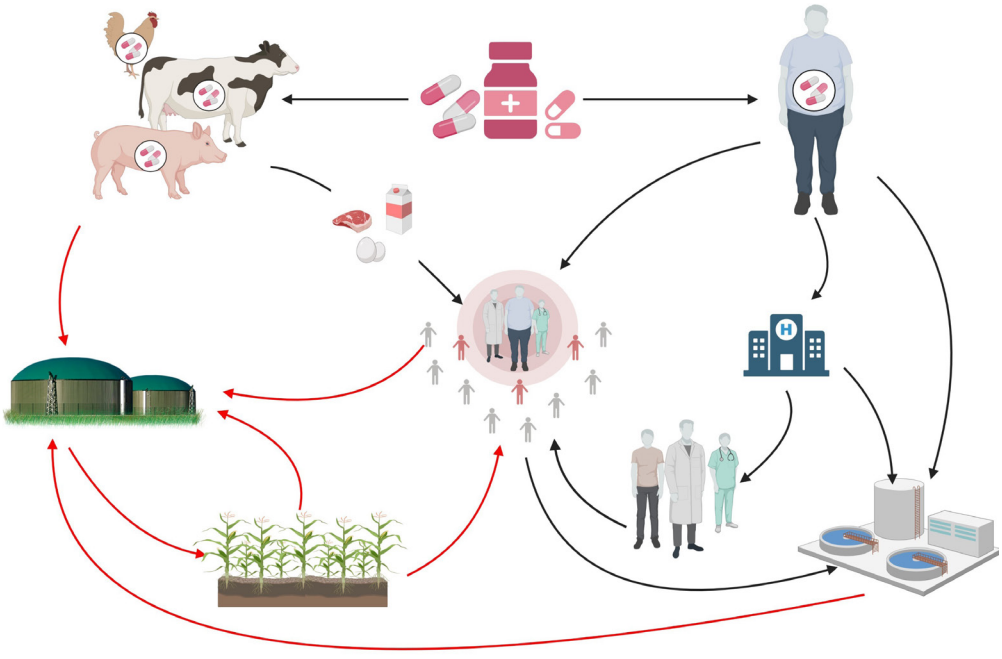
Antibiotic resistance in biogas processes

He Sun



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Cover: Illustration of antibiotic resistance spread in society.
(photo: He Sun. Inspired by https://www.cdc.gov)

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Abstract

Anaerobic digestion (AD) is a well-established technology that can play a key role in development of a sustainable society. In AD, organic wastes such as animal manure, food waste and crop residues are used as substrate and converted to biogas and digestate, which represent green energy and a biofertiliser. Due to intensive use of veterinary antibiotics, antibiotic residues, antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) enter the AD process via the substrates and end up in the digestate. Thus, digestate may represent a source of spread of antibiotic resistance. Antibiotic resistance is one of the greatest global public health challenges of our time and is predicted to cause around 300 million premature deaths by 2050, so countering its spread is critically important. However, research on the antibiotic resistance level in AD is still quite limited. This thesis contributed essential new knowledge by a) identifying ARB communities in digestates originating from food waste, crops and dairy manure; b) assessing antibiotic resistance in plant-based substrates; c) investigating phenotypic and genotypic resistance pattern and resistance transferability of isolated ARB; and d) comparing molecular and culture-dependent methods in evaluation of antibiotic resistance.

Bacillus and closely-related genera such as *Paenibacillus* and *Lysinibacillus* were found to dominate the ARB community isolated from digestate, irrespective of substrate type. Most ARGs identified for these ARB were located on chromosomes, although several ARB strains had extra-chromosomal genomes. Only one was identified as a plasmid (pAMαl), which proved to be non-transferable in plasmid conjugation testing. Thus, the dominant ARB community from the digestates studied likely poses a limited risk of antibiotic resistance spread, although even plant-based substrates were found to contain variant antibiotic resistance components. Combined use of molecular and culture-dependent methods was required to reveal the true antibiotic resistance situation in the AD process.

Keywords: anaerobic digestion, biogas, digestate, antibiotic resistance, antibiotic-resistant bacteria, antibiotic resistance genes, mobile genetic elements.

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Antibiotikaresistens i biogasprocesser

Abstrakt

Anaerob rötning (AD) är en väletablerad teknik som kan spela en nyckelroll i utvecklingen av ett hållbart samhälle. I denna process omvandlas olika typer av organiskt avfall (substrat), som djurgödsel, matavfall och växtrester, till biogas och en rötrest, som representerar grön energi och ett biogödsel. På grund av en intensiv användning av antibiotika kommer antibiotikarester, antibiotikaresistenta bakterier (ARB), resistensgener (ARG) och mobila genetiska element (MGE) in i processen via substraten och hamnar slutligen i rötresten. Användningen av rötresten som biogödsel representera därför en möjlig källa till spridning av antibiotikaresistens. Antibiotikaresistens är en av vår tids största globala folkhälsoutmaningar och förutspås orsaka omkring 300 miljoner förtida dödsfall år 2050, varför det är viktigt att motverka dess spridning. Forskningen om antibiotikaresistens vid rötning av organiskt material är dock fortfarande ganska begränsad.

Denna avhandling bidrar med ny och viktig kunskap genom att a) identifiera ARB i rötrester som härrör från matavfall, grödor och kogödsel; b) analysera antibiotikaresistens i växtbaserade substrat; c) undersöka fenotypiskt och genotypiskt resistensmönster och resistensöverföring hos ARB isolerade från rötrester; och d) jämföra molekylära och odlingsbaserade metoder vid utvärdering av antibiotikaresistens.

Resultaten visade att Bacillus och närbesläktade släkten såsom Paenibacillus och Lysinibacillus dominerade ARB isolerade från rötningsprocesser, oavsett substrattyp. De flesta ARG som identifierades för dessa ARB var lokaliserade på kromosomer, även om flera ARB-stammar hade extrakromosomala genom. En plasmid (pAMal) identifierades för ett isolat men visade sig vara icke-överföringsbar i konjugeringstestning. Slutsatsen är därför att ARB i rötrester troligen utgör en begränsad risk för spridning av antibiotikaresistens. Vidare visade resultaten att en kombinerad användning av molekylära och odlingsbaserade metoder krävs för att tydligt visa omfattningen av antibiotikaresistens i en rötningsprocess.

Nyckelord: anaerob rötning, biogas, antibiotikaresistens, antibiotikaresistenta bakterier, antibiotikaresistensgener, mobila genetiska element.

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Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Sun, H., Bjerketorp, J., Levenfors, J.J. & Schnürer, A., (2020). Isolation of antibiotic-resistant bacteria in biogas digestate and their susceptibility to antibiotics. *Environmental Pollution* 266, 115265.

II. Sun, H., Levenfors, J.J., Brandt, C. & Schnürer, A. Characterization of meropenem-resistant Bacillus sp. FW 1 isolated from biogas digestate (submitted to *Ecotoxicology and Environmental Safety*).

III. Sun, H., Schnürer, A., Müller, B., Mößnang, B., Lebuhn, M. & Makarewicz, O. Antimicrobial resistance in three agricultural biogas plants using plant-based substrates (submitted to *Science and the Total Environment*).

IV. Sun, H., Levenfors, J.J., Brandt, C. & Schnürer, A. Discrepancies in genotypic and phenotypic antibiotic resistance in bacteria isolated from biogas digestate (manuscript).

Paper I is reproduced with the permission of the publisher.

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

I. Investigation, data curation, formal analysis, visualisation, writing original draft.

II. Conceptualisation, methodology, investigation, data curation, formal analysis, visualisation, writing original draft.

III. Conceptualization, methodology, investigation, data curation, formal analysis, visualisation, writing original draft.

IV. Conceptualisation, methodology, investigation, data curation, formal analysis, visualisation, writing original draft.

In addition to Papers I-IV, He Sun contributed to the following papers within the timeframe of this thesis work:

Sun, H., Brandt, C. & Schnürer, A. (2020). Long-read DNA preparation for bacterial isolates. protocols.io 1–5. https://doi.org/dx.doi.org/10.17504/protocols.io.64ghgtw

List of tables

List of figures

Abbreviations

AD	Anaerobic digestion
ARB	Antibiotic-resistant bacteria
ARG	Antibiotic resistance gene
AST	Antibiotic susceptibility testing
CCA	Canonical correlation analysis
HGT	Horizontal gene transfer
HRT	Hydraulic retention time
MGE	Mobile genetic element
SRT	Solids retention time
TS	Total solids
VFA	Volatile fatty acids
WGS	Whole-genome sequence
WWTP	Wastewater treatment plant

tet	Tetracycline resistance gene
erm	Erythromycin resistance gene
sul	Sulfonamide resistance gene
intI1	Class 1 integrase gene
intI2	Class 2 integrase gene

1. Introduction

Anaerobic digestion (AD) is a well-established technology with great potential for expansion and can play a key role in future development of a sustainable society (Kougias & Angelidaki, 2018). In AD, different organic wastes such as animal manure, food waste, sludge from wastewater treatment plants (WWTP), crop residues and dedicated energy crops, *e.g.* maize silage and grass silage in some countries, are degraded anaerobically by a variety of microorganisms working in synchrony and converted to two final products, biogas and digestate (Schnürer & Jarvis, 2018). Biogas is a renewable energy with potential to replace fossil carbon in production of electricity, heat and vehicle fuel (Kougias & Angelidaki, 2018). Digestate is commonly used for fertilisation of farmland, as it contains high concentrations of valuable plant nutrients (nitrogen (N), phosphorus (P), potassium (K), *etc*.). It can thus maintain agricultural productivity at a lower environmental cost compared with fossil-demanding conventional chemical fertilisers (Al Seadi *et al.*, 2013). By using digestate as biofertiliser and returning food produced on the fertilised land to society, utilisation of nutrients is improved and nutrient recycling between urban and rural areas is achieved. However, prior to use of digestate as fertiliser, it is important to determine its quality in terms of nutrient content and levels of chemical and biological contaminants, *e.g.* heavy metals, weed seeds and pathogens (Risberg, 2015). In Sweden, two separate voluntary certification systems, SPCR 120 and Revaq, are currently used, to assure the quality of digestates originating from food or feed materials and WWTP sludge, respectively (Schnürer & Jarvis, 2018). However, neither of these systems requires any form of analysis to determine the levels of organic pollutants, *e.g.* pharmaceutical residues, that can potential enter the AD process via the substrates. Further, no risk evaluation regarding the

potential spread of antibiotic resistance through use of AD digestate as fertiliser is required in either of the current certification systems.

Antibiotic resistance is one of the most significant global public health challenges of our time (CDC, 2019). It is increasingly impairing the effectiveness of antibiotics in the outpatient and inpatient sector, resulting in increased morbidity and mortality but also hospital stays of excessive length and costs (Stewardson *et al.*, 2016). According to a recent report by the United States (U.S.) Center for Disease Control and Prevention (CDC), more than 2.8 million antibiotic-resistant infections occur in the U.S. each year, and more than 35,000 people die as a result (CDC, 2019). Infections with antibiotic-resistant pathogens are also estimated to cause over 33,000 attributable deaths annually (2015 data) in Europe (Cassini *et al.*, 2019). Moreover, antibiotic resistance is predicted to cause around 300 million premature deaths by 2050, with associated losses of between 40 and 100 trillion USD to the global economy (O'Neill, 2014). Veterinary use of antibiotics can result in development of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) in the gut of animals. As a result, antibiotic residues, ARB and ARGs often end up in livestock-derived food and in animal manure (Wichmann *et al.*, 2014; Zhang *et al.*, 2017b; Qian *et al.*, 2018; He *et al.*, 2019). When materials such as animal manure and food waste are used as substrates in a biogas reactor, any antibiotic residues, ARB and ARGs present in the substrates can be degraded to some extent, but are often not eliminated completely (Beneragama *et al.*, 2013; Mitchell *et al.*, 2013; Resende *et al.*, 2014; Zou *et al.*, 2020; Visca *et al.*, 2021). The AD process may consequently represent a route of antibiotic resistance spread when digestate is used for fertilisation, as illustrated in Figure 1. Many studies have investigated the AD-associated antibiotic resistance situation in the AD process, but mainly focusing on biogas processes using animal manure or WWTP sludge as substrates (Ma *et al.*, 2011; Beneragama *et al.*, 2013; Sun *et al.*, 2016, 2019a; Wallace *et al.*, 2018; Zou *et al.*, 2020). Less is known about the antibiotic resistance situation in AD processes using other substrates, *e.g.* food waste and crop materials. Moreover, previous studies on AD-associated antibiotic resistance have mostly been conducted using either molecular analysis or culture-dependent methods. Both methods have their merits and limitations in analysis of the antibiotic resistance situation. However, little is known about the consistency of existing molecular and culture-dependent methods,

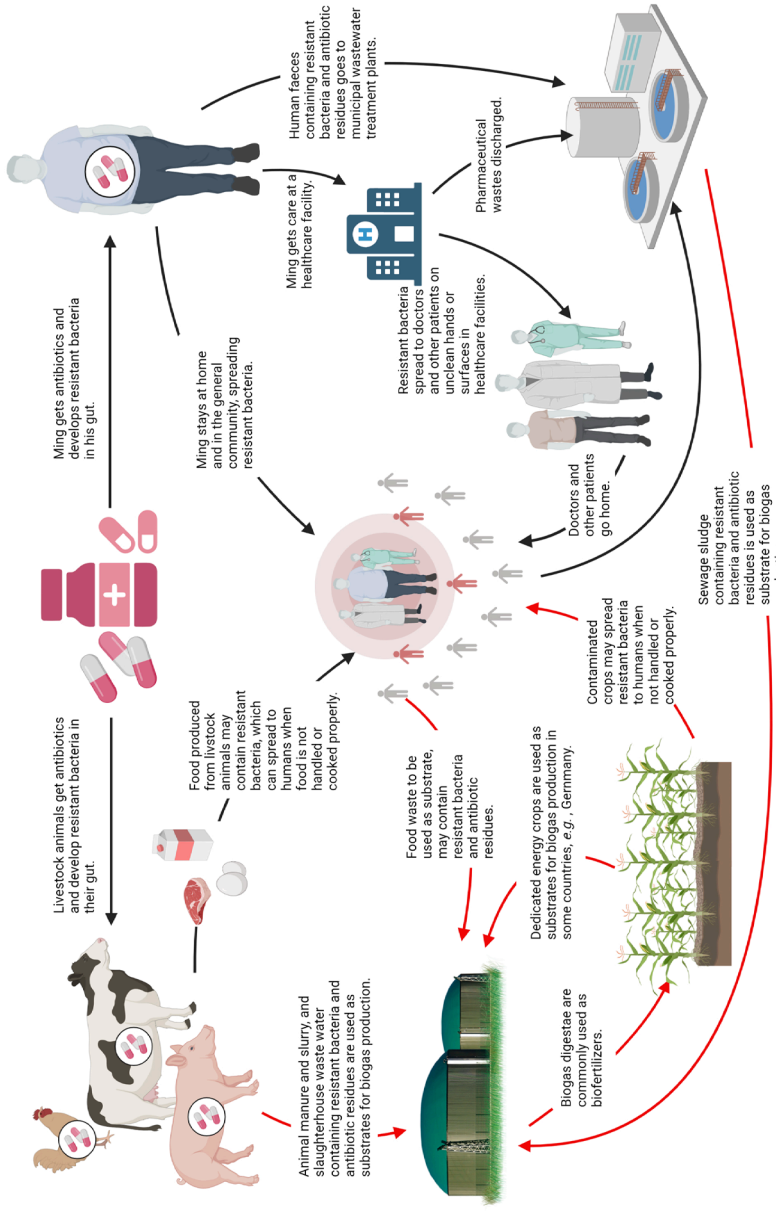


Figure 1. Schematic diagram of antibiotic resistance spread in society. Red arrows indicate possible routes of antibiotic resistance spread via anaerobic digestion (Inspired by https://www.cdc.gov/narms/faq.html and created with BioRender.com).

and an optimised approach, *e.g.* a combination of the two methods, might be needed to gain a fuller understanding of the true antibiotic resistance situation in AD processes.

It is clear that AD digestate represents a potential antibiotic resistance transmission route, but research in this area is still quite limited and more work is needed to fully evaluate the risk of spread of AD-associated antibiotic resistance.

1.1 Aim of the thesis

The aims of this thesis were to gain deeper insights into the AD-associated antibiotic resistance situation and to assess the risk of antibiotic resistance spread from the AD process.

Specific objectives were to:

1. Identify the ARB community in digestates originating from different AD substrates and determine their antibiotic resistance patterns (**I, III**).

2. Investigate the mechanism of antibiotic resistance for any ARB identified in AD digestate and the transferability of such resistance (**II, III, IV**).

3. Evaluate current methods for analysis of antibiotic resistance by comparing phenotypic and genotypic resistance in bacteria isolated from AD digestate (**II, IV**).

2. Antibiotics and antibiotic resistance

Secretion of antibiotic compounds by microorganisms is an ancient and effective method to improve their survival advantage when competing for space and nutrients with other microbes. The emergence of resistance mechanisms to antibiotic compounds is also an ancient natural response (Dcosta *et al.*, 2011; Warinner *et al.*, 2014). Mechanisms that have evolved to overcome antibiotic compounds in co-resident natural environments are considered to constitute intrinsic resistance. However, intrinsically resistant bacteria are not the main focus of antibiotic resistance problems in the world. Instead, the main concern arises from expression of acquired resistance in a bacterial population that was originally susceptible to an antibiotic (Munita & Arias, 2016). Development of acquired resistance can result from mutations in chromosomal genes and/or acquisition of foreign genetic determinants of resistance, likely obtained from intrinsically resistant bacteria present in the environment (Munita & Arias, 2016). In this chapter, antibiotic classes, mechanisms of action and resistance are briefly introduced.

2.1 Antibiotic classes and mechanism of action

Antibiotics are drugs used to treat bacterial infections. They act by either killing bacteria (bactericidal agents) or stopping bacteria achieving growth or reproduction (bacteriostatic agents). Bacteria themselves can be divided into two broad classes, Gram-positive and Gram-negative. Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria because of their unique outer membrane, which prevents certain antibiotics from penetrating the cell (Exner *et al.*, 2017).

There are several ways of classifying antibiotics, but the most common schemes are based on molecular structure and mechanism of action. An overview of different classes of antibiotics on the basis of molecular structure is presented in Figure 2. These classes are named relevant to their molecular structure, *e.g.* β-lactams contain a β-lactam ring (Pandey & Cascella, 2021) and aminoglycosides contain an amino-modified glycoside (Mingeot-Leclercq *et al.*, 1999). Moreover, owing to differences in molecular structure within each class, there are antibiotic subclasses, *e.g.* penicillins, cephalosporins, carbapenems and monobactams in the class of β-lactams.

Regardless of molecular structure, antibiotics can be classified on the basis of mechanism of action into four groups: a) inhibitors of cell wall synthesis (*e.g.* β-lactams and glycopeptides); b) inhibitors of protein synthesis (*e.g.* aminoglycosides and tetracyclines); c) inhibitors of nucleic acid synthesis (*e.g.* quinolones and ansamycins); and d) inhibitors of folic acid metabolism (*e.g.* sulphonamides and trimethoprim) (Figure 3). Antibiotics within the same structural class have a similar mechanism of action, for instance all β-lactams (penicillins, cephalosporins *etc*.) inhibit cell wall synthesis. In contrast, antibiotics with the same mechanism of action can belong to different antibiotic structural classes, for instance inhibitors of protein synthesis include antibiotics belonging to the tetracyclines, aminoglycosides, macrolides *etc*.

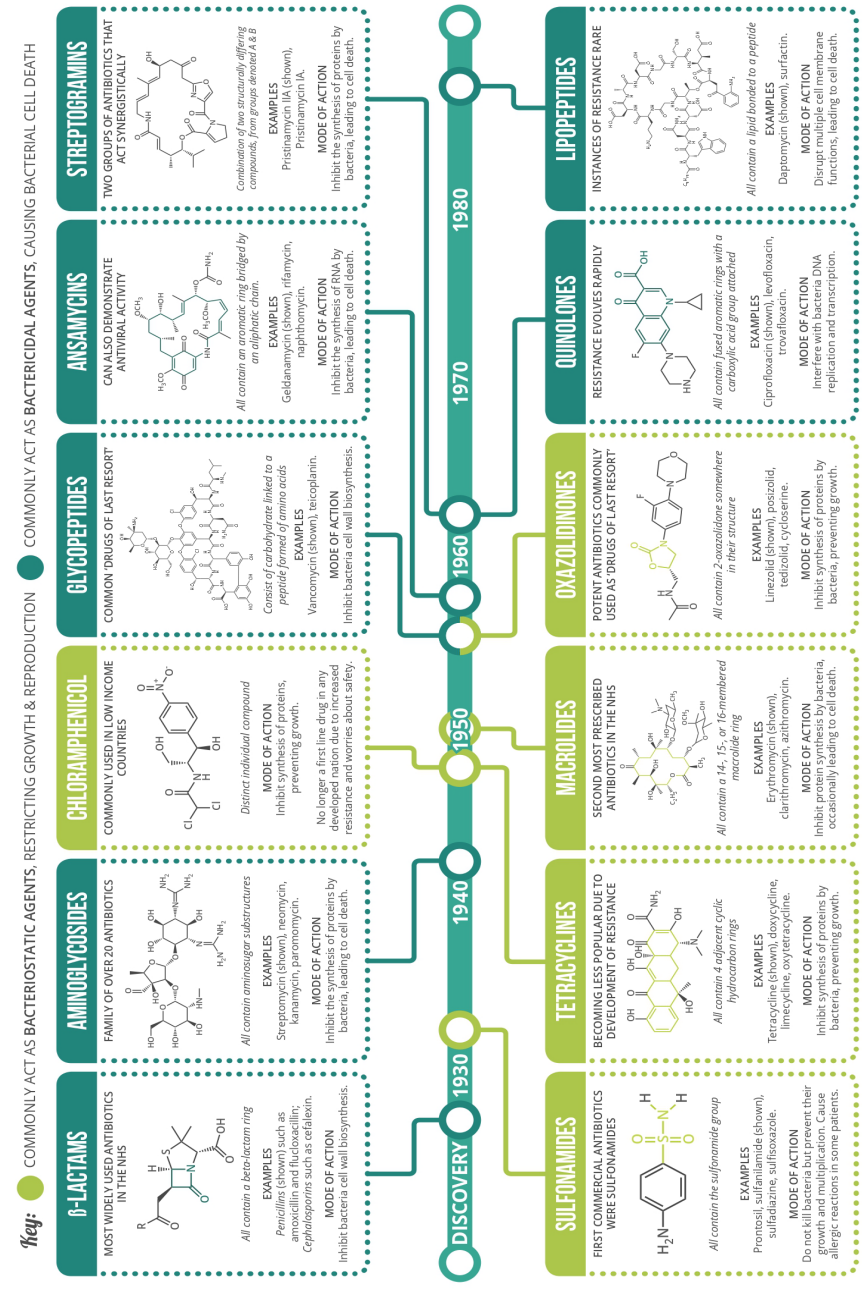


Figure 2. Different classes of antibiotics based on molecular structure (modified from https://www.compoundchem.com/2014/09/08/ antibiotics/).

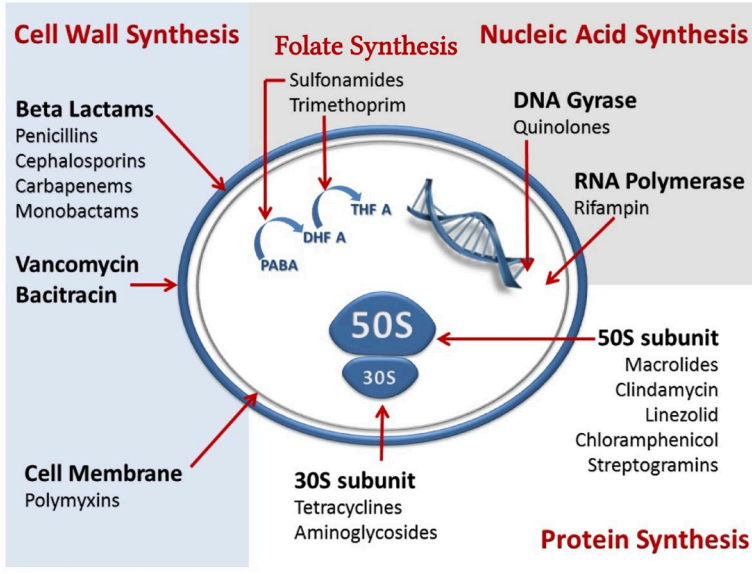


Figure 3. Different groups of antibiotics based on mechanism of action (modified from TheMedSchool.com (2011)).

2.2 Antibiotic resistance

Antibiotic resistance is generally classified into three categories based on mechanism of action (Munita & Arias, 2016): a) modifications of the antibiotic molecule, including chemical alterations or destruction of the antibiotic compound; b) prevention of the compound reaching the antibiotic target, including decreased permeability controlled by cellular outer membrane and efflux pumps extruding the antibiotic compounds; and c) changes in target sites, including target protection which prevents the antibiotic compound from reaching its binding site, and modification of the target site, which decreases affinity for antibiotic molecules.

Acquisition of foreign genes via horizontal gene transfer (HGT) is one of the most important drivers of bacterial evolution, and it is frequently responsible for the development of antibiotic resistance (Munita & Arias, 2016). Bacteria acquire foreign determinants of resistance through three main strategies: a) transformation (incorporation of free DNA), b) transduction (phage mediated), and c) conjugation (bacterial "sex") (Munita & Arias, 2016). In most cases, conjugation uses mobile genetic elements (MGEs), *e.g.* plasmids and transposons, as vehicles for transferring the

genes (Munita & Arias, 2016). Thus, conjugative plasmids and transposons are frequently responsible for the development and dissemination of antibiotic resistance in different environments. In addition, antibiotic resistance genes can be accumulated in MGEs, represented by integrons, which are mostly carried by plasmids or contained within a transposon (Hall & Collis, 1995; Mazel, 2006).

3. Antibiotic resistance in agricultural substrates for anaerobic digestion

In order to tackle the increasing problem of antibiotic resistance, several countries have launched initiatives aiming to reduce veterinary use of antibiotics in animal husbandry and their effects in the nutrient chain and the environment. Such initiatives include the Strama programme in Sweden (Goverment Offices of Sweden, 2016) and DART in Germany (Bundesministerium für Gesundheit *et al.*, 2020). In this chapter, antibiotic resistance in agricultural AD substrates that are associated with veterinary use of antibiotics, *i.e*. animal manure, food waste and crops, is described. Although WWTP sludge is a common substrate for AD, it was not the main focus in this research.

3.1 Antibiotic resistance in animal manure

Veterinary antibiotics are widely used to prevent and treat diseases and, in large parts of the world outside Europe, to promote animal growth in the livestock industry (Massé *et al.*, 2014). Countries all around the world have taken measures to counter the spread of antibiotic resistance. For example, in the European Union (EU), the use of antimicrobials for growth promotion has been banned since 2006. According to the latest report from European Surveillance of Veterinary Antimicrobial Consumption, sales in Europe of antibiotics for veterinary use decreased by more than 34% from 2011 to 2018 (European Medicines Agency, 2020). However, annual sales of antimicrobial agents for use in animals are still considerable, with an estimated total of 6500 tonnes of active ingredient used in 31 European countries in 2018 (European Medicines Agency, 2020). The antibiotics administered to animals are excreted in animal faeces and urine to a large extent, ranging between 10% and 90% of total intake (Kumar *et al.*, 2005). Moreover, the use of antibiotics can result in development of ARB and ARGs in the gut of animals. Consequently, animal manure and slurry are reservoirs of antibiotic resistance (Wichmann *et al.*, 2014; Qian *et al.*, 2018).

Among the agricultural substrates used in AD, animal manures have been the most widely studied in terms of antibiotic resistance (Figure 4). It has been shown that ARB and ARGs are diverse and abundant in different sources of animal manures. Specifically, clinically dangerous pathogens, such as antibiotic-resistant Enterobacteriaceae, antibiotic-resistant *Campylobacter*, methicillin- and vancomycin-resistant *Staphylococcus* and vancomycin-resistant *Enterococcus*, have been identified in dairy operations (CDC, 2019). Moreover, food-borne pathogens, *e.g*. *Escherichia coli*, *Salmonella* spp. *etc*. have been found in dairy and cattle manures (Blau *et al.*, 2005; Carballo *et al.*, 2013; Obaidat *et al.*, 2018). In addition, cattle manures have been found to act as reservoirs for more than 60 different ARGs (Qian *et al.*, 2018), with resistomes varying from herd to herd (Wichmann *et al.*, 2014). Moreover, ARG abundance in swine wastewater samples has been shown to be at least 31-fold higher than in well water and fishpond water (He *et al.*, 2016). According to a recent

review, the most frequently reported genetic elements related to antibiotic resistance in studies of swine, cattle and poultry manure/wastewater are ARGs, *e.g*. *tet* (tetracycline resistance genes), *erm* (erythromycin) and *sul* (sulfonamides), and MGEs, *e.g*. integrons (Pereira *et al.*, 2021). In parallel, tetracyclines, β-lactams (mostly penicillins), macrolides (*e.g*. erythromycin) and sulfonamides were the most commonly used antibiotics in global livestock production between 2015 and 2017 (OIE (World Organization for Animal Health), 2018).

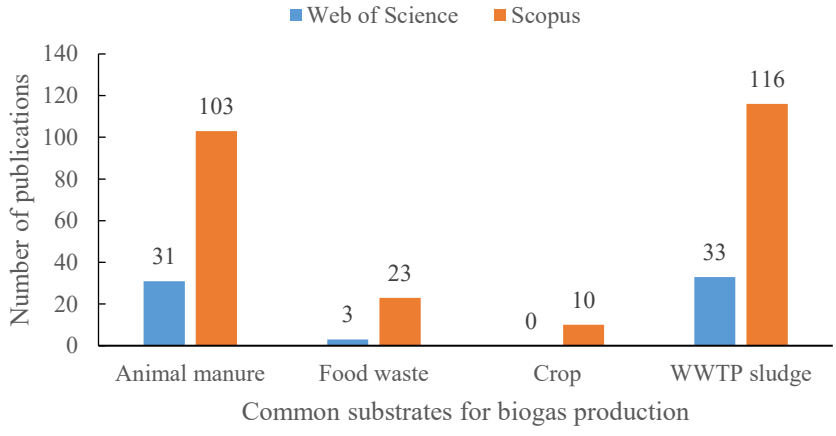


Figure 4. Number of publications on antibiotic resistance in common substrates for biogas production. Results of searches in Web of Science and Scopus using "antibiotic resistan*" AND "anaerobic digestion" AND "manure" or "sludge" or "food waste" or "*crop*" as keywords Plus and keywords, respectively.

3.2 Antibiotic resistance in food waste

Many studies have pointed out the link between use of veterinary antibiotics and presence of antibiotic-resistant pathogens in food from agricultural production (see review by Verraes *et al.*, 2013). Specifically, antibiotic-resistant *Salmonella* spp. have been found in both pork and poultry meat (Depoorter *et al.*, 2012; Van Boxstael *et al.*, 2012), while cephalosporin-resistant *E. coli* has been found in chicken meat (Zou *et al.*, 2011). Presence of these opportunistic pathogens in food poses a direct risk to public health. Moreover, ARGs present in commensal pathogenic or

non-pathogenic strains of bacteria on food represent an indirect risk to public health, since they enrich the resistance pool from where pathogens can pick up resistance traits (Verraes *et al.*, 2013). Similarly to the pattern of ARG types frequently detected in animal manures, the *erm*, *tet* and *sul* genes are also commonly detected in food waste (Zhang *et al.*, 2017b; He *et al.*, 2019; Wang *et al.*, 2021a, 2021b). This can be expected, as antibiotic resistance patterns in animal manure and food waste are driven by the same original pressure, *i.e*. veterinary antibiotic use in the livestock industry.

3.3 Antibiotic resistance in crops and crop residues

Antibiotics are seldom applied to crops to prevent bacteria diseases. For example, the use of antibiotics on crops in the U.S., mainly apple and pear trees, accounted for only 0.26% of total agricultural consumption in 2011(McManus, 2014). No measurable impact of the use of antibiotics in apple orchards has been identified (McManus, 2014).

Instead, an important route of antibiotic resistance spread to crop-related microbes is by growing crops on farmland fertilised with animal manure or digestate (see Figure 1). Several studies have observed transfer of antibiotic resistance from soils to vegetables, *e.g*. lettuce, carrots and radishes (Tien *et al.*, 2017; Zhang *et al.*, 2017a, 2019c). However, the antibiotic resistance situation has not yet been investigated for plant-based AD substrates, *e.g*. crop silage and maize silage. Therefore, these substrates may represent a neglected antibiotic resistance load in biogas production. To address this knowledge gap, one of the studies presented in this thesis examined the antibiotic resistance situation in plant-based AD substrates. The genus *Bacillus* and the closely related genera *Paenibacillus* and *Lysinibacillus* were the dominant ARB isolated from the crop-based substrates studied (Paper **III)**. These bacteria exhibited resistance to a variety of antibiotic classes, including β-lactams, tetracyclines, aminoglycosides *etc*. (**III**). Moreover, ARGs encoding resistance for *e.g*. β-lactams, fluoroquinolones, aminoglycosides *etc*. were identifed in the plant-based AD substrates. Plasmids were also found, with IncFIB being the most frequently identified group in the substrates (n=6), followed by IncW (n=3) and IncK and IncB/O (each n=2). Interestingly, identification of ARGs, especially carbapenemase genes and plasmid groups, was most commonly associated with Gram-negative bacteria, such as the pathogens *E. coli* and

Pseudomonas spp. Therefore, such Gram-negative bacteria were highly likely to have been present on the original crops, although no cultures were made of these (**III)**. In brief, plant-based substrates were found to be associated with antibiotic-resistant components, including culturable Gram-positive ARB and Gram-negative pathogenic bacteria-associated ARGs and plasmids (**III**). This suggests that, in addition to animal manure and food waste, the antibiotic resistance load from plant-based substrates should be taken into consideration in agricultural biogas processing.

4. Factors influencing the degradation efficiency of antibiotic resistance during anaerobic digestion

Apart from the conventional benefits, such as valorisation of organic wastes and green energy production, AD has been found to be effective in reducing antibiotic resistance. The degree of reduction achieved during AD is influenced by different factors, such as operating parameters and pre-/post-treatments (Figure 5). However, in some cases, enrichment of ARGs has also been observed during AD.

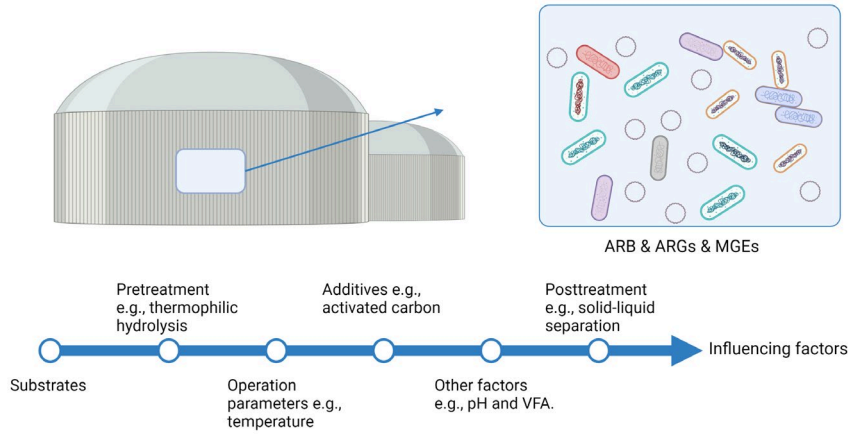


Figure 5. Examples of factors influencing the degradation efficiency of antibiotic resistance. ARB: antibiotic-resistant bacteria; ARGs: antibiotic resistance genes; MGEs: mobile genetic elements (created with BioRender.com).

4.1 Operating parameters

4.1.1 Operating temperature

To achieve successful production of biogas, external heating of AD reactors is required to provide a comfortable environment for microorganisms to grow and function. The temperatures used for AD in agricultural biogas plants are mesophilic (37-42 °C) or thermophilic (50-55 °C) (Schnürer & Jarvis, 2018). In special cases, psychrophilic temperatures (<25 °C) are also used, but mostly for small-scale reactors operated by individual households at rural locations in developing countries (Dhaked *et al.*, 2010).

In general, a higher operating temperature can be assumed to achieve a greater reduction in antibiotic resistance and pathogens and this assumption is supported by many studies. As regards ARGs, thermophilic temperatures have been found to outperform mesophilic temperatures in reduction of tetracycline resistance genes, *e.g. tetA*, *tetO* and *tetX*, when processing WWTP sludge (Ghosh *et al.*, 2009; Diehl & Lapara, 2010). In addition, erythromycin resistance genes, *e.g*. *ermB* and *ermF*, have been found to be reduced only within the thermophilic temperature range, and not at mesophilic temperatures, during processing of WWTP sludge (Ma *et al.*, 2011). A similar trend has been reported for sulfonamide resistance genes, *e.g. sul1* and *sul2*, *i.e.* with greater reductions at thermophilic temperatures than at mesophilic and psychrophilic temperatures, during AD processing of dairy manure (Sun *et al.*, 2016). As regards ARB reduction, thermophilic conditions appear to be better at reducing multi-drug resistant bacteria in co-digestion of dairy manure and waste milk (Beneragama *et al.*, 2013). Moreover, potential pathogens such as members of the phyla Bacteroidetes, Proteobacteria and Corynebacterium are reported to be removed by thermophilic temperatures, but not by psychrophilic and mesophilic temperatures, in dairy manure digestion (Sun *et al.*, 2016). In line with this trend, in Paper **III** a greater reduction in ARGs and plasmids was observed at higher operating temperature, *e.g*. at 42-44 °C and 45-48 °C (biogas plants B and C, respectively) compared with 40-42 °C (biogas plant A), during digestion of crops or crops/poultry manure. One popular explanation for the greater reduction in antibiotic resistance at thermophilic temperatures is a temperature-induced bacterial community shift during AD (Ma *et al.*, 2011; Sun *et al.*, 2016). Compared with mesophilic conditions, the bacterial diversity at thermophilic temperatures is markedly lower, with

a large proportion of mesophilic ARB, *e.g*. members of the Bacteroidetes and Proteobacteria, outcompeted during the process and with a decrease in ARGs carried by these ARB (Sun *et al.*, 2016). While individual ARG hosts can theoretically maintain abundance despite low bacterial diversity, low bacterial diversity diminishes the overall probability of finding other compatible host bacteria through HGT (Ma *et al.*, 2011). Thus, lower diversity of host microorganisms is possibly an important mechanism in ARG reduction in thermophilic digestion. Another possible explanation for the greater ARG reduction at higher operating temperatures is that ARGs may be discarded by host microorganisms (Zou *et al.*, 2020). It has long been known that plasmid-carrying ARB, *e.g*. *Escherichia*, have much higher fitness costs during growth than plasmid-deficient ARB. If the traits expressed by plasmids were not important, for example in a high-temperature but low antibiotic pressure environment, carrying plasmids encoding for antibiotic resistance would thus put bacteria at a competitive disadvantage and they would generally discard the plasmid (Godwin & Slater, 1979; Subbiah *et al.*, 2011). This would be reflected in a reduction in ARG abundance (Zou *et al.*, 2020).

However, inconsistent results have been found in other studies, *e.g*. with mesophilic temperature achieving a greater reduction than thermophilic temperature. For example, in one study *tetC* was found to be enriched in thermophilic digestion of dairy manure, but with no obvious changes at mesophilic temperature (Sun *et al.*, 2016). In two other studies of similar and comparable conditions for WWTP sludge digestion, inconsistent results were obtained for *tetX* reduction, with greater reductions in mesophilic (Ma *et al.*, 2011) or thermophilic conditions (Ghosh *et al.*, 2009; Diehl & Lapara, 2010). According to Ma *et al*. (2011), this difference between studies in results for a particular sludge source (WWTP) possibly derives from differences in operations, *e.g*. insufficient amount and frequency of sludge replacement in the reactor could have imposed feast and famine conditions on the bacterial community, eventually resulting in inconsistency of ARG reduction. This suggests that factors apart from operating temperature may also be important in ARG reduction efficiency in the AD process.

4.1.2 Retention time

In AD processes, retention time is usually referred to as hydraulic retention time (HRT), although solids retention time (SRT) is sometimes used instead. In many cases, HRT and SRT are equal. However, SRT becomes longer than HRT in cases where some digested residues are returned to the process in order to achieve a greater degree of biomass degradation and increased production of biogas (Schnürer & Jarvis, 2018).

Several studies have investigated the effect of retention time on ARG removal during AD. Among these, Ma *et al*. (2011) achieved a greater degree of removal for the tetracycline resistance gene, *tetX*, by increasing SRT in mesophilic digestion of WWTP sludge. Wang *et al.* (2021c) found that the abundance of ARGs, especially ARGs related to aminoglycosides, multidrug and sulfonamide resistance, and of MGEs was more effectively reduced with increasing HRT (64 d compared with 9 d) in co-digestion of pig manure and food waste at a high total solids level (20%). Sun *et al.* (2019a) found that longer HRT, within a set of HRT of 4, 12, 15, 20 and 25 d, increased the removal of ARGs and MGEs during processing of WWTP sludge. In contrast, ARG reduction efficiency has been found to be unaffected by increasing retention time in some other studies (Sun *et al.*, 2016; Zou *et al.*, 2020). A possible reason for greater ARG reduction with increasing retention time is the decreased host range of microorganisms for ARGs, which is supported by findings of lower microbial diversity with longer retention time (Sundberg *et al.*, 2013; Sun *et al.*, 2019a). Notably, the abundance of ARGs and MGEs is driven by ARB reproduction and HGT (Su *et al.*, 2015; Pei *et al.*, 2016). Longer HRT usually leads to oligotrophic conditions within the reactor, resulting in inhibited reproduction of less adaptive ARB and lower probability of encountering a compatible host for mobile ARGs via HGT at lower microbial diversity (Sun *et al.*, 2019a).

Thus, essentially, temperature and HRT may affect the abundance of ARGs and MGEs by a similar mechanism, *i.e*. by shifting the microbial community structure. Of these two operating parameters, canonical correlation analysis (CCA) in a previous study revealed that temperature seemed likely to have a more conspicuous effect than HRT on ARG and MGE profiles (Sun *et al.*, 2019a). Specifically, the effect of HRT on ARG profiles was more obvious for mesophilic digestion than for thermophilic digestion. Thus, different operational factors may impact to varying extents

on the reduction in ARGs and MGEs. Future studies comparing operating parameters, *e.g.* temperature, HRT, pH and volatile fatty acids (VFA), using a large dataset of biogas reactors would be of interest in guiding AD operations to achieve greater reductions in antibiotic resistance.

4.2 Pre-/post-treatments

Pre-treatments are often applied before AD, for purposes such as substrate disintegration or sanitisation, which is commonly needed for food waste but sometimes also for animal manure (Schnürer & Jarvis, 2018). The most common method of sanitisation in biogas plants is heating to 70 °C for one hour for low-risk animal waste, which aims to reduce the abundance by six- and three-log for pathogens and heat-resistant viruses, respectively (Schnürer & Jarvis, 2018). Moreover, in some cases pre-treatments are applied to increase the digestibility of substrates. This is commonly done in WWTP sludge digestion, where pre-treatments such as thermophilic hydrolysis, microwaving and ozone oxidation are applied (Ma *et al.*, 2011; Pei *et al.*, 2016; Tong *et al.*, 2016, 2018). It has been found that most ARGs are reduced after such pre-treatments (Tong *et al.*, 2018). Among the different pre-treatments tested, it has been shown that thermophilic hydrolysis can drastically reduce all types of ARGs and MGEs (Ma *et al.*, 2011; Sun *et al.*, 2019a), and can outperform ozone oxidation in removal of tetracycline resistance genes (Pei *et al.*, 2016), during WWTP sludge digestion. Rebounding of ARGs and MGEs can occur in the subsequent AD process after these pre-treatments, but it has been found that ARB concentrations consistently decrease during microwave pre-treatment and subsequent AD (Tong *et al.*, 2016). Therefore, pre-treatment combined with AD can further reduce ARB, but appears to improve ARG removal only slightly compared with AD *per se*.

To achieve better reductions in antibiotic resistance, post-treatments after AD have been studied, including *e.g.* improving digestate storage, composting and converting digestate to biochar (Gurmessa *et al.*, 2020). It has been found that thermophilic aerobic digestion, subsequent to AD, can effectively further remove ARGs and Class 1 integrase gene (*intI1*) in digestate from WWTP sludge (Min Jang *et al.*, 2019). However, other post-treatments such as stripping off ammonia (Bousek *et al.*, 2018), membrane

distillation (Yan *et al.*, 2019) and composting (Ezzariai *et al.*, 2018), are generally directed at reducing antibiotic residues, rather than ARB, ARGs and MGEs.

4.3 Additives

To improve AD performance, different additives such as granular activated carbon, graphite, iron nanoparticles *etc*. have been evaluated and have been found to give positive results in biogas production (Zhang *et al.*, 2017b; Ma *et al.*, 2019; Wang *et al.*, 2021b). The reason for the positive outcome is considered to be that these additives facilitate contact between acid-degrading bacteria and methanogens (Schnürer & Jarvis, 2018).

In addition to higher production of biogas, additives have also been found to be effective in reduction of antibiotic resistance level and pathogens. For instance, activated carbon has been found to facilitate reductions in ARGs, including tetracycline and sulfonamide resistance genes, and bacterial pathogens in processing of food waste (Zhang *et al.*, 2017b). Among graphite-like substances (*i.e*. graphite, graphene and graphene oxide), graphene has been found to have the greatest effect on removal of ARGs, including $bla_{oxa\text{-}1}$ (resistance to β-lactams), *ermF* and *ermB* (macrolides), *tetQ* and *tetX* (tetracycline), in co-digestion of WWTP sludge and food waste (Wang *et al.*, 2021b). Graphene oxide is reported to be better in removal of MGEs, *e.g*. *intI1*, and other ARGs, *e.g*. *sul1* and *sul2* (sulfonamides), *tetM*, *tetO* and *tetW* (tetracycline) (Wang *et al.*, 2021b). In addition, iron nanoparticles (*e.g*. magnetite nanoparticles, nanoscale zero-valent iron) can reportedly increase removal of ARGs and MGEs in processing WWTP and cattle manure (Ma *et al.*, 2019; Zhang *et al.*, 2020). Microbial community structures in particular are obviously influenced by these additives, with generally lower microbial diversity (Zhang *et al.*, 2017b). Thus, the mechanism of action of these substances is likely decreased biodiversity within the reactor, *i.e*. the main mechanism identified for thermophilic digestion and long retention time.

4.4 Other factors

The effect of other factors in antibiotic resistance reduction, apart from temperature, retention time and additives, has been less well investigated. These factors include: a) substrate properties (*e.g*. total solids (TS) content); b) environmental factors (*e.g*. pH, alkalinity, soluble chemical oxygen demand and heavy metals); and c) intermediate products (*e.g.* VFA and ammonia (NH_3)). Among these, substrate properties have been found to be most strongly correlated with removal of ARGs (Luo *et al.*, 2017; Tong *et al.*; 2018; Zhang *et al.*, 2020; Wang *et al.*, 2021c). For instance, in one study dry anaerobic co-digestion (AcoD) of pig manure and food waste with a TS content of 20% effectively reduced ARGs by 1.24 log copies/g wet sample, while the reduction was only 0.54 log copies/g wet sample in wet AcoD with a TS content of 5% (Wang *et al.*, 2021c). In other studies, heavy metals, pH, VFA *etc*. have also been found to be correlated with ARG removal (Tong *et al.*, 2018; Zhang *et al.*, 2020). In Paper **III** in this thesis, investigating AD processes using mainly crops as substrates, greater removal of ARGs and plasmids was seen at higher NH_3 concentrations, *i.e*. in biogas plants B (916 g L^{-1} on average) and C (955 g L^{-1}) compared with biogas plant A (120 g L^{-1}). Thus the results in Paper **III** suggest that removal efficiency of ARGs and MGEs might be enhanced with increasing NH_3 concentration. This is a reasonable assumption, as high concentrations of NH_3 have been shown to decrease microbial diversity in the AD process (Müller *et al.*, 2016; Peng *et al.*, 2018).

5. Antibiotic resistance in biogas digestate

Although efficient removal of certain ARB, ARGs and MGEs from different substrates has been achieved within a range of AD processes, biogas digestate still poses a risk of antibiotic resistance spread due to the residual resistance components it may contain. Critical questions in this regard are: What residual resistance components are present in digestate? and Are the resistances transferable to soil microbes once digestate is applied as fertiliser? These questions urgently need to be answered to ensure that the digestate does not pose a risk when used as biofertiliser. Thus, antibiotic resistance profiling of biogas digestate is important, and relevant findings are discussed in this chapter.

Figure 6. Biogas digestate from a laboratory-scale reactor processing food waste.

5.1 Antibiotic-resistant bacteria in biogas digestate

In general, ARB can survive AD, with reduced abundance, but enrichment has also been seen in some cases (Table 1). Complete removal of ARB during AD has been reported in a few studies, *e.g.* complete removal of multidrug-resistant bacteria during thermophilic co-digestion of dairy manure and waste milk (Beneragama *et al.*, 2013). In Paper **III** in this thesis, ARB resistant to vancomycin (glycopeptides), ciprofloxacin (fluoroquinolones) and gentamycin (aminoglycosides) were isolated from crop substrates, but not from the subsequent digestate, indicating possible complete removal of such ARB during digestion. In addition to multi-resistant bacteria belonging to Enterobacteriaceae, Staphylococcaceae and Enterococcaceae listed in Table 1, other ARB such as vancomycin-resistant *Enterococcus* (Glaeser *et al.*, 2016), and multi-resistant *Acinetobacter* spp. (Pulami *et al.*, 2020) have been found in digestate from farm-scale biogas plants processing animal manures. ARB in digestate from animal (dairy) manure were also identified in Paper **I** in this thesis, with *Bacillus* and closely related genera such as *Panibacillus* and *Lysinibacillus* being the dominant bacteria among the ARB isolated. These dominant bacteria exhibited diverse resistance to different classes of antibiotics, including β-lactams, tetracyclines and macrolides *etc.* (**I**). In contrast to many studies on ARB in manure digestion, prior to this thesis work no ARB isolation study had been performed on digestate obtained by processing food waste and crops, two other important AD substrates. Thus the studies presented in Papers **I** and **III** were conducted to gain novel insights into these two substrates. As found for dairy manure, *Bacillus* and closely related genera also dominated the bacterial community isolated from digestates processed from food waste (**I**) and crops (**III**). Moreover, they exhibited similar resistance patterns to ARB isolated from manure digestate in Paper **I**, *i.e.* resistance to β-lactams, tetracyclines, macrolides *etc*. Thus, the dominant ARB community isolated from digestate was similar in terms of phylogeny and antibiotic resistance pattern, independent of substrate type (**I, III**).

Table 1. *Resistance pattern and variation in abundance of antibiotic-resistant bacteria (ARB) in anaerobic digestion (AD) processes*

Substrate	ARB resistance pattern in substrate*	ARB resistance pattern in digestate	Reference
Pig manure	Tetracyclines and sulfonamides	Tetracyclines and sulfonamides	(Zou *et al.*, 2020)
Dairy manure and waste milk	Multidrug-resistant[a]	None	(Beneragama *et al.*, 2013)
Cattle manure	Multidrug-resistant	Multidrug-resistant	(Resende *et al.*, 2014)
Animal manure and slurry (*e.g.* cattle, chicken, pig *etc.*).	β-lactams, fluoroquinolone, tetracyclines, trimethoprim/sulfamethoxazole and sulfonamides (for Enterobacteriaceae)	β-lactams, fluoroquinolone, tetracyclines, trimethoprim/sulfamethoxazole and sulfonamides (for Enterobacteriaceae)	(Schauss *et al.*, 2016)
	β-lactams (ceftiofur; amoxicillin and oxacillin), fluoroquinolone, tetracyclines, trimethoprim/sulfamethoxazole and sulfonamides (for Staphylococcaceae)	β-lactams, fluoroquinolone, tetracyclines, trimethoprim/sulfamethoxazole and sulfonamides (for Staphylococcaceae)	
	β-lactams, tetracyclines, trimethoprim/sulfamethoxazole and sulfonamides (for Enterococcaceae)	Tetracyclines, trimethoprim/sulfamethoxazole and sulfonamides (for Enterococcaceae)	
Crops	β-lactams, polymyxins, glycopeptide, fluoroquinolone, aminoglycosides, tetracyclines	β-lactams, polymyxins, tetracyclines	(Paper **III**)
Crops and poultry manure	β-lactams, polymyxins, fluoroquinolone, aminoglycosides, tetracyclines	β-lactams, polymyxins, tetracyclines	

*Green, red and black font represents decreased, increased and not assessed abundance, respectively, of the relative ARB during AD. [a] Presence of antibiotics in substrate, but not in digestate in the same study, indicates complete removal during AD.

Interestingly, very few profiling studies have been performed on ARB cultivated in anaerobic conditions, even though AD refers to anaerobic digestion. Under anaerobic conditions, Derongs *et al.* (2020) isolated *Clostridium perfringens*, an anaerobic spore-forming bacterium, from dairy manure and subsequent digestate, and found that it had multiple resistance, even to imipenme (cabapenemes), which is considered the most reliable last-resort treatment for multidrug-resistant bacterial infections (Meletis, 2016). Tong *et al.* (2016) investigated variations in ARB abundance under anaerobic cultivation and found that ARB (non-identified) resistant to tetracycline and β-lactams were reduced during sludge digestion with microwave pre-treatment. To the best of my knowledge, these are the only previous publications on ARB cultivated from biogas digestate under strict anaerobic conditions. Thus, a profiling study on anaerobic ARB in food waste digestate is currently ongoing as a continuation of this thesis work. Preliminary results show that *Lentilactobacillus* is the most abundant genus, followed by *Paenibacillus*, *Bacillus*, *Clostridium*, *Enterococcus*, *Limosilactobacillus*, *Lacrimispora*, *Lactobacillus*, *Paraclostridium* and *Vagococcus*. These bacteria show resistance to β-lactams, tetracyclines, macrolides *etc*., with similar resistance patterns to the ARB cultivated under aerobic conditions in Papers **I** and **III**.

Some pathogens cultivated from digestates, such as *Escherichia/Shigella* spp., *Staphylococcus* spp. and *Enterococcus* spp. with multi-resistance (Schauss *et al.*, 2016), vancomycin-resistant *Enterococcus* (Glaeser *et al.*, 2016) and multi-resistant *Acinetobacter* spp. (Pulami *et al.*, 2020), pose direct health threats to the environment. For other non-pathogenic ARB, the ARGs they carry must be evaluated in terms of mobility, which can result in transfer of resistance to pathogens. This is discussed in the following section.

5.2 Antibiotic resistance genes and mobile genetic elements in biogas digestate and their transferability to the environment

The fate of ARGs and MGEs throughout the AD process has been widely studied, as this can provide an overview of changes in resistance level and to some extent indicate the transferability of resistance. In order to achieve a greater reduction in ARGs and MGEs, optimisation of AD processes has been attempted (Table 2), using *e.g.* thermophilic digestion (Zou *et al.*, 2020), high solids digestion (TS 22%) (Sun *et al.*, 2019b) and additives, *e.g.* powdered activated carbon (Zhang *et al.*, 2019a) and nano-magnetite (Zhang *et al.*, 2019b). However, most ARGs and MGEs have been able to survive the optimised processes, albeit with reduced abundance.

Among the ARGs subtypes, *sul1* and *sul2* (genes for resistance to sulfonamides) have been found to be present in almost all digestates listed in Table 2. These two genes are mediated by transposons and plasmids, and often found at equal frequencies among sulfonamide-resistant Gram-negative clinical isolates (Rådström *et al.*, 1991; Sköld, 2001). Moreover, the gene *sul1* is mostly found linked to other resistance genes in Class 1 integrons (*intI1*) (Sköld 2001), which is in line with the observed co-occurrence of genes *sul1* and *intI1* in all studies listed in Table 2. Collectively, these findings indicate that genes *sul1* and *sul2* are transferable, and thus that ARB carrying these genes are capable of spreading sulfonamide resistance to other previously susceptible opportunistic pathogens. Other ARGs identified in digestates, such as *tetO* (Luna & Roberts, 1998), *tetM* (Akhtar *et al.*, 2009) and $bla_{CTX\text{-}M}$ (Livermore *et al.*, 2007), have also been found to be associated with MGEs. Furthermore, presence of MGEs such as *intI1* (integrons), *Tn916*/1545 (transposons) and *ISCR1* (insertion sequence common region) (Table 2) provides vehicles for transfer of ARGs, indicating transferability of resistance from digestate. In addition to digestate processed from animal manure and food waste, presence of ARGs and plasmids in digestate derived from crops and crops/poultry manure was investigated for the first time in Paper **III**. Plasmid groups such as IncW, IncK and IncF *etc*. were found in the digestates, and these plasmids have previously been shown to be associated with a wide range of ARGs encoding for different antibiotic classes, including β-lactams, quinolones and aminoglycosides (Galimand *et al.*, 2005; Fernández-López *et al.*, 2006; Lascols *et al.*, 2008; Villa *et al.*,

2010). Thus, digestate derived from digestion of agricultural crops may pose a risk of antibiotic resistance spread.

Table 2. *Presence and variation in abundance of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in anaerobic digestion (AD) processes*

Substrate	Presence in substrate*	Presence in digestate	Reference
Pig manure	sul1, sul2, tetA, tetO, tetX, IntI1	sul1, sul2, tetA, tetO, tetX, IntI1	(Zou *et al.*, 2020)
Cattle manure	sul1, sul2, tetC, tetG, tetW, tetX, ermQ, ermX, qnrA, aac(6')-ib-cr, IntI1, IntI2, ISCR1, Tn916/1545	sul1, sul2, tetC, tetG, tetW, tetX, ermQ, ermX, qnrA, aac(6')-ib-cr, IntI1, IntI2, ISCR1, Tn916/1545	(Sun *et al.*, 2019b)
Swine manure	sul1, sul2, tetG, tetM, tetX, ermB, ermF, mefA, ereA, bla_{CTX-M}, bla_{TEM}, mcr1, IntI1	sul1, sul2, tetG, tetM, tetX, ermB, ermF, mefA, ereA, bla_{CTX-M}, bla_{TEM}, mcr1, IntI1	(Zhang *et al.*, 2019b)
Dairy manure	sul1, sul2, tetC, tetM, tetQ, tetW, tetX, gyrA, IntI1, IntI2	sul1, sul2, tetC, tetM, tetQ, tetW, tetX, gyrA, IntI1, IntI2	(Sun *et al.*, 2016)
Food waste	sul1, sul2[a], tetA, tetM, tetW, tetQ, tetO, tetX, cmlA, floR, IntI1	sul1, tetA, tetM, tetW, tetQ, tetO, tetX, cmlA, IntI1	(Zhang *et al.*, 2017b)
Food waste	sul1, sul2, sul3, tetC, tetM, tetQ, tetX, ermB, mefA, IntI1, tnpA, IS26, ISCR3	sul1, sul2, tetC, tetM, tetQ, tetX, ermB, mefA, IntI1, tnpA, traA[b], IS26, ISCR3	(He *et al.*, 2019)
Chicken manure and food waste	sul1, sul2, tetA, tetB, tetM, tetO, tetQ, tetW, tetX, cmlA, floR, IntI1	sul1,sul2, tetA, tetB, tetM, tetO, tetQ, tetW, tetX, cmlA, floR, IntI1	(Zhang *et al.*, 2019a)

*Green and red font represents, respectively, decreased and increased abundance of the respective gene during AD. [a]Presence of genes in substrate, but not in digestate in the same study, indicates complete removal during AD. [b]Presence of genes in digestate, but not in substrate, indicates emergence of new genes detected in digestate. *sul*, *tet* and *erm* represent sulfonamide resistance genes, tetracycline resistance genes and erythromycin resistance genes, respectively. Other ARGs listed are for resistance to: aminoglycosides (*aac(6′)-ib-cr*), fluoroquinolones (*aac(6′)-ib-cr, gyrA*), macrolides (*ereA*, *mefA*), colistin (*mcr1*), β-lactams (bla_{CTX-M} and bla_{TEM}), chloramphenicol (*cmlA*), phenicol (*floR*). The MGEs are: *IntI*1, *IntI*2, *IS*26, *ISCR*1, *ISCR*3 and *Tn*916/1545.

However, identification of ARGs and MGEs, and even correlation of ARGs and MGEs based on network analysis (Sun *et al.*, 2019b; Wang *et al.*, 2021c), are merely indications of resistance transferability in digestate. Further convincing evidence would be identification of ARGs and MGEs in individual ARB isolated from digestate. To my knowledge, no such studies had been performed prior to this thesis work, but in clinical studies Gram-negative species such as *E. coli* have been found to achieve resistance

transfer via HGT (Nagachinta & Chen, 2008). However, these pathogens are not a dominant community in digestates (Schauss *et al.*, 2016; Zou *et al.*, 2020; **I**). Instead, *Bacillus* appears to be a dominant ARB genus in digestate, but no information is available for this genus regarding antibiotic resistance transferability. Thus, the studies described in Papers **II** and **IV** were conducted to shed some light on this topic. In Paper **II**, a strain of *Bacillus oleronius* that is resistant to β-lactams (ampicillin, ceftazidime, meropenem) and tetracycline was investigated for mechanism of resistance and transferability. A plasmid, pAMαl, was identified as carrying three copies of the *tetL* gene, which explained the tetracycline resistance. However, no genes responsible for resistance to β-lactams were found on the whole genome. Meropenem and tetracycline resistances were tested for transferability, but were found not to be transferable by plasmid conjugation to competent recipient *E. coli* K12xB HB101. Therefore, the strain of *B. oleronius* posed a limited risk of resistance spread to the environment. In Paper **IV**, 18 antibiotic-resistant *Bacillus* and closely-related genera such as *Paenibacillus* and *Lysinibacillus* were investigated in terms of mechanism of resistance and transferability based on whole-genome analysis. Several strains with extra-chromosomal genomes were found, but none was identified as a plasmid. Thus the dominant ARB community likely represents a limited risk of spread of antibiotic resistance.

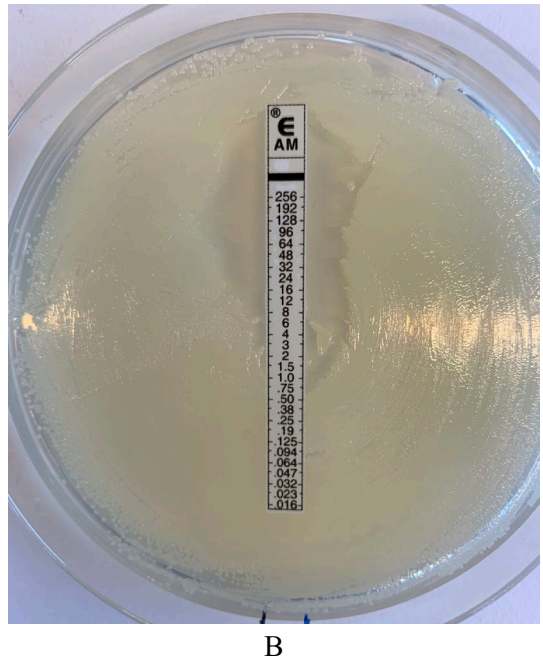
6. Methods for evaluating antibiotic resistance in anaerobic digestion

Assessment of the antibiotic resistance situation in AD processes is mainly conducted using one of two categories of method: molecular analysis or a culture-dependent approach. The culture-dependent method for selection of ARB is reliable in revealing variations in the antibiotic resistance situation during AD. However, culture in the laboratory often underestimates the diversity of ARB compared with cultivation in natural environments, since some bacteria can switch to a viable but non-culturable state under environmental stress (Del Mar Lleò *et al.*, 2003; Zandri *et al.*, 2012). With the development of sequencing technology, the culture-dependent method has gradually been replaced by high-throughput DNA sequencing (molecular analysis). Using molecular analysis, most of the work involved in identification of variations in microbial composition and ARGs can be done by simply extracting DNA directly from AD samples. However, considering the complexity of gene expression and substantial numbers of unknown genes, it is unclear whether molecular analysis can reveal the full antibiotic resistance situation. Thus in this chapter, the consistency in results obtained with molecular analysis and the culture-dependent method is discussed.

Several molecular methods have been used to date for analysis of the antibiotic resistance situation in AD environments, including: a) polymerase chain reaction (PCR) and quantitative PCR (qPCR) (Sun *et al.*, 2019b; Zou *et al.*, 2020), b) DNA chip approaches, such as ArrayMate Reader (Braun *et al.*, 2014) and Resistomap (Muurinen *et al.*, 2017), and c) metagenomic analysis (Zhang *et al.*, 2015, 2019a). All these methods can be used for identification/quantification of ARGs and MGEs in different scenarios, depending on the research aims. Metagenomic analysis is especially popular as it is a high-throughput method that efficiently provides an overview of antibiotic resistance in the AD process in terms of patterns and abundance of ARGs and MGEs, regardless of the cultivability of the bacteria. In addition, correlations can be calculated between microbial community and ARGs based on network analysis, in order to find potential bacterial hosts for ARGs (Zhang *et al.*, 2015, 2019a). However, finding potential hosts for genes based on network analysis was challenged in a recent study, which found no *tetO* gene in *Streptococcus* clones even though there was a significant correlation between the two (coefficient 0.909, $p<0.01$) (Zou *et al.*, 2020).

Notably, molecular analysis is database-dependent, which means that it is limited by recognition of novel genes and thus may overlook existing resistance. Besides, ARGs may be not expressed even though the genes and their promoters are intact (Enne *et al.*, 2006), while in some cases gene expression may not reach sufficient levels to confer resistance (Chen *et al.*, 2003). In cases of non-expression and weak expression of ARGs, the antibiotic resistance situation might be overestimated by a single molecular analysis. Collectively, inconsistency may arise between genotypic and phenotypic resistance. Few comparisons have been made of genotypic and phenotypic resistance. Although not explicitly stated by the authors, inconsistent results were seen in Zou *et al*. (2020), with no targeted *tet* genes (*tetA*, *tetO* and *tetX*) found in tetracycline-resistant bacteria. A study by Pulami *et al*. (2020) found six strains of *Acinetobacter baumannii* susceptible to ciprofloxacin and tetracyclines, but carrying relative resistance genes *abe*M and *ade*IJK. The results from these two studies illustrate some of the limitations of molecular analysis. In Papers **II** and **IV**, direct comparisons of genotypic and phenotypic resistance were made using whole-genome sequence (WGS) analysis and antibiotic susceptibility test (AST) (Figure 7). In Paper **II**, one strain of *Bacillus oleronius* with

A



B

Figure 7. Comparison of genotypic and phenotypic antibiotic resistance. A) Whole-genome sequencing by Oxford Nanopore. B) Antibiotic susceptibility test by E-strip.

phenotypic resistance to β-lactams (ampicillin, ceftazidime and meropenem) and tetracycline was investigated. The ARGs responsible for resistance to tetracycline (*tetL*), rifampicin (*rpfC*) and trimethoprim (*dfrG*) were identified by WGS. For this strain of *Bacillus oleronius*, tetracycline resistance was consistent with identification of *tetL*. However, no genes responsible for β-lactam resistance were found. Moreover, the *Bacillus* strain was susceptible to rifampicin, although *rpfC* was identified. In Paper **IV**, a similar inconsistency was found for every one of 18 bacterial strains isolated from digestate processed from food waste and dairy manure. Note that the molecular method applied in Papers **II** and **IV** was whole-genome sequencing for single isolates, rather than metagenomics for mixed culture (*e.g*. substrates and digestates) and the sequencing quality in terms of sequence coverage and accuracy is higher in WGS than in metagenomics. In brief, WGS analysis is capable of identification of all ARGs collected by databases if present in a bacterial genome. However, this method was unable to match to phenotypic resistance, possibly owing to limitations of databases and expression issues with genes. Thus, the antibiotic resistance situation in the AD process as evaluated by metagenomics may still not be the true situation. For this reason, the culture-dependent method, which is independent of database content and gene expression, is still the gold standard for assessing antibiotic resistance pattern and variations in ARB during AD. In fact, the results in Papers **II** and **IV** indicate that a combination of the two methods is necessary to reveal the full extent of antibiotic resistance in the AD process.

7. Conclusions

The ARB community in digestates originating from dairy manure, food waste and crops was isolated and characterised in terms of phylogenic identity and phenotypic antibiotic resistance pattern. Notably, the ARB community in digestates processed from food waste and crops was identified for the first time. Independent of substrate type, *Bacillus* and closely-related genera such as *Paenibacillus* and *Lysinibacillus* dominated the ARB community. These ARB exhibited resistance to a variety of antibiotic classes, including β-lactams, tetracyclines and macrolides. Crops used as AD substrate were also evaluated for the first time in terms of the antibiotic resistance load they confer to the biogas system. The plant-based substrates were found to be associated with antibiotic-resistant components, including culturable Gram-positive ARB and Gram-negative pathogenic bacteria-associated ARGs and plasmids. Thus, the antibiotic resistance load from plant-based substrates should be taken into consideration in agricultural biogas processing.

Transferability of resistance was evaluated by WGS analysis for a total of 18 strains of ARB (mostly belonging to *Bacillus* and closely-related genera) isolated from digestates based on dairy manure and food waste substrates. Most ARGs identified were located on chromosomes, although several strains were found to have extra-chromosomal genomes. Only one of these was identified as a plasmid (pAMαl), for a strain of *Bacillus oleronius*. One tetracycline-resistant gene, *tetL*, was found on pAMαl, while no gene was identified on any other extra-chromosomal genome. Besides, pAMαl, the only plasmid identified in the dominant ARB community, was found not to be transferable to a competent recipient strain, *E. coli* K12xB HB101, by plasmid conjugation. Collectively,

therefore, the dominant ARB community in AD digestate likely represents a limited risk of spread of antibiotic resistance.

Phenotypic and genotypic antibiotic resistance for the same 18 strains of ARB were compared using antibiotic susceptibility test (Estrip) and WGS analysis. Inconsistency was seen for every single strain, *e.g.* presence of an ARG but phenotypically susceptive, or no ARG present but phenotypically resistant. This inconsistency in antibiotic resistance pattern was observed for different antibiotic classes, including β-lactams, tetracyclines and macrolides. Thus, a combination of molecular and culture-dependent methods may be needed to reveal the true antibiotic resistance situation in AD processes.

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Popular science summary

Anaerobic digestion (AD) is a well-established technology and can play a key role in development of a sustainable society. Through AD, organic wastes produced in industry, agriculture and daily life, such as sewage sludge from wastewater treatment plants, animal manure and slurry from livestock farms, crop residues, and food waste are converted into green energy and a fertiliser. The green energy can be used for production of electricity, heat and vehicle fuel. The fertiliser has a high plant nutrient content and is more environmentally friendly than chemical fertiliser. However, in recent years, this fertiliser has been found to contain antibiotic resistance (AR) elements. AR is one of the most significant global public health challenges of our time, since it can be difficult to find a cure for patients infected with antibiotic-resistant bacterial strains. It is predicted that AR will cause around 300 million premature deaths by 2050. Thus, many countries have taken actions to control the spread of AR. The fertiliser produced from AD represents one potential transmission route of AR and therefore this thesis investigated different questions related to AR in digestate. In particular, it examined antibiotic-resistant bacteria and genes present in organic waste materials and in AD digestate. The results showed that the AR elements present in all original waste types were similar in terms of bacterial community and that they likely represent a low risk of AR spread, due to low transferability. Agricultural crops used for biogas production were found to carry variant AR elements. The best approach for evaluating the AR situation in digestate was found to be a combination of different methods, including identification of both resistant bacteria and antibiotic resistance genes.

Populärvetenskaplig sammanfattning

Biogasprocessen, eller rötning som den också kallas, är en väletablerad teknik som spelar en nyckelroll i utvecklingen av ett hållbart samhälle. Genom denna process omvandlas olika typer av organiskt avfall från industri, jordbruk och vårt dagliga liv, såsom avloppsslam från reningsverk djurgödsel och flytgödsel från djurgårdar, skörderester och matavfall, till grön energi och ett gödselmedel. Den gröna energin kan användas för produktion av el, värme och fordonsbränsle. Gödseln har en hög växtnäringshalt och är mer miljövänlig än konstgödsel. Under de senaste åren har dock detta gödselmedel visat sig innehålla antibiotikaresistens (AR). AR är en av vår tids största globala folkhälsoutmaningar. Om en person är infekterad av en antibiotikaresistent bakterie kan det vara svårt att hitta ett botemedel för denna patient. I värsta fall kan patienten dö av infektionen. Det har uppskattats att AR kommer att orsaka omkring 300 miljoner förtida dödsfall år 2050. Många länder har därför vidtagit åtgärder för att kontrollera spridningen av AR. Gödselmedel som produceras genom rötning, representerar en potentiell överföringsväg för AR, men forskningen inom detta område är fortfarande ganska begränsad och mer arbete krävs för att utreda detta.

Denna avhandling har undersökt olika frågor relaterade till antibiotikaresistens i biogödsel från biogasprocesser, det vill säga vilka är de antibiotikaresistenta bakterierna och generna som finns i de organiska avfallsmaterialen och i biogödslet? Mer specifikt identifierades olika AR-element (bakterier och gener) från olika organiska avfall, gödsel, matavfall och grödor. Resultaten visade att AR-elementen var likartade när det gäller bakteriesamhället, oavsett ursprungliga avfallstyper, och att de sannolikt representerar en låg risk för spridning av resistens, på grund av låg överförbarhet. Resultaten visade också att grödor innehåller AR-element,

vilket tyder på att gödselmedel som produceras från grödor också utgör en potentiell källa för AR-spridning. Slutligen föreslås i denna avhandling att den bästa utvärderingsmetoden för att avslöja den verkliga AR-situationen i biogasprocesser är att använda en kombination av olika metoder, dvs både identifiera antibiotikaresistenta bakterier och gener ansvariga för resistansen.

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Biogas digestate is a nutrient-rich biofertiliser, allowing recycling of nutrients between urban and rural areas, but also representing a potential transmission route for pollutants and antibiotic resistance. In this thesis, a combination of molecular and culture-dependent methods was used to evaluate antibiotic resistance in biogas processes, focusing on antibiotic-resistant bacteria, resistance patterns and transferability. The results indicated that digestates studied pose a limited risk of spread of antibiotic resistance when used as fertiliser.

He Sun received his graduate education at the Department of Molecular Sciences, SLU, Uppsala. He received his Master and Bachelor degrees in Agricultural Engineering at China Agricultural University, Beijing, China.

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