Anaerobic Digestion at Mesophilic and Thermophilic Temperature

With Emphasis on Degradation of Phenols and Structures of Microbial Communities

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Abstract

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During anaerobic digestion of organic wastes, nutrient-rich digestate is produced. This digestate has great potential as a fertiliser on farmland. However, one concern is the content of organic pollutants in the digestate, as these may influence the soil fertility in the long-term perspective. Different organic pollutants have been found in various organic wastes and types of digestate. This thesis describes the effect of temperature on the anaerobic degradation of different organic pollutant, *i.e.* aromatic compounds, and on the structure of microbial communities.

To isolate the impact of temperature on the degradation, investigations were carried out in two laboratory-scale bioreactors that were stable and equivalent in performance apart from process temperature and hydraulic retention time. These reactors have treated the same type of organic municipal household waste for many years. The aromatic compounds were chosen to represent important anaerobic reaction steps and key intermediates. Several of the selected compounds were efficiently degraded at the mesophilic temperature (37 ºC). In contrast, phenols and phthalic acid were not degraded at the thermophilic temperature (55 ºC). This limited degradation at the higher temperature is contrary to the general degradation rate of organic matter, which increases with elevated temperatures. Chemical analysis of the digestate from the laboratory-scale bioreactor operating at thermophilic temperature detected a comparably higher content of phenols, verifying the degradation results. The effect of temperature on phenol degradation was also further confirmed in a study including several large-scale bioreactors.

To investigate the impact of temperature on microbial community structure, phylogenetic analysis was performed with 16S rRNA genes from the archaeal and bacterial communities in the same two laboratory-scale bioreactors as used for the degradation study. This analysis revealed a lower microbial diversity at the higher temperature. This difference in diversity can possibly explain the observed difference in degradation capacity. The microorganisms responsible for the degradation of phenol were studied in enrichment cultures originating from the two laboratory-scale bioreactors. Two different bacteria were suggested to perform the phenol degradation in these cultures, a finding that highlights the influence of temperature on community structure.

Keywords: anaerobic degradation, aromatic compounds, digestate, microbial community, molecular cloning, phenols, temperature, 16S rDNA

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Gå inte alltid dit vägen leder.

Gå istället där ingen väg finns –

Och lämna spår.

Contents

Appendix

Paper I-III

This thesis is based on the following publications, which are referred to by their Roman numerals:

I. Levén, L. & Schnürer A. 2005. Effect of temperature on biological degradation of phenols, benzoates and phthalates under methanogenic conditions. *International Biodeterioration & Biodegradation* 55, 153-160.

II. Levén, L., Nyberg, K., Korkea-aho, L. & Schnürer, A. Phenols in anaerobic digestion processes and inhibition of ammonia oxidising bacteria (AOB) in soil. *Science of the Total Environment* 364, 229-238.

III. Levén, L., Eriksson, A.R.B. & Schnürer, A. Effect of process temperature on bacterial and archaeal communities in two methanogenic bioreactors treating organic household waste. *FEMS Microbiology Ecology* In press.

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My contribution to the papers included in this thesis has been as follows:

I. Participating in planning the project and performed major part of the laboratory work. Main writer of the manuscript.

II. Took part in planning the project and had the main responsibility for performance of the anaerobic degradation study and analysis of results. Main writer of the manuscript.

III. Participating in planning the project. Performed all the laboratory work and data evaluation, and did major part in writing the manuscript.

Introduction

During anaerobic digestion, organic matter is converted to carbon dioxide and methane, *i.e.* biogas, by microorganisms in an oxygen-free environment. This process occurs naturally in swamps, sediments, rice fields, ruminants, hindguts of insects and landfills, and under controlled artificial conditions in anaerobic bioreactors (Barlaz *et al.*, 1989; Schink, 1997; Sowers & Watts, 2006). Due to new regulations from January 2005 prohibiting the deposition of organic wastes in landfills in Sweden (SFS 2001:512), the biogas process is being increasingly used for stabilisation of various organic wastes. Most common is the treatment of sewage sludge, animal manure, slaughterhouse waste and source-separated municipal household waste (Swedish Association of Waste Management, 2005). The anaerobic digestion technique is often applied in either the mesophilic (30-40 ºC) or thermophilic (50-60 ºC) temperature range (Ahring, 1995). A major environmental benefit of the anaerobic digestion process is the production of biogas, a renewable energy source, which can be used as vehicle fuel, for heating and for electricity production. The use of fossil fuel can thereby be reduced, enabling CO_2-levels to be lowered in conformance with the Kyoto protocol.

During anaerobic digestion, not all of the organic matter is completely degraded and will end up in a residual product (digestate), which is also rich in inorganic nutrients. This makes the digestate an excellent complement to manure and commercial fertilisers on agricultural soils (Odlare, 2005). Through this re-circulation of nutrients between urban and rural areas, the waste can be considered a reusable resource. However, it is important that the recycled digestates do not contain pathogens and/or chemical contaminants that may build up to deleterious levels in the soil. Unfortunately, different organic pollutants have been found in digestates. When considering the risks associated with the use of digestate as fertiliser, information on the character and the fate of the pollutant in anaerobic bioreactors and in soil is of importance.

Aim and outline of the thesis

The main degradation pattern of organic matter in the anaerobic digestion process has been well investigated. It is also known that the performance of the anaerobic digestion process, and thus the degradation of organic pollutants, is influenced by the composition of the microbial community present. However, little attention has been given to the effect of temperature on the development of microbial communities and on the degradation of specific aromatic compounds. These two topics were therefore selected for investigation in this thesis.

In an initial investigation, the anaerobic degradation of different aromatic compounds was studied in laboratory-scale bioreactors degrading organic household waste at mesophilic and thermophilic temperatures (**Paper I**). The set of aromatic compounds studied represented important anaerobic degradation reactions or key intermediates. The results revealed that temperature had a strong impact on the degradation of phenolic compounds. This difference in degradation capacity of phenols due to temperature was subsequently verified in large-scale bioreactors (**Paper II**). In order to investigate the effect of process temperature on microbial community structure, phylogenetic analysis of the bacterial and archaeal communities was performed on material from the laboratory-scale bioreactors used in first study (**Paper III**). The result from these analyses revealed a possible link between microbial diversity and degradation capacity of aromatic compounds. The microorganisms responsible for the degradation of phenol were further studied in enrichment cultures originating from the two laboratory-scale bioreactors operating at mesophilic or thermophilic process temperature.

Anaerobic digestion

History

The use of anaerobic digestion for treatment of wastewater and stabilisation of solid waste is nothing new; it has been used since the latter part of the 19th century. In rural parts of China and India, simple reactor constructions have long been used to treat manure and agricultural wastes with the main purpose of recovering energy for cooking and lighting (Gijzen, 2002; Fig. 1). Anaerobic digestion of sewage sludge in large-scale plants using more advanced technology was introduced in the 1860s in France (McCarty, 2001). However, it was not until the 1970s that anaerobic treatment received more attention in terms of both research and technological development. This interest arose, as a consequence of increased environmental awareness and escalating energy costs. Furthermore, at the end of 1980s, co-digestion processes treating a mixture of different types of waste, including manure, food waste and organic household waste, were introduced *e.g.* in Denmark (Ahring, 2003). Today, a number of different anaerobic digestion techniques are in use for different types of substrate and development is still in progress (Gijzen, 2002).

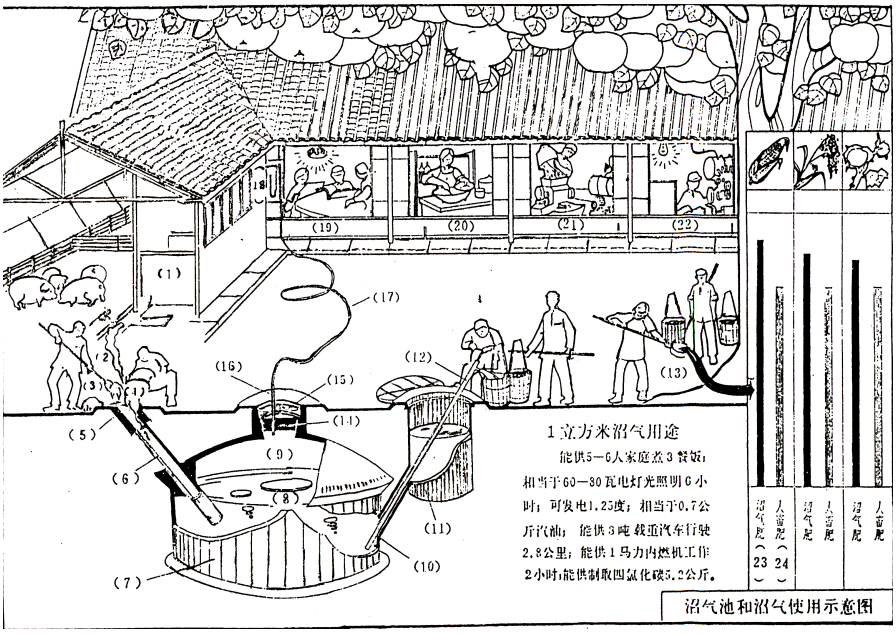

Fig. 1. An overview of a Chinese-type reactor producing biogas for cooking and lighting. Original picture published in Journal 'Biogas in China' by National Office for Biogas Development and Extension, People's Republic of China.

Anaerobic digestion in Sweden

In Sweden, the history of biological anaerobic treatment of organic material started about 40 years ago. At that time the technique was mainly used for stabilisation of sewage sludge. However, after the energy crisis in the 1970s, interest increased in the production of biogas from organic materials other than sewage sludge, such as manure and industrial wastewater. From the middle of the 1990s, several biogas plants treating organic solid wastes from the food industry and food waste have been in operation in Sweden (Swedish Association of Waste Management, 2005). Thanks to environmental taxes, investments support, environmental objectives and items of legislations such as the prohibition on landfilling of organic wastes (SFS 2001:512), both the number of biogas plants in Sweden and their capacity are steadily increasing (Swedish Environmental Protection Agency, 2005). Excluding the processes used for sewage sludge treatment, there are around 15 large-scale anaerobic bioreactors in Sweden today, treating 244 000 tonnes of organic material annually (Swedish Environmental Protection Agency, 2005). The substrate usually consists of different organic materials such as organic municipal solid waste, slaughterhouse waste, industrial waste, and cow and swine manure, and is treated in different combined mixes (co-digestion). At present, around 10% of Swedish source-separated household waste is biologically treated and the national target is to treat 35% of the food waste biologically by 2010. Extended waste management in the municipals councils will possibly double the use of biological treatment within a few years (Swedish Association of Waste Management, 2005). In addition to the co-digestion plants, there are about 11 farm-based plants mainly treating manure. The interest in using agricultural crops in both industrial and farm plants for production of biogas is presently increasing. Preliminary calculations have indicated that as much as 85% of the potential for biogas production in Sweden in the future, will originate from agricultural crops (Norberg, in press).

Different anaerobic digestion techniques are used for treatment of various organic materials. In Sweden, the continuously stirred tank bioreactor (CSTR) is the most commonly used technique for solid wastes. In these completely mixed reactors with continuous or intermittent loading, different organic wastes are commonly treated by co-digestion. Co-digestion of wastes of different composition generates higher gas production compared to sewage sludge or manure solely (van Lier *et al.*, 2001). Furthermore, some wastes are not suitable for treatment as a sole substrate. For example, fat- and protein-rich wastes can cause problems in the degradation process due to the release of inhibitory concentrations of ammonia and fatty acids. These inhibitory effects can be reduced by dilution with other types of wastes (Ahring, 2003). In addition to substrate composition, temperature is an important process parameter for management of biogas processes. In Sweden, CSTRs commonly operate at mesophilic or thermophilic temperature.

Process temperature

In the beginning of the anaerobic digestion era, no heating or mechanical mixing was applied in bioreactors, resulting in low conversion efficiency and gas yield. Development of bioreactors operating at mesophilic (30-40 ºC) and thermophilic

(50-60 ºC) temperatures was a consequence of increased knowledge about reaction rates and growth optima for the anaerobic microorganisms active in the digestion process (Fig. 2; McHugh *et al.*, 2006). Anaerobic digestion at thermophilic temperature has a higher bioconversion rate and a shorter treatment time than digestion at lower temperature (Zábranská *et al.*, 2000; van Lier *et al.*, 2001). The increase in gas production at the higher temperature can compensate for the additional costs related to heating (Hartmann & Ahring, 2006). Another advantage of digestion at thermophilic temperatures is the comparatively stronger hygienisation effect, *i.e.* more efficient killing of pathogens present in the waste (Zábranská *et al.*, 2000; Sahlström, 2003; Bagge *et al.*, 2005). In comparison with thermophilic bioreactors, the mesophilic process requires less energy for heating and has a slower conversion of the organic material. This process is also commonly less affected by inhibitory effects of ammonia released during the mineralisation of proteins (Angelidaki & Ahring, 1994; Sanchez *et al.*, 2000). At present, the interest in anaerobic treatment under psychrophilic conditions (<20 ºC) is increasing due to its considerably lower heating costs (McHugh *et al.*, 2006). New and modified bioreactors successfully facilitate the use of this low temperature technique (Connaughton *et al.*, 2006).

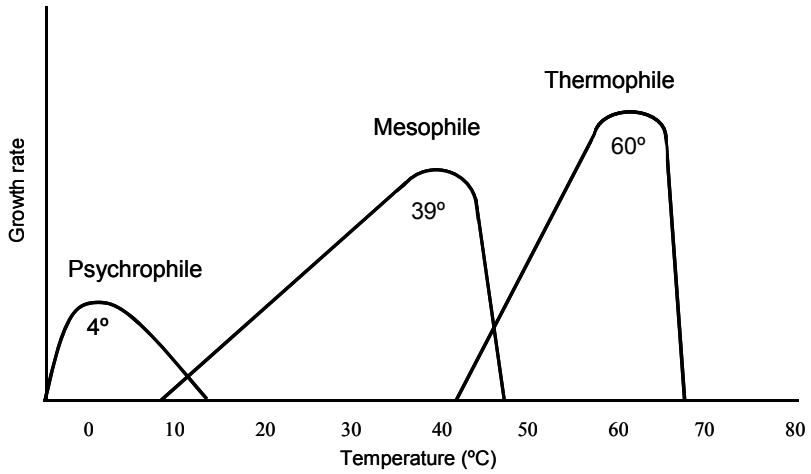

Fig. 2. Relation of temperature to growth rates for a psychrophilic, mesophilic and thermophilic microorganism. Modified after Brock, Biology of Microorganisms (Madigan & Martinko, 2006).

Laboratory bioreactors

Much knowledge concerning anaerobic digestion processes is gained from the use of reactors in bench-, lab- or pilot-scale. Small-scale bioreactors is possible to use to imitate large-scale biogas plants, because identical process performance to large-scale reactors are easily obtained in the laboratory (Leksell, 2005). There are several advantages in performing studies on a smaller scale. It is possible to have replicates, to use less advanced technology and to lower the experimental costs. Furthermore, the same substrate can be used during the entire experimental period and the characteristics of the input substrate can be better controlled. The composition of the substrate can consist of only a few components or be of a more

complex character such as organic municipal solid waste. For the studies performed in this thesis (**Paper I** and **III**), two semi-continuous laboratory-scale reactors treating organic municipal solid waste at 37 ºC or 55 ºC were used (Fig. 3). After a stabilisation period, these reactors operated with parameters in conformity with large-scale bioreactors in Sweden. These processes were stable in performance and equivalent in terms of *i.e.* volatile solid reduction, gas yield and methane content, but differed in temperature and hydraulic retention time.

Fig. 3. The two laboratory-scale bioreactors used in experimental work, treating source sorted organic municipal household waste at mesophilic or thermophilic temperature.

Methanogenic degradation of organic matter

The complete anaerobic degradation of organic matter is a complex process involving a number of steps and microorganisms with different metabolic capacities (Fig. 4; Zinder, 1984). In the first step, polymers such as lipids, proteins and polysaccharides are converted to monomers through hydrolysis. This degradation is performed by the action of extracellular enzymes produced by hydrolytic and fermentative bacteria. The monomers produced, *i.e.* amino acids, sugars and fatty acids, are then primarily fermented to volatile fatty acids, alcohols, hydrogen (H_2) and carbon dioxide (CO_2) by different groups of fermentative bacteria. In the third step, proton-reducing acetogenic bacteria produce acetate, hydrogen and carbon dioxide through anaerobic oxidation of the fermentation products. This step is endergonic under standard conditions and can only be performed if these organisms are operating in a syntrophic relationship with hydrogenotrophic methanogens (Stams, 1994; Schmitz *et al.*, 2005). Acetate can also be formed from hydrogen and carbon dioxide through the activity of hydrogen-oxidising acetogenic bacteria. The reverse reaction, *i.e.* the oxidation of acetate to CO_2 and H_2, can be performed under certain conditions by some acetogenic bacteria in syntrophic cooperation with hydrogen-transforming methanogens (Zinder & Koch, 1984; Schnürer *et al.*, 1996; Hattori *et al.*, 2000; Balk *et al.*, 2002). Methanogenesis is the last step in the anaerobic digestion, with two main possibilities to produce methane. One involves the acetotrophic

methanogens, which have acetate as substrate, whereas the hydrogenotrophic methanogens use the hydrogen and carbon dioxide produced (Zinder, 1984).

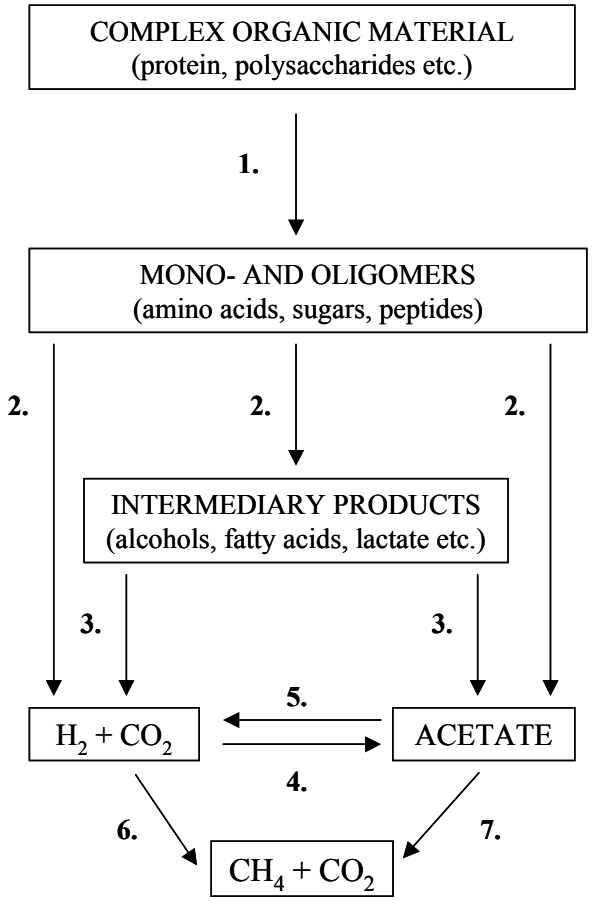

Fig. 4. Carbon flow during anaerobic degradation of complex organic material to methane and carbon dioxide: 1. Hydrolysis; 2. Fermentation; 3. Anaerobic oxidation; 4. Hydrogen oxidation; 5. Syntrophic acetate oxidation; 6. Hydrogenotrophic methanogenesis; 7. Acetotrophic methanogenesis. Modified after Zinder (1984).

During formation of biogas from complex organic material, only a small amount of energy is available for microbial growth compared to when oxygen or terminal acceptors other than carbon dioxide are used (Schink, 1997). Most of the energy (86%) is recovered in the end product, methane. Roughly only 10% of the potential energy is available to the fermentative bacteria, while 90% is conserved in the volatile fatty acids (Westermann, 1996). Of this 90%, only 4% is available to the methanogens. This low energy yield forces the microorganisms that are active during the methanogenic degradation into very close syntrophic relationships (Schink, 1997). Through this syntrophic cooperation, the thermodynamic restrains are altered and otherwise unfavourable reactions become favourable and can proceed. The energy provided for the syntrophic consortium is

generally higher than if a fermentative bacterium were to solely utilise the same substrate (Stams, 1994).

Anaerobic digestate as a fertiliser

Anaerobic digestate is a suitable fertiliser for agricultural soil (Fig. 5). The digestate contains organic matter and plant nutrients (N, P, K and Mg) and these positively affect soil quality by improving the soil structure, increasing the water-holding capacity and stimulating the microbial activity (Marinari *et al.*, 2000; Debosz *et al.*, 2002). The end result is not only an increase in soil quality, but also higher crop yields and better grain quality in comparison with unfertilised soil, or equivalent effects after application of artificial fertiliser (Odlare, 2005). However, the disposal of the residual product is one of the main problems for anaerobic treatment plants in Sweden today. Although a voluntary certification system for anaerobic digestate and composts was introduced in 1999 (Swedish National Testing and Research Institute, SPCR120), many trade associations and farmers are still reluctant to use this product on agricultural soil (ARLA, Swedish Farmers Association (LRF); pers. comm.). This negative attitude depends mainly on previous alarming reports on high concentrations of heavy metals and organic pollutants in sewage sludge. In addition, fear for spreading heat-resistant pathogens restricts the use of digestate on farmland. For waste of animal origin, including manure, a hygienisation process (70 ºC for 1h) is recommended to kill pathogens. However, spore-forming pathogenic microorganisms, such as *Clostridia* and some fungi, can survive this treatment (Bagge *et al.*, 2005; Schnürer & Schnürer, 2006). In order to certify a digestate, the concentrations of plant nutrients, heavy metals and pathogens present have to be declared. At present, no declaration of organic pollutants is required (Certificate protocol: SCPCR120).

Fig. 5. Distribution of anaerobic digestates on agricultural soil according to the general practice for liquid manure application used by farmers in Sweden. (Photo: Lena Rodhe)

Organic pollutants in digestate

Organic industrial wastes, manure and organic household wastes may contain a variety of organic pollutants, often with an aromatic structure (Angelidaki *et al.*, 2000). In fact, dioxin-like compounds (Engwall & Schnürer, 2002; Olsman *et al.*, 2002), polyaromatic hydrocarbons (PAH) (Angelidaki *et al.*, 2000), pesticides, PCBs (Nilsson, 2000), chlorinated paraffins (Nilsson *et al.*, 2001), phthalates (Angelidaki *et al.*, 2000; Nilsson *et al.*, 2000; Hartmann & Ahring, 2003) and phenolic compounds (Angelidaki *et al.*, 2000; **Papers I** and **II**) have been found in different digestates. The presence of these compounds can result in a digestate not suitable for use as fertiliser on agricultural soils. These contaminants can originate from pesticide traces on fruit and vegetables or additives in plastic material, or may come from contamination in the collection and transport chain of the waste to the biogas plant (Nilsson, 2000; Hartmann & Ahring, 2003). However, some toxic compounds may also be formed during the anaerobic degradation of larger organic compounds. One example of such compounds is phenols, commonly produced during biodegradation of both xenobiotic compounds and naturally occurring aromatic amino acids and aromatic polymers, *e.g.* humic acids, lignins and tannins in plant material (van Schie & Young, 1998). The presence of pollutants after the anaerobic treatment of organic waste indicates that they may not be efficiently degraded during this process. Furthermore, analyses of digestate from the mesophilic and thermophilic bioreactors described in this thesis have revealed an impact of process temperature on the content of pollutants. For example, the highest levels of dioxin-like compounds have been detected in digestate from the mesophilic bioreactor (Engwall & Schnürer, 2002). In addition, results reported in this thesis showed a higher content of phenols in the digestate from the thermophilic bioreactor (**Paper I**).

Effects of organic pollutants on soil microorganisms

Addition of pollutants to a soil ecosystem may pose a serious threat to microbial functions and hence the productivity and sustainable use of the soil (Pell & Torstensson, 2002). One way to investigate effects of toxic compounds on the microbial transformation of carbon and nitrogen in soil is to use different assays such as basal respiration, substrate-induced respiration (SIR), potential denitrifying activity (PDA) and potential ammonia oxidation (PAO) (Pell & Torstensson, 2002). The ratio of basal respiration and SIR reveals how efficiently organic carbon is converted into biomass by heterotrophic bacteria, and can be used as an indicator of stress caused by chemical contaminants (Jones & Ananyeva, 2001). PDA and PAO assays address both a function and a specific functional group. The target organisms in the PAO, the autotrophic ammonia oxidising bacteria (AOB), are highly sensitive to environmental disturbances. For that reason, they have frequently been used as indicator organisms for studying the effects of different pollutants applied on the soil, as well as different agronomic treatments (Hastings *et al.*, 1997; Pell *et al.*, 1998; Chang *et al.*, 2001; Petersen *et al.*, 2003; Nyberg *et al.*, 2004).

Addition of digestates to soil causes a general stimulation of soil microorganisms, due to the presence of inorganic nutrients and organic matter (Petersen *et al.*, 2003). This stimulation might hide the underlying effects of pollutants on specific microbial groups (Nyberg *et al.*, 2004). However, the effects of pollutants can be isolated by extracting the organic fraction of the digestate and in turn using the extract in soil microbial tests (Nyberg *et al.*, 2004; **Paper II**). Using this approach, organic extracts of digestates from large-scale bioreactors and swine manure have been shown to inhibit AOB activity, indicating the presence of toxic organic substances (Nyberg *et al.*, 2004; Nyberg *et al.*, 2006: **Paper II**). Furthermore, there was a clear positive relationship between the degree of inhibitory effects on AOB activity and the content of phenols in the digestate (**Paper II**). Activity measurement with addition of pure phenols also supported the hypothesis that phenols in the digestate were the cause of inhibition (**Paper II**). Negative effects of phenols on microbial activities have previously also been reported for other microorganisms (Dyreborg & Arvin, 1995; Varel & Miller, 2001; Olguin-Lora *et al.*, 2003), illustrating the toxicity of these compounds. A possible source of phenols in the digestate examined was swine manure. Swine manure is known to contain phenolic compounds (Wu *et al.*, 1999) and the phenol content in the digestate was higher with a higher input of swine manure (**Paper II**).

Risks in using digestate as a fertiliser

The presence of organic pollutants in digestate can cause disturbances that may imply reduction of microbial diversity and functional redundancy in soil. This can impair resilience to perturbations in the soil (Girvan *et al.*, 2005). When considering whether application of toxic compounds present in digestate represents a risk to the soil, several issues are of importance; the concentration, the degradation pathway and the chemical and physical properties of the compound, *e.g.* its chemical reactivity, water solubility, volatility and sorption capacity (Ejlertsson *et al.*, 1996; Alexander, 1999). Furthermore, the character and degradation capacity of the soil, as well as the long-term perspective, should also be considered (Bergström & Stenström, 1998; Enwall *et al.*, 2005; Girvan *et al.*, 2005). Persistent pollutants accumulating in soil might affect the soil fertility in the long-term perspective, in contrast to rapidly degraded compounds with formation of non-toxic intermediates.

Application of phenol-containing digestate to soil gave rise to concentrations (0.01-3.11 µg phenols g-1 d.w. of soil, **Paper II**) that are close to the threshold value set by the Swedish Environmental Protection Agency for contaminated soil (4 µg g-1 d.w. of soil). However, the phenols present in these digestates (Fig. 6) were rapidly degraded in soil and their negative effects on soil bacteria (**Paper II**) will therefore most likely fade and not cause any long-term effects in soil. To enable risks associated with organic pollutants to be estimated in general terms, analysis of such compounds should be included in the certification protocol. Only by identifying and measuring the concentration of the pollutants is it possible to evaluate the quality of the digestate. Furthermore, by optimising the anaerobic digestion process towards a more efficient degradation of different organic pollutants, the amount of toxic compounds in the digestate could be reduced and the risks minimised.

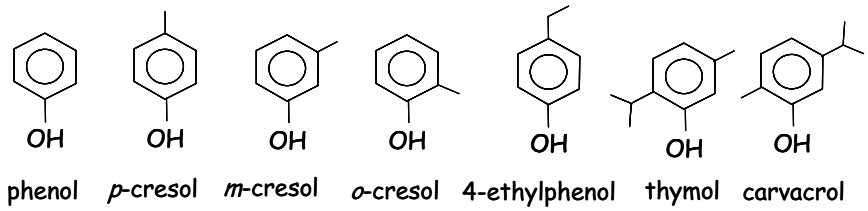

Fig. 6. The main phenols found in digestates from anaerobic digestion processes degrading a mix of organic wastes.

Anaerobic degradation of aromatic compounds

Laboratory degradation studies

Aromatic compounds are the second most abundant group of organic components in the biosphere after carbohydrates, and many are considered to be pollutants (Field *et al.*, 1995). Thus, to get a general picture of the fate of organic pollutants in anaerobic bioreactors the aromatic compounds are interesting to study. In order to investigate the capacity of the anaerobic digestion processes to degrade these compounds, a set of different compound structures were used, namely benzoate, 2,3,5-trichlorobenzoate (triCBa), phthalic acid, methylphthalate, ethylphthalate, resorcinol, phenol, pentachlorophenol (PCP), 4-nitrophenol, 4-ethylphenol and *o*-, *m*- and *p*-cresol (**Paper I**). These were selected to represent key metabolic intermediates and important anaerobic reaction steps (dehalogenation, dehydroxylation, nitro-group reduction, deamination, carboxylation, decarboxylation and ester hydrolysis). A similar approach has previously been used by Ejlertsson *et al.* (1996).

Batch cultures

The anaerobic degradation studies in **Paper I** and **II** were performed by dilution of digestate from laboratory- or large-scale bioreactors in anaerobic liquid medium. This is a commonly used method for these kinds of investigations. In small diluted anaerobic batch cultures it is easy to monitor the degradation over time, as samples can easily be repeatedly taken with a syringe from the bottles (Fig. 7). It is probably also easier to detect intermediates as the degradation rate decreases in the dilution culture compared to the original bioreactor. Dilution cultures also facilitate the measurement of methane, produced during mineralisation of the compound *i.e.* methane produced in excess to endogenous background levels.

Fig. 7. Diluted anaerobic batch cultures containing anaerobic medium and inoculum from a bioreactor or an enrichment culture.

A disadvantage with dilution cultures is that cell aggregates may become disrupted during preparation, thereby reducing the degradation efficiency. Disruption of spatial juxtaposition of syntrophic bacteria and their methanogenic partner inhibits important oxidation reactions (Schink, 1997; McMahon *et al.*, 2001). In addition, incubation in liquid media containing nutrients can affect the members of the microbial community differently compared to the *in situ* situation. Thus, in the interpretation of the results from the degradation study, one has to be aware that the conditions in the small diluted anaerobic batch cultures differs in some aspects from the prevailing situation in the reactor. The degradation potential might be either over- or underestimated. However, the results obtained in the initial degradation study reported in this thesis were confirmed by chemical analysis of the digestate of the two laboratory-scale bioreactors and additionally through a verifying degradation study in several large-scale bioreactors (**Papers I** and **II**).

Extraction methods

Before suitable extraction methods were identified for these investigations, several methods and techniques were evaluated for the various compounds. To confirm the degradation of a specific compound the recovery is important to consider. It should be high enough to ensure the disappearance as degradation and not just an inability to extract the compound. Unfortunately, exchange values are seldom mentioned in degradation studies, sometimes making it difficult to evaluate methods and results. However, in **Papers I** and **II**, it was possible to achieve recoveries above 80% by developing efficient extraction methods. The most difficult compounds to extract are those with lipophilic properties, such as heavily chlorinated compounds, which bind to the organic matter. Using freeze-drying, an increase in pH, organic solvents, and sonication, satisfactory extraction of such compounds (PCPand triCBa) was achieved (**Paper I**). For more hydrophilic compounds, dissociation through changes in pH transforms the compounds into charged form and makes them easier to extract from the samples. This method was used in **Papers I** and **II** for phenol, phthalic acid, benzoate and cresols. In the extraction of nitrophenol, resorcinol and ethylphenol, not only a change in pH but also a change in polarity, through addition of methanol, was needed to reach the required recovery values (**Paper I**).

Analysis techniques

With chemical analysis of aromatic compounds, both disappearance and production of intermediates and final products can be studied. In order to detect and quantify compounds with different characters, different techniques are optimal. One such technique is chromatography, where chemical interactions with the column are used to separate the compounds and their degradation products. For example, water-soluble compounds are easily analysed using high performance liquid chromatography (HPLC). In this thesis phenolic compounds were analysed with this technique (**Paper I and II**), performed in accordance with Karlsson *et al.* (1999). A gas chromatography-mass spectrometry (GC-MS) method developed by Schmidt-Bäumler and colleagues (1999) was used in order to separate and analyse compounds with a more lipophilic character, *i.e.* pentachlorophenol and trichlorobenzoate (**Paper I**). Gas chromatography was also applied for the analysis of methane (**Paper I**).

Labelled carbon-13 and carbon-14 can also be used for studying microbial metabolism of different compounds. Different techniques such as nuclear magnetic resonance (NMR) and scintillation counting are available. NMR is based upon the magnetic properties of the nucleus in an atom and not only can it detect isotopic differences, but it also gives an indication of the position of the atom. These properties have been utilised to investigate degradation pathways in previous studies (Schnürer *et al.*, 1996; Elshahed *et al.*, 2001), as well as in **Paper I**. With scintillation counting, confirmation of mineralisation of a compound is possible by measuring the amount of methane or carbon dioxide produced from a labelled organic compound (Schnürer *et al.*, 1994; Girvan *et al.*, 2005; Karakashev *et al.*, 2006). By labelling techniques it is possible to detect very low concentrations of the added compound, intermediates and/or the product formed. These methods are more sensitive than the approach with chromatography. However, one drawback is that both NMR and scintillation counting are more expensive to perform.

Factors influencing degradation

In anaerobic degradation, aromatic compounds are mainly transformed by different reductive reactions and commonly require a consortium of bacteria (Field *et al.*, 1995; Heider & Fuchs, 1997). As previously mentioned, the collaboration between microorganisms increases the energy yield obtained during anaerobic degradation and facilitate the degradation (Schink, 1997). The anaerobic microorganisms obtain energy from degradation of aromatic compounds either through fermentation or respiration (Heider & Fuchs, 1997). The degradation can be influenced by several environmental factors: Firstly, the amount of energy obtained during degradation is dependent on the final electron acceptor (NO_3^- > MnO_2 > FeOOH > SO_4^{2-} > S > CO_2). For example, the pathway used for the degradation of benzoate is not the same for nitrate reducers as for syntrophically fermenting bacteria. This difference is probably caused by the strict energy constraints on syntrophic metabolism compared to anaerobic respiration with nitrate as the final electron acceptor (Elshahed *et al.*, 2001; Schink, 2006). Secondly, the number and characters of the functional groups facilitate or impede the nucleophilic mechanism commonly occurring in anaerobic degradation (Field *et al.*, 1995). For example, chloro- and nitro-groups are known to facilitate the anaerobic degradation of the aromatic ring due to their electron-withdrawing capacity, in contrast to electron-donating groups such as amino-groups (Field *et al.*, 1995; **Paper I**). Functional groups can also negatively affect degradation by creating steric hindrance for enzymes (Alexander, 1999). In addition, the position of the functional groups influences the degradation pathway and the potential of the aromatic compound to be degraded, as for *o*-, *m*- and *p*-cresol (Londry & Fedorak, 1992: **Paper I**). A third important factor influencing the degradation pathway is the composition of the microbial community. For instance, the presence of different microbial populations exhibiting specific dehalogenating enzymes results in various degradation pattern of PCB dechlorination in sediments (Borja *et al.*, 2005). Yet another important factor is temperature, which was the specific focus in this thesis and which is discussed in the following section.

Effect of temperature

Temperature can influence the selection of different microbial communities or directly affect the enzyme activity, and hence it has an impact on the degradation of different aromatic compounds (Kohring *et al.*, 1989; Wu *et al.*, 1996). In addition, the bioavailability of an organic compound can be enhanced at elevated temperatures. Bioavailability is especially important as regards sorption of lipophilic compounds to organic matter (Field *et al.*, 1995; Ejlertsson *et al.*, 1997). If sorption occurs, the compound may become unavailable to microorganisms and enzymes. A possible explanation for the increase in bioavailability at higher temperatures is enhanced degradation of solid organic matter to which these compound are adsorbed (Hartmann & Ahring, 2003). These factors might explain the differences in degradation seen in the two laboratory-scale bioreactors used in this thesis (**Paper I**). In these processes, a more efficient dechlorination of highly chlorinated PCBs and a higher production of dioxin-like compounds have been shown at the lower process temperature (Engwall & Schnürer, 2002; Olsman *et al.*, 2006). Furthermore, in these bioreactors the degradation capacity of monoaromatic compounds was limited at the thermophilic temperature compared to the mesophilic temperature (**Paper I**). A clear difference was shown for phthalic acid and different phenols, which were degraded at the mesophilic temperature, but not at the higher temperature. The lower degradation capacity of phenols at 55 ºC was subsequently confirmed in large-scale bioreactors (**Paper II**). In these processes phenols was degraded very slow or not at all. In contrast, the transformation of methylphthalate and ethylphthalate into phthalic acid, was higher at the thermophilic temperature than at the mesophilic temperature (**Paper I**). Moreover, the reaction rate of chemical degradation of dimethylphthalate and diethylphthalate increased with increasing temperature (results not shown), which correlates with the established knowledge about rate and temperature.

Degradation pathways

By different fermentative bacteria, aromatic compounds are channelled through peripheral pathways into three central intermediates: phloroglucinol, resorcinol and benzoyl-CoA. Most commonly, anaerobic degradation of aromatic compounds proceeds via the formation of benzoyl-CoA (Schink *et al.*, 2000; Fig. 8). Under methanogenic conditions, this activated aromatic compound is further transformed through the benzoyl-CoA pathway via cyclohexane carboxylate and cyclohex-1-ene carboxylate, *i.e.* ring reduction, and β-oxidation to acetate, carbon dioxide and hydrogen (Fig. 9) (Elshahed *et al.*, 2001; Elshahed & McInerney, 2001). During the degradation of benzoate, bacteria belonging to delta *Proteobacteria* (genus *Syntrophus*) and *Desulfotomaculum* (subcluster Ih) are involved, commonly in cooperation with methanogens (Mountfort & Bryant, 1982; Wallrabenstein *et al.*, 1995; Jackson *et al.*, 1999; Qiu *et al.*, 2006). Interesting observations indicate an alternative pathway transforming benzoate to acetate and methane, but not via cyclohexene carboxylate, in a syntrophic conversion process with a hydrogenotrophic methanogen (Qui *et al.*, unpublished results).

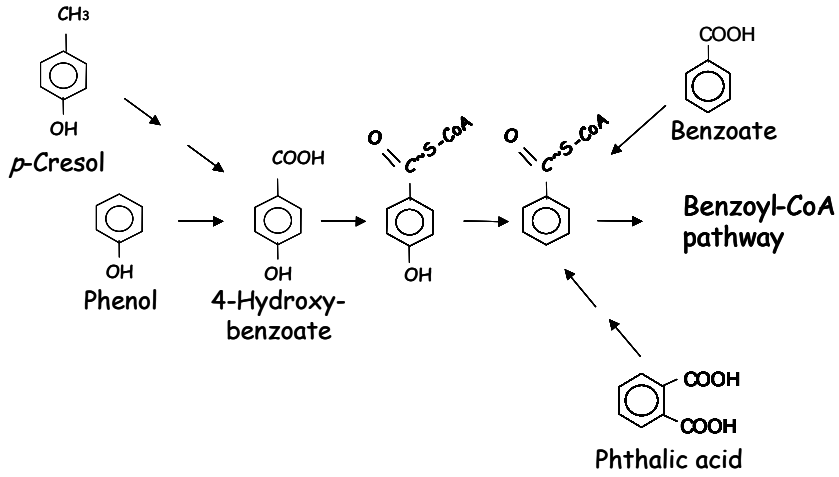

Fig. 8. Peripheral reactions transforming some aromatic compounds to benzoyl-CoA

Degradation of phenol under methanogenic conditions

Phenol can be completely oxidised by pure cultures using anaerobic respiration with nitrate, ferric ion and sulphate as the terminal electron acceptor (Bak & Widdel, 1986; Tschech & Fuchs, 1987; Kuever *et al.*, 1993; Lovley *et al.*, 1993; van Schie & Young, 1998). Under methanogenic conditions, the mineralisation process is complex and can proceed through different pathways and require a consortium of various microorganisms. So far, two possible pathways for mineralisation of phenol have been reported; either via 4-hydroxybenzoate into the benzoyl-CoA pathway or via caproate (Fig. 9). The degradation of phenol to benzoate is well documented and has been shown in several methanogenic consortia (Knoll & Winter, 1989; Kobayashi *et al.*, 1989; Béchard *et al.*, 1990; Sharak Genthner *et al.*, 1991; Karlsson *et al.*, 1999; **Papers I** and **II**). Furthermore, isolated organisms also demonstrate formation of benzoate from phenol (Juteau *et al.*, 2005; Qui *et al.*, unpublished results). On the contrary, although caproate has been identified as an intermediate, neither the bacteria responsible for the production nor the degradation pathway is presently identified (Fang *et al.*, 2006). The overall mineralisation of phenol via benzoate only occurs at very low hydrogen partial pressures, whereas the transformation of phenol to benzoate is possible even at high hydrogen levels (Knoll & Winter, 1989; Kobayashi *et al.*, 1989; Karlsson *et al.*, 1999). During the first step of phenol conversion, *i.e.* the carboxylation, an enzyme (phenol carboxylase/4-hydroxybenzoate decarboxylase) with a reversible activity is involved. This reaction is thermodynamically unfavourable at pH 6.5 and 37 ºC (Li *et al.*, 2000). However, the second step, dehydroxylation of 4-OHB to benzoate, is exergonic and may allow conservation of energy (Karlsson *et al.*, 1999).

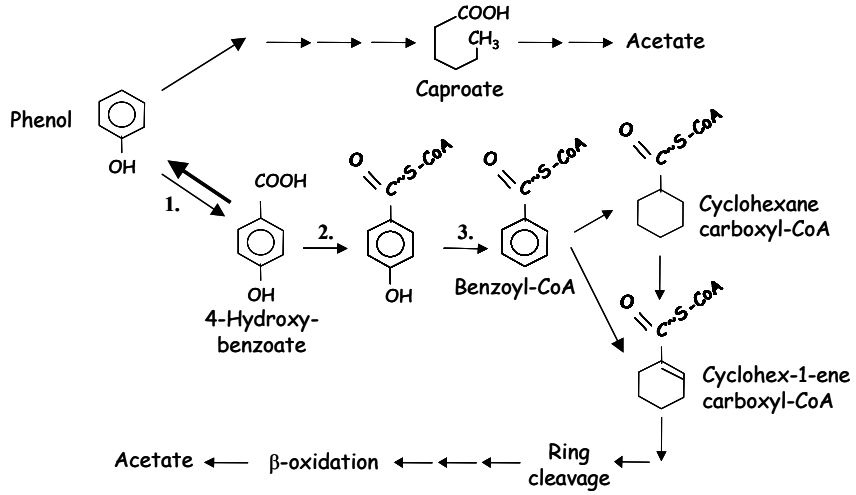

Fig. 9. Two alternative degradation pathways for phenol under methanogenic conditions, either via caproate or via 4-hydroxybenzoate and benzoyl-CoA pathway. Enzymes involved in transformation of phenol; 1. 4-hydroxybenzoate decharboxylate/phenol carboxylate; 2. 4-hydroxybenzoate-CoA ligase; 3. 4-hydroxybenzoate-CoA reductase.

Effect of temperature on degradation of phenol

Even though phenol degradation have been reported at thermophilic temperature (Karlsson *et al.*, 1999; Fang *et al.*, 2006), the majority of known phenol-degrading consortia and isolated organisms are mesophilic (Qiu *et al.*, unpublished results; Knoll & Winter, 1989; Kobayashi *et al.*, 1989; Béchard *et al.*, 1990; Sharak Genthner *et al.*, 1991; Karlsson *et al.*, 1999; Juteau *et al.*, 2005). This thesis presents that phenol was degraded slowly or not at all at the higher temperature (**Papers I** and **II**). One possible explanation for this limitation can be that some enzyme(s) involved in the degradation of phenol to benzoate is sensitive to temperature. The fact that phenol degradation in the thermophilic laboratory-scale process was activated after lowering the temperature from 55 to 48 ºC strengthens this hypothesis (**Paper I**). The effect of different environmental parameters on the enzyme(s) can be examined by measuring enzyme activity. However, the impact of temperature on enzyme activity is difficult to measure for phenol degradation, since one of the possible temperature-sensitive enzymes (phenol carboxylase/4-hydroxybenzoate decarboxylase) has a strong reversible activity (Li *et al.*, 2000: Fig. 9). The results from the laboratory-scale bioreactors were further confirmed by a more efficient degradation of phenol after lowering the temperature in batch cultures, started with material from thermophilic large-scale bioreactors. (**Paper II**). Interestingly, at 55 ºC no accumulation of benzoate occurred during phenol degradation, indicating simultaneous turnover of benzoate or an alternative pathway (**Paper II**). Accordingly, Fang *et al.* (2006) suggest that phenol is transformed via caproate instead of benzoate at thermophilic temperature.

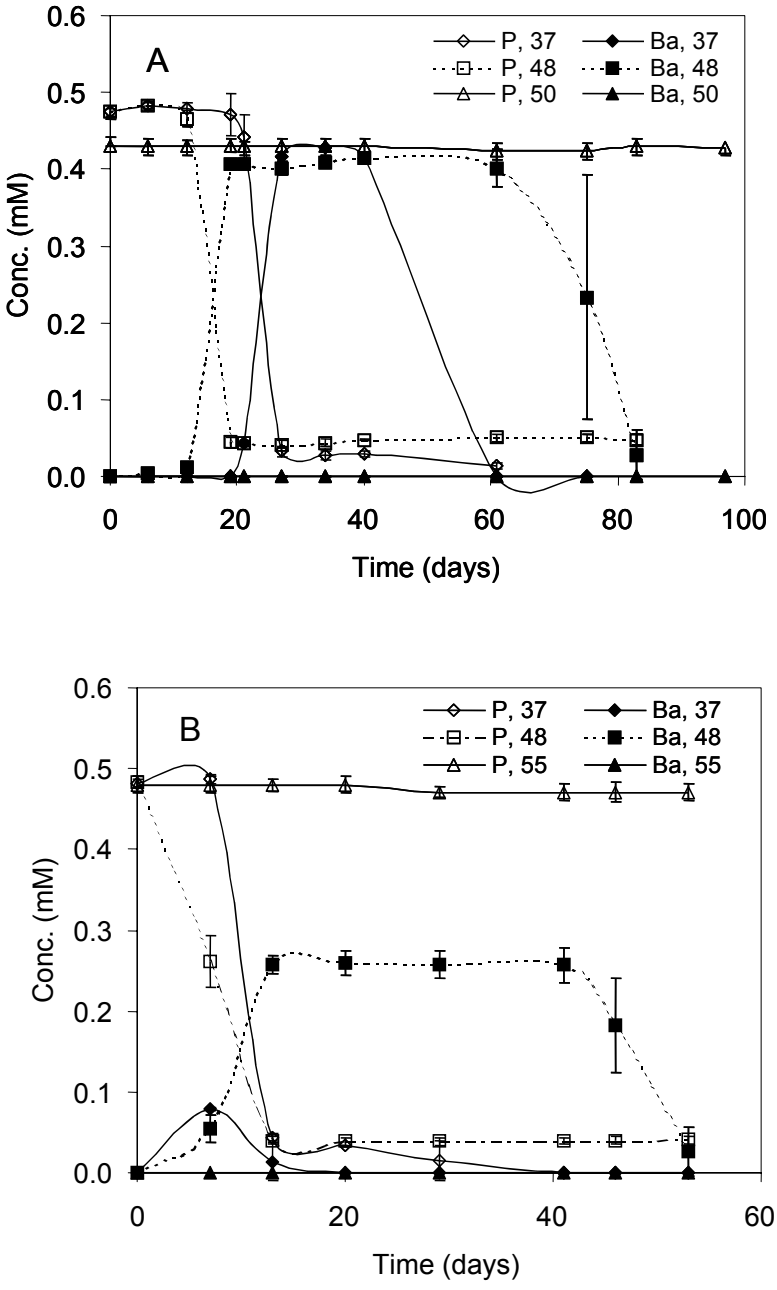

Fig. 10. Anaerobic degradation of phenol via benzoate in thermophilic (A) and mesophilic (B) batch cultures at different incubation temperatures. The inoculum was taken from the two laboratory-scale bioreactors described in this thesis.

Temperature not only affects the transformation of phenol, but also the degradation of the intermediate benzoate (Fig. 10). Benzoate was found to accumulate when the incubation temperature was changed in comparison the original process temperature (**Paper II**), likely due to disturbance of the methanogens, or activation of another degradation pathway or organism. Disturbed methanogens can cause accumulation of acetate and hydrogen, with a subsequent increase in benzoate (Knoll & Winter, 1989; Kobayashi *et al.*, 1989; Karlsson *et al.*, 2000). In these conditions, the conversion of phenol to benzoate is initially not affected, but if benzoate accumulates to high concentrations, conversion of phenol is also inhibited (Knoll & Winter, 1987; Wang & Barlaz, 1998). Transformation of benzoate recovers when levels of acetate and hydrogen eventually decrease (Knoll & Winter, 1989).

Microbial diversity in anaerobic bioreactors

The different steps and the main microbial groups in anaerobic digestion of organic material are well understood, but details concerning the prevailing microbial community have long been considered a 'black box'. Knowledge about the microbial community of the process is essential for further optimisation and better management of bioreactors. Traditionally, analysis of bacterial communities has involved identification of pure cultures isolated on laboratory media. However, only a small proportion (0.1-10%) of all bacteria can be cultivated in the laboratory (Torsvik *et al.*, 1996; von Wintzingerode *et al.*, 1997; Head *et al.*, 1998). Difficulties in cultivating anaerobic bacteria may arise from their limited substrate range, slow growth rates and the need for syntrophic relationships to obtain energy for growth. Therefore, there is also a need for other approaches focusing on cultivation-independent methods. The rapid development of new techniques has made it possible to investigate the diversity of microorganisms in various ecosystems. Different methods can be used targeting functional genes, phospholipids or the rRNA gene (Hofman-Bang *et al.*, 2003; Sundh *et al.*, 2003; Hallin *et al.*, 2006) . The 16S rRNA gene has become the most commonly used molecule for studying microbial communities and is now used routinely in microbial ecology. Since the ribosomal RNA molecule is widely spread in all cellular life forms and contains both conserved and variable regions, it is a suitable molecule for analysis of complex microbial environments (Woese, 1987; Hofman-Bang *et al.*, 2003). Both active and inactive organisms can be identified in methods targeting the rRNA gene in complex microbial environments. However, in an anaerobic bioreactor with continuous flow of organic material, there is a general washout of inactive organisms.

The rRNA gene has been used in several studies investigating complex microbial communities in anaerobic digestion processes. Different techniques targeting this molecule are available, such as fluorescent *in situ* hybridisation (FISH), molecular cloning or different fingerprinting methods (Hofman-Bang *et al.*, 2003). In FISH, general or specifically designed probes with fluorescent labelling are used to show the spatial distribution and morphology of uncultured cells and can also be used in cell counting. However, it is not possible to target unknown organisms by using this technique without knowledge of their specific DNA sequence. Fingerprinting methods such as temperature and denaturing gradient gel electrophoresis (TGGE / DGGE), terminal restriction fragment length polymorphism (T-RFLP), and single-strand conformational polymorphism (SSCP) can separate the many different DNA sequences obtained after amplification of extracted DNA from an environmental sample. These methods are often used to compare and estimate the level of diversity and monitor changes in community structure in anaerobic bioreactors (Hofman-Bang *et al.*, 2003). Although fingerprinting methods have the advantage that they often are fast and simple, molecular cloning and construction of clone libraries provides a higher resolution and hence also more information (Hallin *et al.*, 2006).

In this thesis, the microbial community structures in the mesophilic and thermophilic laboratory-scale bioreactors were analysed using molecular cloning,

construction of clone libraries and sequencing (**Paper III**). Comparing sequences of 16S rRNA genes from microbial communities with sequences available in the database generally allows phylogenetic characterisation of different organisms. However, most sequences in the database from anaerobic bioreactors arise from unidentified bacterial clones making the identification a difficult task. In the clone libraries from the mesophilic and thermophilic laboratory-scale reactors, the obtained sequences were possible to group in phyla, but most of the sequences did not affiliate with any known bacteria (**Paper III**). This made it difficult to completely elucidate their functional properties in the processes. This result illustrates the need for additional basic research with cultivation techniques, in order to increase our knowledge of the physiological and functional properties of bacterial communities from complex methanogenic environments.

Effect of temperature on microbial diversity

To isolate the impact of temperature on the development of anaerobic microbial communities, bioreactors that are stable and equal in substrate and performance (apart from temperature) are preferable. This approach has been adopted in a few previous studies (Sekiguchi *et al.*, 1998; Pender *et al.*, 2004; Hernon *et al.*, 2006; **Paper III**). In this regard, the microbial community analysis reported in this thesis is unique, as it represents the only example of a process treating a complex solid waste (**Paper III**). All previous studies were performed in bioreactors with retained biomass treating a liquid substrate. With only these few studies to compare, it is difficult to draw general conclusions about the effect of temperature on bacterial groups. However, in these previous studies anaerobic bioreactors contain lower species richness of both *Bacteria* and *Archaea* at thermophilic temperatures compared with mesophilic (Sekiguchi *et al.*, 1998; Pender *et al.*, 2004; Karakashev *et al.*, 2005; Hernon *et al.*, 2006). Comparing the microbial community structures in the mesophilic and thermophilic laboratory-scale bioreactors, it is clear that even when treating more complex substrate similar result was obtained (**Paper III**; Fig. 11). Furthermore, *Clostridia* have been shown to represent a higher fraction of the total community at the higher temperature (**Paper III**; Fang *et al.*, 2004; Fang *et al.*, 2006; Hernon *et al.*, 2006). The is probably a consequence of activation of clostridial spores at elevated temperatures (Mead, 1992). Some bacteria have only been detected at mesophilic temperatures, such as *Actinobacteria* and *Spirochaetes* (Sekiguchi *et al.*, 1998; Dollhopf *et al.*, 2001; Chouari *et al.*, 2005; Hernon *et al.*, 2006; **Paper III**). This difference in microbial diversity between the bioreactors operating at mesophilic and thermophilic temperature (**Paper III**) can possibly explain the difference in degradation capacity observed for the same bioreactors (**Paper I**). In the thermophilic reactor, with limited degradation capacity of aromatic compounds, the microbial diversity was lower than in the mesophilic process, having a broader degradation capacity.

When comparing diversity in complex ecosystems such as anaerobic bioreactors, it is important to be aware that functional stability may not be the same as stable community structure. Relatively stable microbial community structures have been observed in bioreactor systems with stable performance (LaPara *et al.*, 2000; LaPara *et al.*, 2002; Gentile *et al.*, 2006). However, despite stable reactor

processes, some systems have been shown to harbour unstable microbial community structures (Fernandez *et al.*, 1999; Zumstein *et al.*, 2000), with fluctuations in individual populations. It is currently unclear why there is a difference in stability of microbial communities between different processes. There are indications that large-scale bioreactors (LaPara *et al.*, 2000; LaPara *et al.*, 2002) and more complex substrates (Zoetendal *et al.*, 1998) develop more stable community structures. Furthermore, the presence of functionally more flexible populations might also contribute to more stable communities (Gentile *et al.*, 2006). However, in methanogenic ecosystems, functions and essential enzymes are dispersed between various organisms, and hence fluctuations are likely to occur over time. In spite of these population fluctuations, microbial community studies have shown general differences in diversity between mesophilic and thermophilic bioreactors (Sekiguchi *et al.*, 1998; Pender *et al.*, 2004; Karakashev *et al.*, 2005; Hernon *et al.*, 2006: **Paper III**).

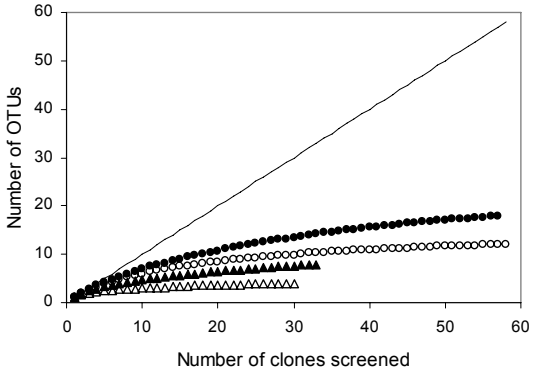

Fig. 11. Rarefaction curve showing the number of operational taxonomic units (OTUs) detected within the total number of screened mesophilic 16S rDNA clones (black symbols) and thermophilic clones (white symbols). Circles represent bacterial clones and triangles *Archaea*. The solid line indicates the maximum possible diversity observed in a clone library.

Important additional aspects to consider when discussing diversity and process stability include rare species and minor populations. Phylogenetic studies often focus on dominant species, as these are more easily detected. However, the ecologist Berlow (1999) proposes that rare species and minor populations can also be of great importance when spatial and temporal variations occur. By studying only major populations there is a possible risk that microorganisms important for degradation of specific compounds, such as pollutants, may be excluded. The laboratory-scale bioreactors investigated in this thesis illustrate the importance of minor groups for the degradation of specific compounds. For example, even though phenolic compounds were degraded, the community analysis revealed no obvious phenol-degrading organism (**Paper III**). Thus, both the dominant and the minor populations should be examined in order to better understand the process and optimise the performance of the anaerobic bioreactor.

Phenol degraders

Isolates of phenol-degrading bacteria

A number of anaerobic phenol-degraders have been isolated from denitrifying, iron-reducing and sulphate-reducing environments (Bak & Widdel, 1986; Tschech & Fuchs, 1987; Kuever *et al.*, 1993; Lovley *et al.*, 1993; Boopathy, 1995; van Schie & Young, 1998; Shinoda *et al.*, 2000; Mechichi *et al.*, 2002; Shinoda *et al.*, 2005). Under methanogenic conditions, phenol transformation has been reported in several different consortia (Knoll & Winter, 1989; Kobayashi *et al.*, 1989; Sharak Genthner *et al.*, 1991; Karlsson *et al.*, 2000; Zhang *et al.*, 2005; Fang *et al.*, 2006). However, only three phenol-degrading bacteria have been isolated under these conditions; *Sedimentibacter hydroxybenzoicum* (previously known as *Clostridium hydroxybenzoicum*; Zhang *et al.*, 1994), *Cryptanaerobacter phenolicus* (also known as 'Strain 7'; Juteau *et al.*, 2005) and *Syntrophorhabdus aromatica* (Qui *et al.*, unpublished results).

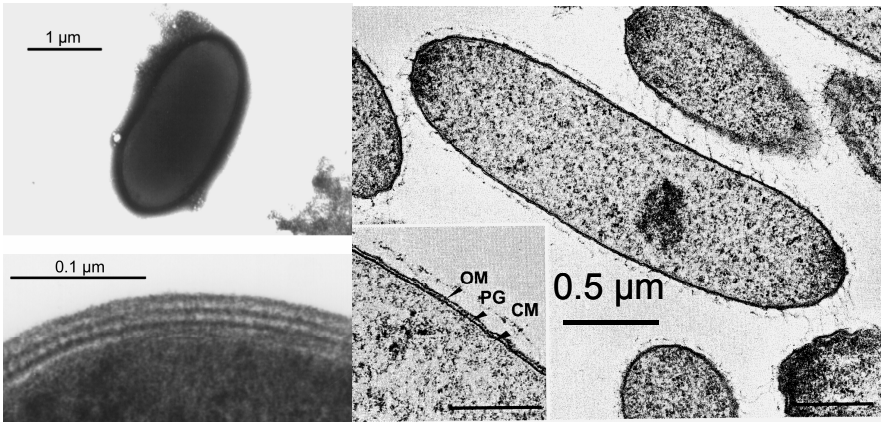

Fig. 12. Electron micrographs of the phenol-degraders, *Cryptanaerobacter phenolicus* (left) and *Syntrophorhabdus aromatica* (right). Micrographs kindly provided by Professor Juteau and Professor Sekiguchi.

These bacteria are all mesophilic rod-shaped bacteria, but metabolise phenol differently; *C. phenolicus* can only convert phenol into benzoate in the presence of unknown growth factor(s), whereas *S. aromatica* transforms phenol to acetate and methane in cooperation with a methanogen. Without the methanogen, *S. aromatica* can convert phenol to acetate in the presence of a quinone as the final electron acceptor. On the other hand, phenol carboxylation in *S. hydroxybenzoicum* is only revealed with whole-cell suspension and cell extracts (Zhang & Wiegel, 1994). *C. phenolicus* and *S. aromatica* are affiliated with members of *Desulfotomaculum* (subcluster Ih) and the group TA in delta *Proteobacteria*, respectively (Fig. 13). Both these cluster of anaerobes have the ability to degrade aromatic compounds such as phenol, benzoate, 4-hydroxybenzoate and phthalate isomers (Qiu *et al.*, 2004). *S. hydroxybenzoicum* is assigned to the *Clostridia* family *Peptostreptococcaceae*.

The isolation of a phenol-degrader in pure cultures under methanogenic conditions may be obstructed by its use of only a limited substrate range, the need for syntrophic relationships and unknown growth factors (Schink 1997; Karlsson *et al.*, 2000; Juteau *et al.*, 2005; Qui *et al.*, unpublished results). Difficulty in isolating phenol-degraders on a solid medium has been experienced in several studies (Knoll & Winter 1989; Juteau *et al.*, 2005; Levén and Schnürer, unpublished results; Qui *et al.*, unpublished results). Therefore, isolation by dilution in liquid media is a common approach. In order to achieve successful isolation in liquid media, different steps can be tried. Firstly, it might be possible to minimise the number of microorganisms in favour of the target bacteria by using selective inhibitory components such as antibiotics or heat treatment (Li *et al.*, 1996; Karlsson *et al.*, 2000; Juteau *et al.*, 2005). A second approach is to add an excess of a known methanogenic or sulphate-reducing partner to the liquid. This approach can possibly enhance the growth of the syntrophic phenol-degrader and facilitate the isolation (Schink, 1997). Thirdly, through removal, exchange or pre-digestion of alternative carbon sources, *e.g.* cysteine and yeast extract, unwanted microorganisms might also be diluted out from the enrichment culture at the same time as essential unknown growth factors can be provided (Li *et al.*, 1996; Karlsson *et al.*, 2000; Juteau *et al.*, 2005). Finally, centrifugation on a Percoll gradient can sometimes be necessary for the separation of two organisms before isolation (Juteau *et al.*, 2005). Thus, the isolation of phenol-degraders is difficult and requires many steps and years of work. Sometimes pure cultures are not obtained despite repeated attempts with different approaches (Levén and Schnürer, unpublished results; Karlsson, pers. comm.).

Methanogenic, phenol-degrading co-cultures

Instead of isolation in pure culture, the presence of phenol-degraders can be confirmed by the use of the molecular rDNA technique. This technique has been used in a few studies to characterise microbial communities degrading phenol at different temperatures. All studies but one (Levén & Schnürer, unpublished results) were performed in two upflow anaerobic sludge blanket (UASB) reactors with phenol as the only carbon source (Fang *et al.*, 2006; Fang *et al.*, 2004; Zhang *et al.*, 2005). These two UASB bioreactors were both seeded with the same inoculum, thereafter the microbial communities were allowed to develop at different process temperature (Fang *et al.*, 2006; Fang *et al.*, 2004; Zhang *et al.*, 2005). Fang *et al.* (2006) were not able to identify any phenol-degraders at thermophilic temperature, although phenol was mineralised via caproate. However, by further investigating the sequences from this study it was clear that one clone (TPD-48) was affiliated with the group TA in delta *Proteobacteria* (Fig. 13), containing bacteria degrading aromatic compounds.

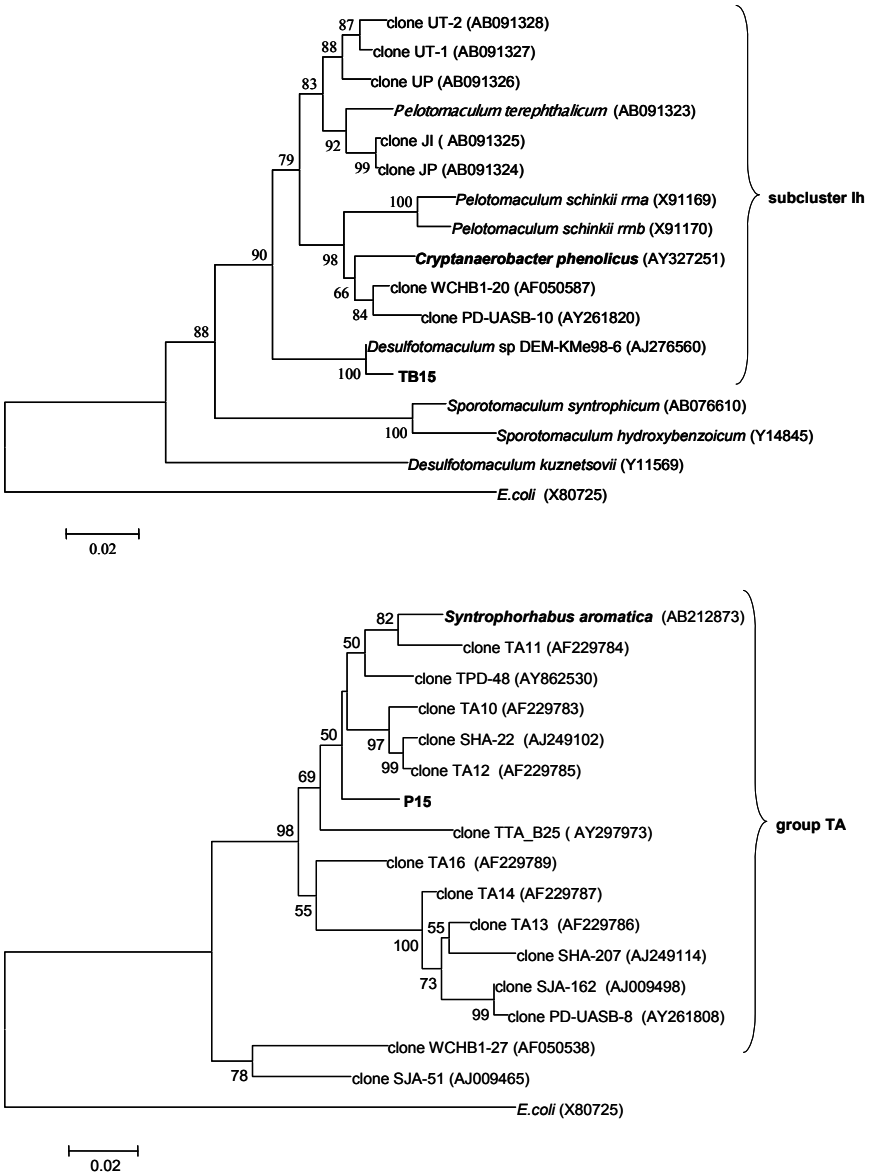

Fig. 13. Phylogenetic relationships in the group *Desulfomaculum* subcluser Ih and the group TA in delta *Proteobacteria*, based on neighbour-joining analysis of partial 16S rDNA sequences. Bootstrap values are shown for nodes that had >50% support in a bootstrap analysis of 1000 replicates. The accession number is given in brackets and *Escherichia coli* is used as outgroup.

In the UASB reactor at ambient temperature, phenol was degraded via benzoate, possibly by a bacterial clone (clone PD-UASB-10) with 97% sequence identity to *Cryptanaerobacter phenolicus* (Fang *et al.*, 2004; Zhang *et al.*, 2005: Fig 13). Thus, different phenol-degraders seem to be present at the different process temperatures. To obtain further knowledge of phenol degradation under methanogenic conditions two enrichment cultures were characterised. These cultures originated from the anaerobic bioreactors treating organic household wastes at 37 ºC or 55 ºC used in degradation and microbial community studies (**Paper I** and **II**). All enrichment cultures were incubated at 37 ºC, since no degradation of phenol occurred at the original thermophilic temperature. This finding of two phenol-degrading enrichment cultures, containing a mesophilic and a 'thermophilic' phenol-degrader respectively, is presented exclusively in this thesis (Table 1).

The enrichment cultures were obtained through repeated serial dilution in liquid media without cysteine. Further attempts to minimise the number of microorganisms in the culture using different approaches were not successful. In the mesophilic co-culture where phenol is transformed into methane, preliminary results from ocular examination and cloning showed four morphological and phylogenetically different bacteria (Table 1) and one methanogen. The most dominant bacterial shape was a rod with blunted ends (Fig. 14). The culture also contained three additional rod-shaped bacteria, one with pointed ends. Detection of a mesophilic clone (P15) affiliated with members of group TA in delta *Proteobacteria* (Fig. 13), verified the presence of a probable phenol-degrader. Sequence identity of 95% to the recently isolated *Syntrophorhabdus aromatica* (Qui *et al.*, unpublished results), which is capable of converting phenol to acetate in syntrophic associations with hydrogenotrophic methanogens, also support the identification of this clone as capable of phenol degradation. In the mesophilic culture, the irregular coccus with fluorescent capacity probably corresponded to *Methanoculleus palmaeoli* (99% sequence identity) and represents the likely hydrogen scavenging partner organism.

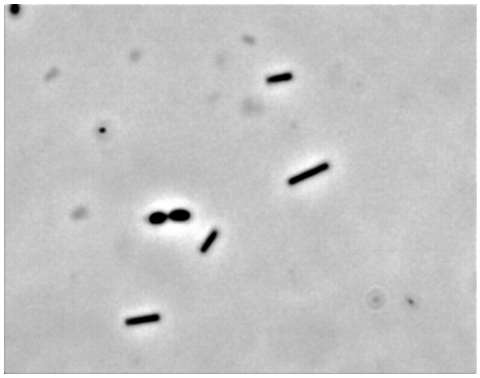

Fig. 14. Phase-contrast of two of the bacteria present in the mesophilic enrichment culture grown on phenol (0.5 mM); the most dominate rod shape with blunted ends and rods with pointed ends.

Table 1. Affiliation and distribution of bacterial 16S rRNA gene sequences analysed from the mesophilic (P) and thermophilic (TB) enrichment cultures.

Putative division	*OTU*	*% of total clones*	*Closest sequence/microorganism*	*Accession no.*	*Sequence identity%*
MESOPHILIC					
Delta Proteobacteria	P15	50	*Syntrophorhabdus aromatica*	AB212873	95
Bacteroidetes	P17	21	Bacteroides sp 22C	AY554420	99
Spirochaetes	P12	21	Clone B-9		99
Thermotogae	P5	7	Clone M79	DQ661210	99
THERMOPHILIC					
Firmicutes, Clostridia	TB74	20	Anaerobaculum mobile	AJ243189	100
	TB33	20	Bacteroides sp 22C	AY554420	100
	TB1	17	Clone PPC78A	AY542483	99
	TB15	8	Desulfotomaculum sp DEM-Kme98-10	AJ276557	99
	TB8	3	*Tepidanaerobacter syntrophicus*	AB106353	100
	TB53	2	*Coprothermobacter proteolyticus*	X69335	99
	TB66	1	Clone LS4-242	AB234271	99
Bacteroidetes	TB2	5	Clone AHU23	AB092908	98
	TB23	2	Clone 012D03_B_SD_P15	CT573914	99
Epsilon Proteobacteria	TB38	10	*Wolinella succinogenes*	M88159	99
Thermotogae	TB41	1	*Petrotoga mobilis*	Y15479	90
	TB75	6	Clone M79	AY692052	99
Spirochaetes	TB10	2	Clone E3	AY426468	99
	TB87	1	Clone BHB20	AB248649	99
Deferribacteres	TB20	1	Clone mle1-42	AF280863	99
Unknown	TB93	1	Clone EUB38-2	AY693827	98

Higher species richness was found in the 'thermophilic' co-culture (Table 1). This was most likely an effect of slow growth rate and thereby fewer dilution steps compared to the mesophilic culture. The most dominant group of thermophilic clones and the dominant bacterial shape resembled the rod-shaped *Anaerobaculum mobile* (100% sequence identity) (Menes & Muxí, 2002). This organism is known as a moderately thermophilic, peptide-fermenting bacterium with the ability to ferment yeast extract. Two methanogens, one with acetotrophic activity (*Methanosarcina* sp) and one with hydrogenotrophic activity (*Methanoculleus* sp), were identified among the clones. The likely phenol-degrader in this 'thermophilic' culture is a clone (TB15) assigned to *Desulfotomaculum* (subcluster

Ih), known to contain organisms with phenol-degrading capacity (Fig. 13). During the enrichment of this culture, the ability to degrade benzoate was lost, which was manifested as an accumulation of benzoate. Therefore, the 'thermophilic' phenol-degrader is only transforming phenol into benzoate.

In general, clone sequences found in the mesophilic and the 'thermophilic' enrichment cultures were not the same, suggesting development of unique phenol-degrading microbial community structures due to temperature. The phylogenetic analysis confirmed the presence of one conceivable phenol-degrader in each culture. However, the close relationship (97-99%) to uncultured *Thermotogales* M79 and *Bacteroides* sp. 22 in both cultures indicates the importance of these organisms, for degradation of more complex material such as yeast extract. These bacteria can through such degradation also support the phenol-degrader by production of growth factors. As benzoate was formed as an intermediate in both enrichment cultures, it appears as though the same pathway was used. Thus, the hypothesis that the limited degradation of phenol at 55 ºC is due to the presence of temperature-sensitive enzymes may still hold. On the other hand, activation of a thermotolerant mesophilic phenol-degrader cannot be completely excluded. It is also possible that both these explanations are true.

Concluding remarks

The process temperature in anaerobic bioreactors was shown to have a strong impact on the degradation of organic pollutants, with a lower degradation capacity at higher temperature. As a consequence, digestate produced at this thermophilic temperature contained the highest levels of some organic pollutants, *e.g.* different phenolic compounds. These phenolic compounds inhibited indicator organisms (AOB) in soil, implying that environmental disturbances are possible. In addition to temperature, the composition of the ingoing substrate also influenced the concentration of phenols in digestates, with a positive correlation to the fraction of swine manure. The limited degradation of organic pollutants at the thermophilic temperature could likely be explained by lower diversity in the microbial community compared to that in the reactor at mesophilic temperature. The observed difference between phenol degradation in enrichment cultures originating from the bioreactors operating at mesophilic or thermophilic temperature can be explained by the fact that two different bacteria were responsible for this degradation. Another possible explanation is that the activity of enzyme(s) involved in anaerobic phenol degradation is strongly regulated by temperature.

This thesis provides some new insights into anaerobic degradation of phenolic compounds. Nevertheless, new questions have also arisen from this work, some of which are discussed below.

How do phenols affect microorganisms in anaerobic digestion processes?
In this thesis, phenols identified in digestates caused inhibition of AOB activity in soil. Furthermore, in previous studies these compounds were shown to have negative affects on methanogens active in the anaerobic digestion process. If the activity of the methanogens is disturbed, anaerobic degradation of organic matter is probably also negatively affected. Hence, in order to optimise the digestion process, it is important to obtain information concerning the effect of phenols on the structure and activity of the methanogenic community.

How can anaerobic degradation be optimised to decrease organic pollutants in the digestate?
At present, it is not easy to give any general recommendations concerning an optimal process temperature to minimise the presence of toxic compounds in the digestate. One reason is that apart from degradation of toxic compounds, there are other important factors that have to be considered when optimising a process and its temperature. However, the results presented in this thesis, in combination with earlier research, show that degradation of different compounds react differently to temperature. One possible approach to get around this problem could be to run two processes in series, at different temperatures. This would increase the possibility of degrading a variety of organic pollutants. Thus, it would be interesting to monitor different pollutants during parallel anaerobic treatments in single and two-reactor systems.

Which organic pollutants should be included in certification analyses?
Identification of toxic compounds in the anaerobic digestates studied in this thesis suggests that it is important to include these compounds in certification protocols. It is possible that different compounds should be targeted depending on the type of waste treated. For instance, if the feedstock contains source-separated organic municipal household waste (OMSW) or waste from restaurants, it is important to investigate the presence of pesticides and fungicides. Monitoring programmes run by the Swedish National Food Administration could perhaps recommend important compounds for such analysis. Another group of compounds that deserves attention is the phthalates, which are often found in OMSW due to contamination with plastic material. Furthermore, the content of phenols in the digestate is of interest when swine manure is included as substrate in the biogas plant. Finally, the presence of animal pharmaceuticals should be investigated if slaughterhouse waste and manure are treated in the bioreactor. Application of these compounds to the soil can cause an increase in the amount of soil microorganisms resistant to antimicrobial drugs. This is highly undesirable as it increases the risk of creating public health problems.

Will phenols affect microorganisms in soil?
Even though a greater understanding of the fate of phenols in anaerobic digestion processes has been reached in this thesis, there are still many questions to answer concerning the effects of phenols in the soil. These include *e.g.* whether the negative effect shown in the PAO assay is persistent or whether microorganisms can recover after rapid degradation of the phenols; whether microorganisms other than AOB in soil are negatively affected after application of digestate containing high levels of phenols; and whether there are any effects on crop yield in the short-term and long-term perspective.

How is it possible to determine the function of uncultured bacteria?
In microbial ecology, a fundamental question concerns which microorganisms within a complex microbial consortium perform which function. As mentioned in this thesis, the isolation of phenol-degraders in pure culture was difficult. Further investigations in this area could involve linking the phenol-degrading function with a specific organism in the enrichment culture. A potential technique would be to use labelled compounds in stable-isotope probing (SIP) or FISH-MAR (fluorescent *in situ* hybridisation in combination with microautoradiography).

Summary in Swedish

Vid nedbrytning av organiskt avfall vid syrefria förhållanden produceras biogas (metan och koldioxid) och en näringsrik rötrest. Biogasen är energirik och kan användas som drivmedel till fordon, samt till el- och värmeproduktion. Den näringsrika rötresten är ett bra alternativ till konventionella gödselmedel och ger både förbättrad markkvalitet och skörd. Kvaliteten på rötresten och dess effekter på miljön och människan i ett långt tidsperspektiv är viktiga frågor att beakta. I Sverige kan rötresten certifieras och levereras som biogödsel om nivåer av metaller, näringsämnen för växter och patogena mikroorganismer är inom uppsatta mål. Analys av organiska föroreningar ingår inte i certifieringen, vilket är vore angeläget eftersom ett flertal olika aromatiska föroreningar har identifierats i rötresten.

I avhandlingen identifierades fenoler i olika rötrester och dessa föreningar visades också inverka negativt på markmikroorganimser (**artikel I och II**). Vidare observerades att olika biogasprocesser har olika förmåga att bryta ner dessa substanser, vilket har stor betydelse för den slutliga koncentrationen av fenoler i rötresten. Processtemperaturen visade sig ha en stark inverkan på nedbrytningen (**artikel I och II**). De vanligaste processtemperaturerna för rötning av organiskt material är 37ºC (mesofil) och 55ºC (termofil). Trots att nedbrytningen generellt går snabbare vid den högre temperaturen var förmågan att bryta ner fenoler sämre i dessa processer.

Eftersom mikrobiell aktivitet påverkar effektiviteten i nedbrytningen är det viktigt att öka kunskapen om hur olika processparametrar, såsom temperaturen, inverkar på sammansättningen av det mikrobiella samhället. Det mikrobiella samhället i reaktorn med mesofil processtemperatur hade högre diversitet, med ett större antal olika arter och en jämnare fördelning mellan arterna, jämfört med reaktorn med termofil processtemperatur (**artikel III**). Möjligen kan detta förklara den högre nedbrytningskapaciteten av fenoler vid den lägre processtemperaturen. En annan möjlig förklaring till den försämrade fenolnedbrytningen vid 55ºC är en hämning av enzymaktiviteten pga. temperaturen.

För att uppnå en fullständig nedbrytning av fenol krävs det att ett flertal mikroorganismer samarbetar. Nedbrytningen sker i tre steg: 1) omvandling av fenol till bensoat; 2) klyvning och nedbrytning av den aromatiska ringen; och 3) slutlig omvandling till metan. Hittills har endast ett fåtal fenolnedbrytare isolerats från reaktorer med metanogena förhållanden. Orsaken är att fenolnedbrytarna växer långsamt, är svårisolerade och behöver andra mikroorganismer för att kunna bryta ner fenol. Med molekylära metoder analyserades två fenolnedbrytande organismsamhällen. Dessa kulturer härstammar från den mesofila respektive termofila reaktorn som tidigare beskrivits. De fenolnedbrytande samhällenas mikrobiella sammansättning var olika och innehöll fenolnedbrytare av olika karaktär, vilket återigen visar på betydelsen av temperaturen för utvecklingen av det mikrobiella samhället.

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