Composition of Microbial Communities in Composts

A Tool to Assess Process Development and Quality of the Final Product

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Abstract

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The potential of microbial community fingerprinting methods to provide information about compost maturity in composting facilities was investigated.

Studies in a pilot-scale reactor equipped with independent control of oxygen content and temperature showed that low oxygen contents or low temperature had no dramatic effects on overall development of microbial community structure, determined with the PLFA (phospholipid fatty acid) method, although the process was generally delayed. Analyses of PLFAs typical for Actinobacteria, however, revealed that these bacteria were favoured when the maximum temperature was 40ºC. Actinobacteria populations constituted almost 50% of the microbial community during later stages of the reactor experiments, when only relatively complex, recalcitrant compounds remained, suggesting that some Actinobacteria may be suitable as indicator organisms for mature compost.

Studies in a full-scale composting system showed that Actinobacteria constituted a relatively low, but constant proportion at roughly 10% of the microbial community. Analyses of Actinobacteria species composition using PCR-DGGE (denaturing gradient gel electrophoresis) targeting 16S rRNA genes demonstrated that members of *Corynebacterium* were present at early stages and that thermo-tolerant Actinobacteria, *e.g. Thermobifida*, *Streptosporangium, Saccharomonospora* and *Saccharopolyspora*, were found throughout the long thermophilic phase. During the stage of decreasing temperature, the community included both thermo-tolerant and mesophilic Actinobacteria.

The ester-linked fatty acid (EL) method for describing microbial community structure was shown to provide information related to aspects of maturity, and is potentially a relatively simple and fast method of assessing compost maturity. Combination of signature lipid and nucleic acid-based analyses greatly expanded the specificity and scope for assessing microbial community composition in composts.

Keywords: Actinobacteria, composting, EL, organic waste, PCR-DGGE, PLFA

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Für meine Mutti

The challenge is to create harmony with the means
at our disposal in order to get best possible society.
Of course we can never be entirely successful in this,
but we must keep on trying.

För att få njuta av det bästa möjliga samhället,
måste vi skapa harmoni med alla oss tillgängliga metoder.
Det är klart att man aldrig kan lyckas perfekt,
men vi måste försöka hela tiden.

Niillä keinoin, jotka meillä on, meidän on luotava harmoniaa
jotta saamme niin hyvän yhteiskunnan kuin suinkin.
Tietenkään se ei voi koskaan onnistua täysin,
mutta koko ajan täytyy yrittää.

Alvar Aalto (1972)

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Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Steger, K., Jarvis, Å., Smårs, S. & Sundh, I. 2003. Comparison of signature lipid methods to determine microbial community structure in compost. *Journal of Microbiological Methods* 55, 371-382.

II. Steger, K., Eklind, Y., Olsson, J. & Sundh, I. 2005. Microbial community growth and utilization of carbon constituents during thermophilic composting at different oxygen levels. *Microbial Ecology* 50, 163-171.

III. Steger, K., Jarvis, Å., Vasara, T., Romantschuk, M. & Sundh, I. Effects of different temperature management on the development of Actinobacteria populations during composting. (Submitted).

IV. Steger, K., Sjögren, Å., Jarvis, Å. & Sundh, I. Development of compost maturity and Actinobacteria populations during full-scale composting of organic household waste. (Manuscript).

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The author's contribution to the papers has been as follows:

I. Participated in the planning of the study. Did major part of laboratory work, data evaluation and writing of the manuscript.

II. Planned the study together with the co-authors. Did the laboratory work and data evaluation together with Eklind. Major writer of the manuscript.

III. Planned the study with supervisors Sundh and Jarvis. Major part of the laboratory work, data evaluation and writing of the manuscript. Started the molecular work during a stay at Romantschuk's laboratory.

IV. Major part of the planning of the study. Performed and evaluated the molecular work in collaboration with Sjögren. Did the final data treatment and was major writer of the manuscript.

Introduction

"…..compost making may well be as much an artful science as wine making." This comment (Logsdon, 1989) gives an idea of the demands on the management of the composting process for obtaining a high quality product. Good quality compost can be used as a fertilizer or soil conditioner on agricultural fields, a substitute for peat in horticulture, and as a microbial additive and suppressive agent against plant pathogens (Eklind *et al.*, 1998; Hoitink & Boehm, 1999; Odlare, 2005). Thus, the use of compost enhances the environmental sustainability of agriculture by decreasing chemical inputs and increasing soil organic matter levels.

There are many definitions of composting, but a general description is that it is the biological degradation of heterogeneous organic material to a humus-like stable product under aerobic, moist and self-heating conditions. The process is performed under controlled conditions to achieve the major objectives:

- Conversion of potentially putrescible waste into a beneficial product.
- Disinfection of the original material that might be contaminated with pathogens.
- Bioremediation of hazardous waste.

The history of composting in a broad sense started in ancient times when Greeks and Romans used organic wastes that had rotted for long times (Epstein, 1997). Animal and food wastes were composted before being used as fertilizers in the early civilizations of South America, China and India. The myth that composting is a "natural process" that can be left to itself has impeded the development of composting for a long time (Miller, 1993). Until industrial times, the focus was directed towards the uses of compost, and little interest was shown in process control criteria. Research on composting seems to have begun in the 1880s with one of the earliest publications appearing in the United States where results from an agricultural experiment station were presented (Epstein, 1997). Between the years 1924 and 1931, the concept of large-scale composting was introduced and improved. A mixture of food wastes, "night soil" (human excrement) and a neutralizing alkaline agent was aerated through turning and water was added in order to manage the process (Howard, 1935). In Europe, the first full-scale facility was established in the Netherlands in 1932 using a modification of the Howard method. Since then, the number of composting facilities has increased greatly, especially during the 1980s and 1990s.

The increase in waste generation, concomitant with the increase in economic growth has been a major incitement for the establishment of composting plants . In the European Union, both the gross domestic product GDP and the amount of municipal waste grew by 19% between 1995 and 2003 (12/05 EU-Legislation http://europa.eu.int/comm/environment/waste.htm). Landfilling of organic waste has been prohibited in the EU since January 2005 (Council Directive 1999/31/EC

on the landfilling of waste). This has stimulated biological waste treatment via composting and anaerobic digestion, and around 15% of organic waste is presently treated in this way in Europe (ECN/Orbit e.V. http://www.compostnetwork.info/). However, it has been estimated that approximately 40% of urban and industrial wastes could be treated biologically. To achieve this considerable potential, the final products of the treatment have to fulfil environmental and market requirements. In turn, this encourages the producers to develop composts of high quality.

At present, the maturity of compost cannot be easily determined by a single, easy, rapid and reliable test for all types of waste materials (Mathur *et al.*, 1993). The established tests often have conceptual deficiencies, are complicated to perform and expensive to apply reliably for rapid testing of composts at a variety of composting facilities. Compost maturity tests can be divided into those that evaluate chemical or microbiological parameters. The first category includes the measurement of pH, electrical conductivity, C/N ratio of the organic matter, cation exchange capacity, the ratio of humic to fulvic acids, reheating or final temperature drop test, and phytotoxicity tests. Some of the microbiological tests are based on respirometry measurements, others on the formation of enzymes such as cellulases, amylases and proteinases. Others are based on ATP measurements as a direct measure of metabolic activity. In this thesis, the measurement of the microbial biomass and analysis of the microbial community structure are investigated as potential indicator methods to determine compost maturity. Since the relationship between maturity and microbial community structure is not well known, one objective of this thesis was to identify groups of indicator microorganisms that would enable the determination of compost maturity.

Due to the quality criteria for heavy metals, organic pollutants and the hygiene requirements, mixed municipal solid waste (MSW) is becoming more unusual in Europe; source separation is currently the state-of-the-art. The introduction of a quality assurance system for compost plants necessitates source-separation and influences the other stages of the waste treatment, e.g. plant engineering, compost production and application, marketing and public relations work. To fulfil the requirements for the properties of the final product, an established quality assurance system is the first step, and then a quality label or certificate will be given to compost products; this is similar to wine labels which designate the origin and quality of the product.

The basic concept of composting

Many factors are involved in the complex process of composting, and they affect to a greater or lesser extent the direction of the process. Figure 1 depicts the basic concept of composting, where the microorganisms have an essential role in the decomposition of organic matter. Despite the heterogeneous nature of organic source materials it can be divided into the following major elements: carbohydrates, proteins, fats, hemicellulose, cellulose, lignin and mineral matter (Epstein, 1997). The first three groups of organic matter, which are very susceptible to decomposition, include compounds like sugars, starches, pectin,

fatty acids, lipids, amino acids and nucleic acids. Hemicellulose, cellulose and lignin, on the other hand, are much more resistant to decomposition, and mineral matter is mainly unaffected by the process. The organic matter serves as a substrate for microorganisms and generates energy for the anabolic reactions, e.g. biosynthesis of cellular constituents. These macromolecules (polysaccharides, proteins, nucleic acids, lipids) are the building blocks for microbial growth, which in turn is the final result of both catabolic and anabolic reactions.

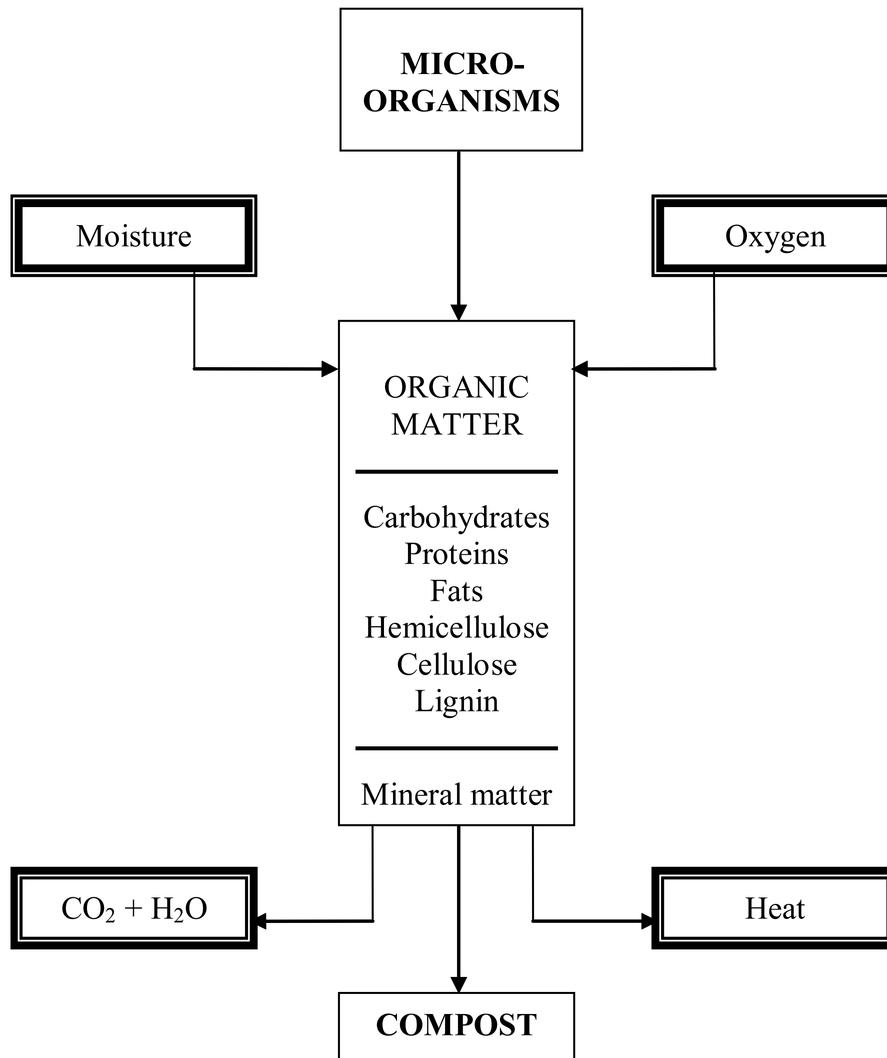

Fig. 1. Basic concept of the composting process (based on Epstein, 1997).

Oxygen is an essential component for the metabolism of aerobic microorganisms. Oxygen is supplied by active mechanical aeration, convective air flow (= passive aeration) and physical turning of the compost mass (Epstein, 1997). Oxygen diffusion can be limited by a moisture content exceeding 60%, because the free

pore space may be blocked by water (Haug, 1993). Excessively wet compost material becomes anaerobic, which inhibits the growth of aerobic microorganisms. Low moisture content, on the other hand, can also limit microbial activity, because water is the essential medium in which nutrients diffuse and the availability of nutrients may become limited. Anyhow, inhibition of microbial growth and activity affects the degradation of organic compounds.

As a result of microbial respiration, heat is liberated and the temperature rises, if the heat is retained in the composting mass. Moreover, carbon dioxide and water are released as decomposition products. Respiratory CO_2 is evolved during microbial activity, and the change in CO_2 emissions reflects the metabolic activity during the composting process. In the beginning, readily available carbon is utilized and released CO_2 increases to a peak almost simultaneously with peaks in temperature and moisture released (Wiley & Pierce, 1955). As the process continues, however, the rate of CO_2 evolution decreases as the availability of carbon decreases, leading to reductions in metabolic activity. One of the main objectives of composting is the effective decomposition of organic matter in the shortest possible time, and knowledge of basic aspects of composting is essential to be able to control and manage the process efficiently.

The heart of the composting process

A clear understanding of composting ecology provides the best basis for developing process control strategies (Miller, 1993). The identification of microorganisms is an important prerequisite to determine microbial community structures and metabolic activity pathways within the composting ecosystem.

Some of the first microbiological studies investigated the effects of temperature on the presence of microorganisms and on the efficiency of the compost process. It was noted that self-heating during composting was due to biological processes (Browne, 1933). Another early study concluded that inoculation with thermophilic populations led to a higher decomposition rate at higher temperatures than inoculation with mesophilic populations (Waksman & Cordon, 1939). Furthermore, it was found that bacteria, actinomycetes and thermophilic fungi were all active in compost at 50°C; however, at 65°C, fungi were rare, and at 75°C, spore-forming bacteria were the predominant organisms (Waksman *et al.*, 1939). These and other early studies (Webley, 1947; Forsyth & Webley, 1948; Chang & Hudson, 1967) stressed the importance of temperature on the development of microbial populations in the compost environment.

Based on the development of temperature, the composting process can be divided into the mesophilic phase (temperatures below 45°C), the thermophilic phase (temperatures above 45°C), and finally the curing phase, which is characterized by a decrease in temperature (Fig. 2).

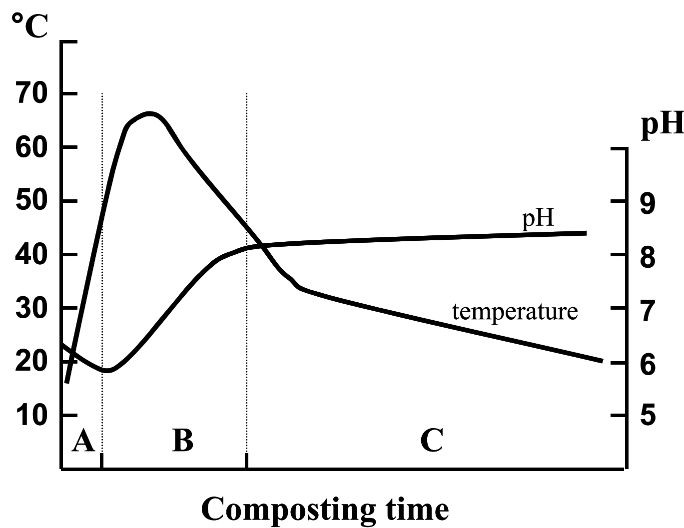

Fig. 2. Schematic description of different phases of the composting process based on the development of temperature and pH: A-mesophilic; B-thermophilic; C-curing phases.

The general relationships of temperature and pH as a function of composting time have been depicted in many ways and the shape of the curves varies with the original material to be composted and the applied conditions. However, the initial stage is characterized by the growth and activity of mesophilic organisms, such as fungi, yeast, Gram-negative and lactic acid bacteria (Finstein & Morris, 1975; Miller, 1993; de Bertoldi, 1998; Ryckeboer *et al.*, 2003b). A consequence of the activity of acid-producing bacteria is a short-term decrease in pH (Fig. 2). Generally, the activity of the mesophilic community leads to an increase in temperature. In the next phase, thermophilic temperatures are reached and organisms adapted to these conditions (e.g. *Bacillus* spp., *Thermus thermophilus* and *Thermoactinomyces* sp.) take over the degradation process (Strom, 1985a,b; Blanc *et al.*, 1998; Song *et al.*, 2001). The growth and activity of non-thermotolerant organisms, including pathogens and parasites, are inhibited during the thermophilic phase. During this stage, the pH stabilizes in a range of 7.5 to 8.5 (Jeris & Regan, 1973), because short organic acids have been consumed and nitrogen mineralised (Beck-Friis *et al.*, 2003). The final curing stage is characterized by the development of a new mesophilic community. As the temperature declines, mesophiles and moderate thermophiles reappear, including fungi and actinomycetes (Chang & Hudson, 1967; Finstein & Morris, 1975). The latter group of microorganisms is nowadays referred to as Actinobacteria, probably to emphasize that they are not fungi, but belong to the Gram-positive bacteria. The term Actinobacteria will be used further in this thesis. The recolonization of a mesophilic community might be more a matter of nutritional factors than a result of temperature. Since easily degradable compounds have been depleted in the early stages of composting, microorganisms present in the later stages have to be able to degrade compounds less susceptible to mineralization.

This means that they need certain enzymes to degrade cellulose, hemicellulose and lignin. Actinobacteria are commonly believed to play a significant role in the degradation of relatively complex, recalcitrant compounds (Goodfellow & Williams, 1983). The ability of Actinobacteria to degrade lignocelluloses implies that this group of bacteria may be suitable as potential indicator organisms for compost maturity.

Objectives of the study

The biological conversion of organic material during composting occurs concurrently with dynamic changes in the microbial assemblage. Thus, the composition of microbial communities is to some extent interrelated with aspects of maturity and quality of the compost. Therefore, standardized methods to follow the microbial succession have the potential to provide information about compost maturity. The main objectives of this thesis were to:

- investigate the potential of different microbial community fingerprinting methods to yield information about compost maturity.

- determine the effects of different environmental conditions, e.g. oxygen contents and temperatures, on the development of microbial populations.

- increase knowledge about the interactions between changes in the composition of the organic matter and microbial populations dynamics.

- investigate the development of Actinobacteria populations under different composting conditions, and also their potential to characterize the final compost product.

Signature lipid analyses for determining microbial biomass and community structure

Biochemical methods for microbial biomass measurements overcome the disadvantages of classical methods, like viable counts and most-probable-number (MPN) estimations, which depend on growth and are therefore affected by the problem of microbial selection. Suitable assays for determining microbial biomass can be based on the presence of universal cellular components including adenosine triphosphate (ATP), constituents of microbial cell walls and membrane components. The measurement of such signature constituents requires (a) a component which only occurs in living microorganisms; (b) a component which exists in fairly uniform concentrations in the cell; and (c) a component which can be analysed quantitatively with appropriate sensitivity (Tunlid & White, 1992). ATP analysis has been widely used as a tool for total biomass determination in environmental systems (Holm-Hansen, 1973; Karl & LaRock, 1975; Sparling & Eiland, 1983; Pinkart *et al.*, 2002). The amino acids muramic acid and D-alanine are present in all bacterial cell walls, whereas diaminopimelic acid (DAP) is common in all Gram-negative bacteria, but only in some Gram-positive bacteria (Schleifer & Kandler, 1972). For fungal biomass, glucosamine has been used as a signature constituent of the cell wall and ergosterol as a membrane component (Grant & West, 1986). Other membrane components that have been used to estimate microbial biomass include the lipopolysaccharides (LPS), which are unique polymers in the outer cell membrane of Gram-negative bacteria, and the phospholipids (PL), which constitute the main structural components of the cell membrane in all living cells (Nichols *et al.*, 1987; Balkwill *et al.*, 1988). The analyses of these signature lipid molecules are summarised in Figure 3.

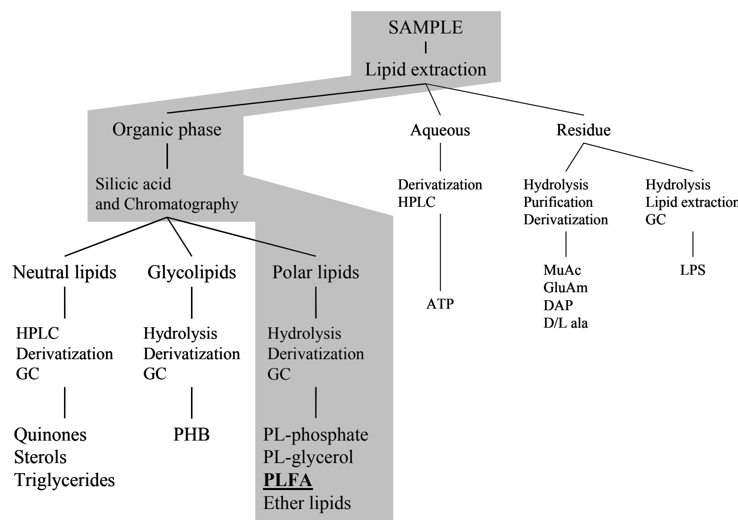

Fig. 3. Schematic flow diagram for lipid analyses of natural microbial communities, and the pathway for PLFA analysis shaded in gray (based on Tunlid & White, 1992).

Many microorganisms store intracellular compounds during periods of nutritional stress or unbalanced growth. The presence of these storage compounds can be used as an indicator of the metabolic status of the community (Vestal & White, 1989). For example, polybetahydroxyalkanoate (PAH) is synthesized by many bacteria during nutritional starvation (Findlay & White, 1983). Triglycerides are another kind of storage polymer, which are formed by microeukaryotes, such as fungi and protozoa. The ratio of triglyceride glycerols to phospholipids has been monitored to indicate the metabolic status of these organisms (Gehron & White, 1982). During nutrient starvation, some bacteria change the configuration of monounsaturated phospholipid fatty acids (PLFA) from *cis* to *trans*. Hence, a shift in the *trans*:*cis* ratio is associated with starved or stressed bacteria in natural environments (Guckert & White, 1986). A more detailed description of the PLFA method and its application to determine microbial biomass and community structure is provided in the following section.

Phospholipid fatty acid analysis (PLFA)

The content of phospholipids has been used as an estimate of microbial biomass in different environments. Figure 3 depicts the extraction of lipids in general, and the phospholipids in the polar fraction of the organic phase in particular. The measurement of phospholipid (PL) concentrations fulfils the requirements of biomarkers for viable microbial biomass to a high degree:

- PL occur in the membrane of all living cells, but not in the storage products of microorganisms (Kates, 1964).
- PL are rapidly degraded to neutral lipids upon cell death (White *et al.*, 1979b; Tollefson & McKercher, 1983; Harvey *et al.*, 1986).
- PL are a relatively constant proportion of the cell dry matter content (White *et al.*, 1979a).
- PL can be quantitatively extracted and reasonable estimates of the microbial biomass can be obtained using conversion factors (White *et al.*, 1979a).

Apart from the archaeans, which have special kinds of PL (Koga *et al.,* 1998), the phospholipid molecules consist of three units: a polar head group, a glycerol skeleton and two long-chain fatty acids. Different groups of organisms contain different fatty acids in their structural lipids. The composition of fatty acids from the phospholipids (PLFA) therefore reflects the microbial community structure of environmental samples. The PLFA method has been used to monitor the composition of microbial communities in various environments, such as agricultural soils (Zelles *et al.*, 1992; Reichardt *et al.*, 1997; Bossio *et al.*, 1998), peatlands (Sundh *et al.*, 1997), desert soils (Steinberger *et al.*, 1999) and other diverse systems (Webster *et al.*, 1997; Lewis *et al.*, 2000; Silvey *et al.*, 2000). Several studies have used the analysis of PLFA to describe the microbial succession in different compost systems (Herrmann & Shann, 1997; Klamer & Bååth, 1998; Carpenter-Boggs *et al.*, 1998; Eiland *et al.*, 2001; Sundh & Rönn, 2002).

Comparative analyses of monocultures and environmental isolates have revealed that subsets of a microbial community can be identified by specific signature PLFAs (Tunlid & White, 1992). Fungi, for example, contain saturated even number-chained and polyenoic fatty acids (Wassef, 1977; Federle, 1986). Methyl-branched fatty acids on the 10th carbon atom are characteristic for many actinomycetes (Kroppensedt & Kutzner, 1978; Brennan, 1988; Macnaughton & O'Donnell, 1994). Bacteria typically synthesize iso- and anteiso-branched, and cyclopropane fatty acids (Kates, 1964; Tunlid & White, 1989; Vestal & White, 1989). Table 1 gives an overview of typical PLFAs in different groups of organisms.

Table 1. *Examples of signature phospholipid fatty acids for microorganisms* [#]

Gram-negative bacteria	16:1ω5, 16:0, 18:1ω7, cy17:0, cy19:0
Sulphate-reducing bacteria	i17:1ω7c, 17:1 ω6, 10Me16:0
Methane-oxidizing bacteria Type I	16:1ω8c, 16:1ω6c, 16:1ω5c, 16:1ω5t
Type II	18:1ω8c, 18:1ω8t
Gram-positive bacteria/Thermophiles	i14:0, i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, 17:0, i18:0, i19:0
Actinomycetes	10Me16:0, 10Me17:0, 10Me18:0
Fungi	18:2ω6, 18:3ω6, 18:3ω3
Eucaryotes	14:0, 16:0, 18:1ω9c, 18:0

[#] Fatty acids are designated by standard nomenclature as total number of carbon atoms followed by a colon and the number of double bonds, with the position counted from the aliphatic end indicated by "ω". The suffixes "c" and "t" refer to the *cis* and *trans* configurations, respectively. Methyl branching at the *iso* and *anteiso* positions and at the 10th carbon atom from the carboxylic end are named by the prefixes "i", "a" and "10Me", respectively. Cyclopropane fatty acids are denoted by the prefix "cy".

In this thesis, the protocol for PLFA analysis was based on modifications of the Bligh and Dyer method (1959), in which a mixture of chloroform, methanol and water (1:2:0.8) was originally used to extract lipids from brain tissue. Frostegård *et al.* (1991) used a citrate buffer instead of water, and improved the extraction efficiency by 38% in soil samples. The one-phase mixture is split into two phases by adding chloroform and citrate buffer to a final proportion of 1:1:0.9 of chloroform, methanol and citrate buffer. The organic phase containing the lipids was separated on silicic acid columns into neutral, glyco- and polar lipids by eluting with chloroform, acetone and methanol respectively (King *et al.*, 1977). The phospholipids in the polar fraction were treated by mild alkaline methanolysis according to Dowling *et al.* (1986). The resulting fatty acid methyl esters (FAME) were extracted with a hexane/chloroform (4:1) mixture and dried under a stream of nitrogen. Samples were dissolved in heptane and transferred to GC vials. The separation and quantification of FAME was performed on a gas chromatograph with flame ionization detector (GC-FID) on a Hewlett Packard 6890 instrument equipped with a HP-1MS capillary column (30m x 250μm x 0.1μm, cross linked methyl siloxane), following the procedure of Virtue *et al.* (1996). However, a different temperature program was established: after two minutes at the injection temperature of 50°C, the oven temperature was increased at 30°C/min to 110°C, then at 3°C/min to 220°C and finally at 10°C/min to 320°C where the temperature was maintained for ten minutes. The fatty acids were quantified by correlating the

peak areas to the peak area of the internal standard 19:0 (nonadecanoic acid methyl ester).

The identification of FAMEs was performed on a GC-MS (mass spectrometry) instrument where the retention times were compared with those obtained for standard fatty acid mixtures. In general, about 35 to 45 fatty acids were identified depending on the sample size and the detection sensitivity. The samples were derivatised with dimethyl disulfide (DMDS) in order to determine the position of double bonds and the amount of monounsaturated fatty acids, according to Nichols *et al.* (1986). The DMDS adducts were analysed with the GC-MS set in selective ion monitoring (SIM) mode which allows the relative quantification of pre-selected ions that are specific for the derivatives. The temperature program differed slightly from the GC-FID program: the increase from 110 to 220°C was performed at a rate of 4°C/min and the final holding at 320°C was 20 min.

Whole cell fatty acid analyses

Fatty acids in the phospholipids have proved to be useful chemical measures of the microbial community structure in ecological studies. A drawback with PLFA analysis is the fact that it is comparatively time-consuming, since the lipid extraction includes several separation and overnight steps (Bligh & Dyer, 1959) and the fractionation of the lipids with solid phase extraction can take several hours, depending on the number of samples to be analysed and on the amount of sample applied to the columns. Simpler methods have been developed to determine the fatty acid composition of microorganisms, such as the ester-linked method (EL) introduced by Drijber (2001, personal communication) or the microbial identification system (MIDI) introduced by Sasser (1990). Both methods extract microbial fatty acids in one single step, but they are non-specific, meaning that they do not separate the polar lipids from the other lipids. Thus, fatty acids from neutral fats, glycolipids and phospholipids are extracted by the EL and MIDI methods, not only from living microbial cells, but also from dead organic matter. In this study, the extraction procedures for both methods followed the protocol of Schutter & Dick (2000) who applied EL and MIDI in a comparative study of different soils. Other studies have used either the EL (Ritchie *et al.*, 2000) or MIDI method (Cavigelli *et al.*, 1995; Ibekwe & Kennedy, 1999; Petersen *et al.*, 2002) to describe microbial communities in soils.

Briefly, the EL method comprises a mild alkaline methanolysis, similar to the one in the PLFA procedure. During this step, the ester-linked fatty acids are released and methylated. A neutralisation step follows, before the FAMEs are partitioned into an organic phase of hexane. Finally, the samples are dissolved in heptane for GC analyses. The MIDI protocol consists of saponification, methylation, extraction and a cleanup step. During the saponification in 3.75M NaOH at 100°C, the cells are lysed and fatty acids are cleaved from the cell lipids and converted to sodium salts. In the next step, fatty acids are converted to FAMEs by incubation in 6M HCl:MeOH (1:0.85) in a 85°C water bath. A 1:1 mixture of hexane:methyl-*tert*-butyl ether is used to extract the FAMEs. Finally,

the organic phase is cleaned from acidic residues with a mild base and the samples are dissolved in an organic solvent for GC analyses.

Comparison of PLFA and whole cell fatty acid analyses

One aim of this study was to investigate the extent to which the two simpler whole cell fatty acid analyses provide information which is as relevant and valuable as the PLFA method. Furthermore, we wanted to apply all three methods to compost and compare the fatty acid profiles to answer the question as to how efficient the simpler methods EL and MIDI are in determining the microbial community structure in compost (paper I). For this, we wanted to use compost material of different ages derived from the same process, which should be characterised by dynamic changes in both the organic material and microbial community structure. These samples were obtained from the composting process in our laboratory reactor, which is described more in detail later.

Samples from the starting material (day 0), the early mesophilic stage (day 3), the thermophilic phase (day 7) and the final mesophilic phase (days 24 and 45) were analysed with PLFA, EL and MIDI. The large amount of data derived from the fatty acid analyses was analysed with multivariate strategies in order to assess the major patterns of variation. Principal component analysis (PCA) was used to follow the changes in fatty acid patterns over time due to the different extraction methods used (Fig. 4).

In general, the temporal patterns for bacterial fatty acids obtained with the three fatty acid extraction methods were strikingly similar (paper I). It can be concluded from the results of this methodological study that the simpler EL and MIDI methods describe the microbial community structure in the later stages of composting almost as well as the PLFA method. The deviation of the early EL and MIDI data from PLFA (Fig. 4a) can be explained by the considerably higher fatty acid concentrations due to non-specific extraction of fatty acids from organic matter in the original waste (paper I). It has been suggested that the MIDI/PLFA ratio is an indication of the background contribution from non-microbial material (Petersen *et al.*, 2002). However, later in the process, the difference in fatty acid concentrations between PLFA and the two simpler methods is no longer so large, and this "background ratio" decreased as the process continued (Fig. 4a).

Fatty acids common in eukaryotic cells (e.g. 16:0, 18:2, 18:1ω9, 18:0) formed a cluster at the start of composting (Fig. 4b). These fatty acids probably derive to a large extent from plant and animal remnants in the original waste (Sundh & Rönn, 2002), and account for a large part of the fatty acids extracted with the EL and MIDI methods. These eukaryotic fatty acids are also dominant at the start of composting according to PLFA, but at much lower concentrations. Other important fatty acids in the beginning of the process were straight-chain, monounsaturated fatty acids which are typical of Gram-negative bacteria (Wilkinson, 1988). This group of bacteria is, along with the fungi, characteristic in the initial mesophilic phase of composting (Herrmann & Shann, 1997; Klamer & Bååth, 1998; Eiland *et al.*, 2001). When the thermophilic phase is reached, Gram-

positive bacteria take over the degradation process and a shift towards iso- and anteiso-branched, cyclopropane and 10 methyl-branched fatty acids is demonstrated (Fig. 4b).

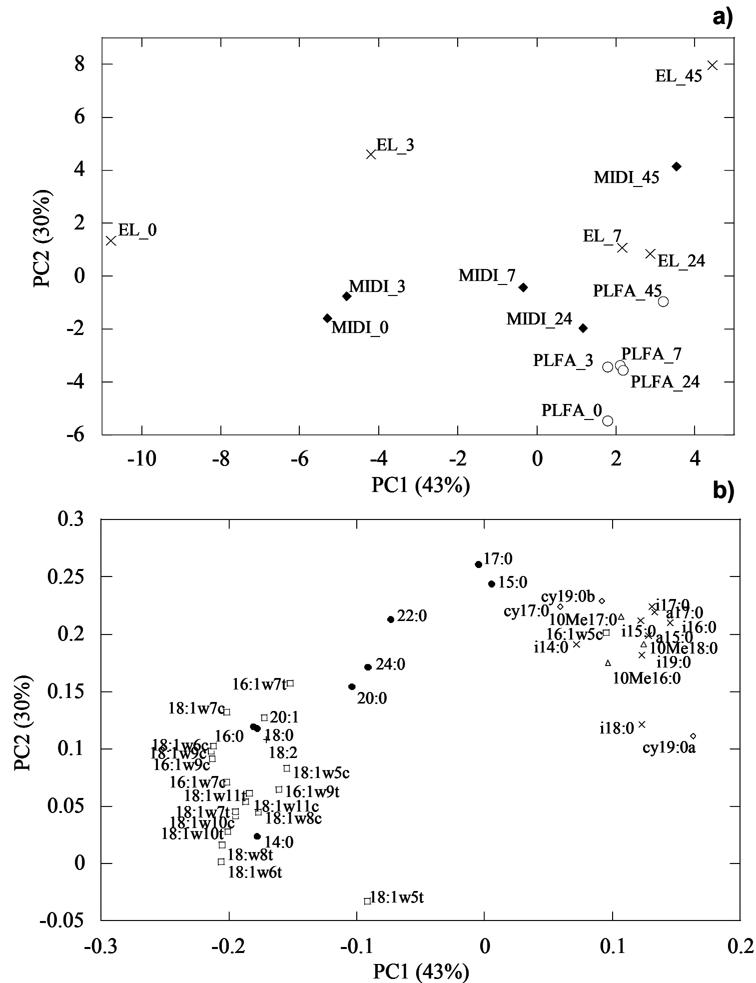

Fig. 4. PCA showing variation in the fatty acid patterns obtained using three different extraction methods. (a) Scores plot (numbers denote the sampling day). (b) Loadings plot for individual fatty acids (symbols for fatty acids: • saturated; □ monounsaturated; + polyunsaturated; × branched saturated; Δ 10Me branched; ◊ cyclopropane).

Our results demonstrate that at later stages of composting, EL and MIDI mainly detect fatty acids from viable microorganisms, like PLFA. Although both EL and MIDI are faster methods than PLFA analysis, EL has the advantage that it involves less hazardous laboratory work than the MIDI method. Moreover, the harsh saponification step of the MIDI procedure may lead to losses of fatty acids due to some oxidative modifications of the lipid material. For these reasons, the EL method was preferred to MIDI for further analyses in this thesis.

A similar development in fatty acid composition was observed in the studies of the effects of different oxygen contents and temperature regimes on the microbial community in the compost reactor (papers II and III). However, the analyses of more samples over the entire period of composting contributed to a better description of the changes in microbial community structure during the process. Furthermore, the studies revealed that the proportion of fatty acids deriving from microorganisms in the EL fraction was notably higher at the end of composting than in the beginning of the process, when the background contribution from non-microbial material was predominant. This suggests that the EL method gave a good description of the microbial succession and the microbial community structure in the later stages of the process. Thus, the relatively simple and fast EL method can give valuable information about compost maturity.

Molecular analyses for characterising microbial diversity

Assessment of the microbial diversity in environmental systems requires culture-independent approaches. In this respect the universal nucleic acids DNA and RNA are powerful target molecules in microbial ecology. The extraction of nucleic acids and the use of 16S/18S ribosomal RNA gene sequences gives information on which groups and species are present and allows the characterization of microbial community structure. Changes in either the overall microbial community structure or in the prevalence of phylogenetic subgroups are monitored. The application of fingerprinting techniques such as temperature or denaturing gradient gel electrophoresis (TGGE or DGGE), terminal restriction fragment length polymorphism (T-RFLP), or single-strand conformational polymorphism (SSCP) gives information about the types of microorganisms present in the environment (van Elsas *et al.*, 2006). In contrast to the signature fatty acid analyses, molecular techniques can give information on the species level, but the quantitative importance of the species is not revealed, unless advanced techniques, like real-time PCR are used. This chapter discusses useful approaches to directly extract DNA from complex environments like composts and the use of 16S ribosomal RNA gene-based PCR coupled to DGGE analysis to describe the microbial diversity in composts.

Nucleic acid extraction

The procedures for direct extraction of DNA or RNA from environmental samples have been developed from often laborious, manual methods, to commercially available kit-based protocols. Besides bead beating for disrupting the starting material, these procedures include various treatments with detergents for lysis of cell membranes, addition of solvents for separation of the nucleic acids from other cell components, and enzymatic treatment for recovery of the nucleic acid in purified form (Nicholl, 2002). Such extraction kits designed to obtain DNA from soils and sediments have been successfully used for compost samples, e.g.

MOBIO Power Soil™ DNA Kit or FastDNA® SPIN Kit for Soil (Dees & Ghiorse, 2001; Marshall *et al.*, 2003; Franke-Whittle *et al.*, 2005).

In our studies, we used the FastDNA® SPIN Kit for samples from the compost reactor (paper III), whereas other protocols were also used for samples from the full-scale facility (paper IV). DNA extraction from compost samples was especially problematic in the latter study. Humic substances are major components of organic matter, that are known to inhibit restriction endonucleases and *Taq* DNA polymerase (Tsai & Olson, 1992; Tebbe & Vahjen, 1993). The particularly high levels of humic substances in aged composts were obvious after using the FastDNA® SPIN Kit procedure: the DNA extracts of our late full-scale samples (9-13 months) were rather brownish, indicating that humic compounds were co-purified with DNA. Additional treatments are necessary to remove these highly complex compounds from environmental DNA, including for example:

- Hydroxyapatite columns (Torsvik, 1980)
- Cesium chloride density centrifugations (Holben *et al.*, 1988)
- Hexadecyltrimethyl ammonium bromide treatment (Zhou *et al.*, 1996)
- Sephadex G-200 columns (Tsai & Olson, 1992)
- Polyvinylpolypyrrolidone treatment (Picard *et al.*, 1992)
- Guanidine thiocyanate (Jaatinen *et al.*, 2004)

In our full-scale study, we applied the extended FastDNA® SPIN Kit procedure with guanidine thiocyanate treatment to remove humic acids from our late compost samples. Several washing steps with guanidine thiocyanate were tested, but each additional washing led to a lower recovery of DNA and only minor changes in humic acid content (measured as A_{230nm}). Similar unsatisfactory results were obtained when the DNA extracts were purified with high and low salt solutions by using Elutip-d columns (Schleicher & Schuell, Germany) or with the freezing-thawing procedure (Hallin & Lindgren, 1999). Finally, we decided to use the MOBIO Power Max™ DNA Kit for obtaining DNA from the aged compost samples (paper IV). This commercially available kit allows the use of larger sample volumes (up to 10g). This method was much more efficient for our samples with a high content of humic substances. The DNA recovered was obtained in rather large volumes (ca. 5ml) and the humic acid content was low, so that the compost DNA was suitable for further molecular analyses.

Polymerase chain reaction (PCR)

The extracted DNA is theoretically a mixture of genomic DNA from all microorganisms that were present in the compost. Using polymerase chain reaction (PCR), specific genes can be amplified in sufficient numbers for further analysis. Amplification and sequence analysis of the gene encoding 16S ribosomal RNA is one such commonly used method. The primer design is crucial for obtaining PCR results that are unambiguous and readily interpretable, since it determines the specificity of the primers.

In this study, we used nested PCR approaches with sets of specific and universal bacterial primers targeting fragments of 16S rRNA genes specific to the group Actinobacteria (Table 2). Initially, we followed Heuer *et al.* (1997), using the Actinobacteria-specific primer F243 and the universal bacterial reverse primer R1378 in the first-round PCR, while the resulting amplicons were used as templates in the final PCR step with primers F984 and R1378 (paper III). A second nested PCR approach was performed with the universal bacterial primers fD1 (Weisburg *et al.*, 1991) and 926R (Muyzer *et al.*, 1995) in the first-round. The amplified ca. 870 bp fragment of the 16S rRNA gene was then used as template for the PCR with Actinobacteria-specific primers (Stach *et al.*, 2003), which resulted in a ca. 600 bp fragment (paper IV).

Table 2. *Sequences of specific and universal primers used in the molecular studies*

Primer (Reference)[#]	*16S rDNA target*	*Primer sequence (5' - 3')*
F243 (A)	Actinobacteria	gga tga gcc cgc ggc cta
R1378 (A)	Eubacterial	cgg tgt gta caa ggc ccg gga acg
F984 (A)	Eubacterial	aac gcg aag aac ctt ac
fD1 (B)	Universal	ccg aat tcg tcg aca aca gag ttt gat cct ggc tca g
926R (C)	Universal	ccg tca att ctt ttr agt tt
S-C-Act-235-a-S-20 (D)	Actinobacteria	cgc ggc cta tca gct tgt tg
S-C-Act-878-a-A-19 (D)	Actinobacteria	ccg tac tcc cca ggc ggg g

[#] References for the PCR primers are: (A) Heuer *et al.* (1997); (B) Weisburg *et al.* (1991); (C) Muyzer *et al.* (1995); (D) Stach *et al.* (2003).

The original intention was to use the Actinobacteria-specific primers designed by Stach *et al.* (2003) in a direct PCR approach, but these primers did not yield any amplification products with our compost samples, probably due to their high specificity. Therefore, a nested PCR which amplified general bacterial genes prior to the amplification of genes specific to Actinobacteria was the strategy of choice (paper IV). However, if possible, the direct PCR is preferred to the nested approach, since it is less biased.

The amplification cycle, which is achieved by using concerted temperature programs, consists of 1) denaturation of template DNA, 2) annealing of primers to target DNA and 3) primer extension steps. The first step is performed at a high denaturing temperature (often 94°C), which requires a thermostable DNA polymerase, such as the frequently employed *Taq* polymerase isolated from the thermophilic bacterium *Thermus aquaticus*. The second amplification step is more specific, since the annealing temperature of the primer set depends on the primer configuration. In our PCR approach with primers designed by Heuer *et al.* (1997), for example, we used 62°C in the first and 57°C in the second PCR as annealing temperatures (paper III). For the second PCR approach, an annealing temperature

of 55°C was used for the first-round PCR with universal bacterial primers, but for the second round with the Actinobacteria-specific primers a so called touch-down thermal cycle was employed, meaning that the initial annealing temperature of 72°C was decreased by 0.5°C in each successive cycle to 67.5°C (paper IV). This strategy of rather high annealing temperatures contributes to an increase in specificity of the PCR reaction. Using an extension temperature of 72°C, a non-specific hybridization of primers to non-target DNA rarely occurs and this results in a homogeneous PCR product.

Besides the choice of primers and thermal conditions of amplification, the composition of the reaction mixture was also of importance for successful PCR reactions. In the study with samples from the compost reactor, the use of BSA (bovine serum albumin) in the PCR mixture prevented interference from humic acids (paper III). In the full-scale study, on the other hand, the use of BSA in the reaction mixture did not prevent interference reactions. The protein T4 gene 32 (Schwarz *et al.*, 1990) was used instead of BSA and this resulted in sufficient amplification products (paper IV). In line with this, it has been reported that the T4 gene 32 protein can be added to enhance PCR product yield, especially for long amplicons, and to detect low numbers of genes and stabilize single-stranded DNA during primer annealing (Tebbe & Vahjen, 1993).

Denaturing gradient gel electrophoresis (DGGE)

The PCR products were generated with sets of conserved primers that are specific for the group of Actinobacteria. Subsequently, the amplified products were separated to give a picture of this specific bacterial community. We achieved this by applying the fingerprinting technique denaturing gradient gel electrophoresis (DGGE). DNA fragments of the same length, but with different nucleotide sequences, are separated in a polyacrylamide gel containing a linearly increasing gradient of denaturants (formamide and urea). The separation is based on the decreased mobility of the partially melted DNA strands, meaning that migration of the DNA fragment stops when a region of sufficient denaturant is reached. To prevent complete melting of the molecules in the denaturing gradient, a GC-clamp (a 40 bp stretch containing high guanine + cytosine content) is attached to the 5'-end of one primer. Differences in melting properties are to a large degree controlled by differences in the sequences of the molecular types. The resulting band pattern reflects, in principle, different species of microorganisms, with each band representing a different molecular type. Individual bands can be excised and sequenced. By comparing the sequences with those of known species that are available in appropriate databases, the actual species present in the community can be determined (Fig. 5).

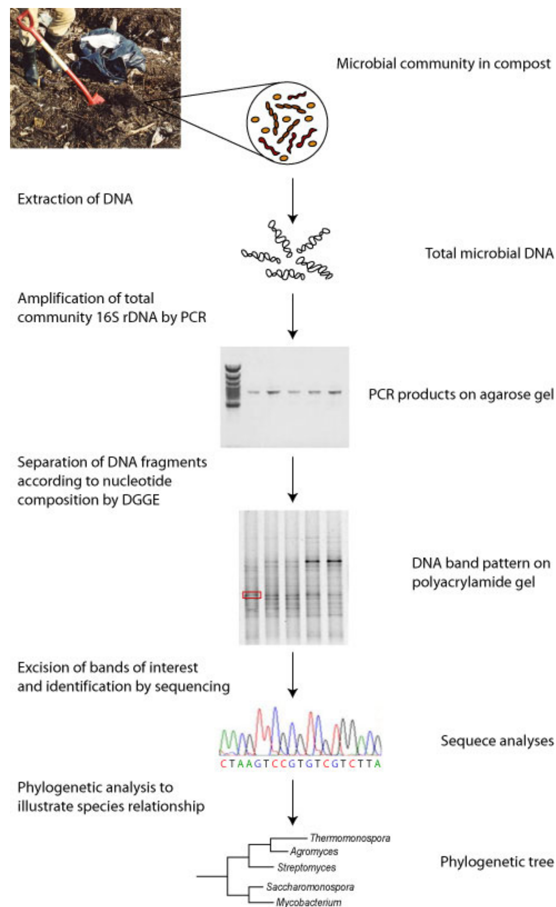

Fig. 5. Schematic illustration of different steps in biodiversity analysis of microbial communities (illustration by Å. Sjögren).

The DGGE method has been used to determine the microbial community structure in different compost systems (Kowalchuk *et al.*, 1999; Ishii *et al.*, 2000; Pedro *et al.*, 2001; Cahyani *et al.*, 2003; Marshall *et al.*, 2003), but not with any special focus on Actinobacteria. Our molecular studies of the development of Actinobacteria populations in composts from pilot and full-scale facilities followed the protocols of standard procedures (Muyzer *et al.*, 1993). A GC-clamp was attached to the F984 primer in the reactor study (paper III) and to the forward primer S-C-Act-235-a-S-20 in the full-scale study (paper IV). The DGGE gels were run under constant voltage for 16 h, and afterwards the gel was stained by SYBR gold. The bands were visualized under UV light and gel pictures were analysed using relevant computer programs. The treatment of excised bands, their sequencing and the phylogenetic analyses are described in detail in papers III and IV.

Impact of environmental conditions on microbial community development

The laboratory reactor and the compost substrate

An advanced experimental reactor was constructed at the Department of Agricultural Sciences at SLU, Uppsala (Sweden) between 1996 and 1998 to support systematic studies of the effects of environmental parameters on carbon and nitrogen turnover in the compost process (Fig. 6). A detailed description with a schematic illustration of the reactor system has been published by Smårs *et al.* (2001), but a brief summary of the main components is given here.

Fig. 6. The laboratory reactor for composting experiments with source-separated organic household waste.

The reactor has a volume of approximately 200 L. It is thermally insulated and equipped with a radial fan to obtain a uniform temperature and to distribute the gas flow evenly throughout the compost matrix. 17 transducers are placed at different heights in the compost material and in the gas stream to monitor and control the process temperature. Additionally, the reactor is equipped to enable independent control of oxygen levels and continuous monitoring of gas flow and concentrations of CO_2, NH_3 and N_2O in the compost atmosphere. Water is added

once a day to compensate for moisture losses due to drying of the material as a result of heat production and aeration. The evaporated water was trapped as condensate in a cooler, which serves as a part of the regulation system. The material in the reactor is mixed by tilting the reactor 90° to a horizontal position and then rotating it by hand. A few revolutions back- and forwards daily served the purpose of mixing the material to balance out moisture gradients, expose new surfaces, break established channels for preferential airflow and preserve the structure of the material to simplify aeration.

The same substrate was used, and the same procedure for the preparation of the material was performed, for all experiments in the compost reactor. In 1995, source-separated organic household waste was collected from the treatment plant in Uppsala, Sweden (Eklind *et al.*, 1997). Before chemical characterization, the material was cleaned from plastic bags and other contaminants, and milled in a cutting mincer. The material consisted of 65.8% water, 25.6% organic matter and 8.6% ash. The chemical composition of the organic matter is summarised (Tab. 3).

Table 3. *Chemical composition of the organic matter in the household waste material used in the reactor experiments (Eklind et al., 1997)*

Components	***% of organic matter***
Sugar	1.6
Cellulose	15.6
Hemicellulose	3.2
Lignin	9.9
Starch	13.2
Crude fat	15.0
Crude protein	6.4
Lactic acid	1.5
Acetic acid	0.6
Ethanol	0.5
Unidentified	32.5

The material was divided into portions of ca. 24 kg, packed in polyethene plastic bags and stored at -20°C for future use. Prior to starting a composting run in the reactor, ca. 40 kg of the material was thawed slowly for 3-4 days and mixed with wheat straw and water to adjust the C/N ratio to 22 and the water content to 65% w/w (Smårs *et al.*, 2001). Sampling from the reactor was performed in such a way that the reactor was turned to mix the material immediately before a sample was taken. The material was transferred to plastic bags and stored at -20°C prior to chemical and biological analyses. The sampling procedure was repeated, so that three replicates were taken on each occasion.

Influence of oxygen content

The importance of oxygen for microbial activity and the entire process is obvious, since composting is an aerobic process. The effects of different oxygen concentrations on the development of microbial populations in the composting reactor were studied (paper II). Moreover, the interactions between growth of the microbial community and turnover of major classes of the organic matter were

determined. The decomposition dynamics of six different carbon compound classes (cellulose, hemicellulose, fat, starch, lignin and sugar) were correlated with the changes in microbial biomass and community structure determined by lipid fatty acid analyses. The total PLFA concentration was used as a measure of viable microbial biomass, to identify the periods of greatest increase in microbial biomass.

Results of the PLFA analysis were converted to living microbial biomass by assuming that 1 g dry weight of bacterial cells contains 100 µmol of PLFA (White *et al.*, 1979a), and that carbon constitutes 50% of the dry cell mass. The increase in the standing stock of microbial biomass, together with data on the total rate of organic matter degradation, allowed estimations of microbial growth yield during periods of high activity and biomass increase. For the periods of highest activity and biomass increase, the microbial growth yields based on the relation to substrate consumption ranged between 22 and 28% among the three compost processes (paper II), and there was no substantial difference among the treatments. The results from our reactor system fall within the lower range of growth yields commonly reported in soils (Anderson & Domsch, 1986; Frey *et al.*, 2001).

The chemical analyses of the carbon compound classes revealed delayed decomposition at the lower oxygen concentrations of 2.5% and 1.0% compared to the process at 16% O_2 (paper II). Cellulose constituted the largest fraction of the waste/straw mixture, followed by hemicellulose. Lignin was not significantly degraded in any of the three treatments during the 22-to 25-day experimental period, whereas the monomeric sugars were already completely degraded at the onset of the increase in microbial biomass. The starch and fat fractions were the main substrates utilized during the rapid growth of microbial biomass (Fig. 7).

The delay in peak microbial activity corresponded with a delay in temperature increase (Beck-Friis *et al.*, 2003). Rapid growth of thermophilic bacteria caused the rapid increase in microbial biomass in all three processes, as revealed by increased concentrations of the group of PLFAs that indicate the biomass of thermophiles. When the total PLFA concentrations were expressed as a function of CO_2 evolved, the patterns were very similar in the three treatments, indicating a similarity in microbial growth and succession. On the other hand, multivariate analysis of relative PLFA concentrations resulted in slight, but significant differences in fatty acid composition among the three treatments (paper II).

With respect to the faster carbon turnover at higher oxygen supply, it seems probable that for many populations in the reactor, nutrient limitation was reached earlier at 16% O_2 than at lower oxygen supply. The shift from *cis* to *trans* isomers of monounsaturated fatty acids under conditions of stress (Guckert *et al.*, 1986; Virtue *et al.*, 1996) can probably explain the increased significance of 18:1ω7t at lower O_2 concentrations, since a low oxygen supply is considered a stressful milieu for an aerobic microbial community.

In general, quite similar processes with respect to both organic matter decomposition and growth in microbial biomass were achieved at 16, 2.5 and 1.0% O_2 in the compost atmosphere. Although the process was delayed at lower oxygen levels, a low but continuous gas transport through the compost material was maintained even at such low O_2 concentrations as 2.5 or 1.0%.

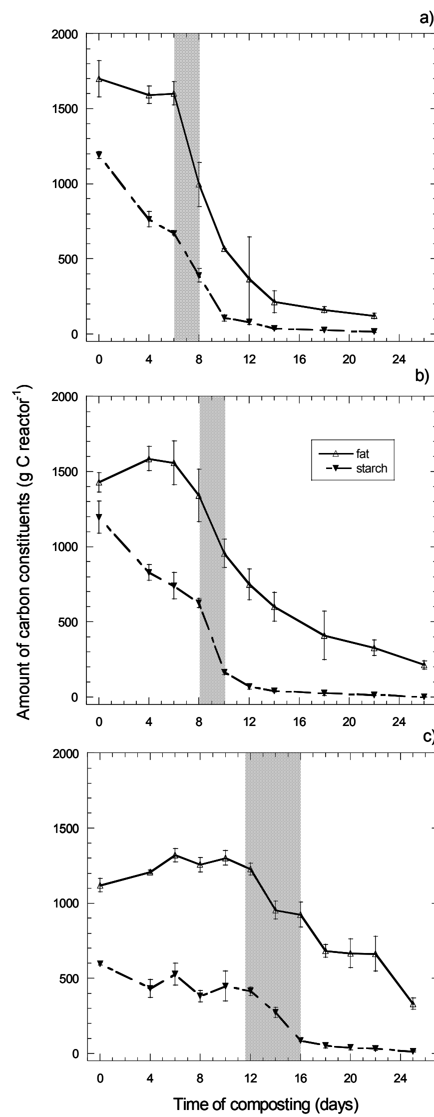

Fig. 7. Decomposition of the starch and fat fractions during composting at (a) 16% O_2, (b) 2.5% O_2 and (c) 1.0% O_2 in the compost gas (shaded intervals indicate the periods of highest microbial biomass increase).

In large-scale composting systems with more heterogeneous gas transport through the material, such low oxygen levels indicate that anaerobic conditions could soon arise. Another important aspect of lower oxygen levels is the possibility of emission of methane, a greenhouse-gas and undesirable product of anaerobic microbial activity during composting (Beck-Friis *et al.*, 2003). Thus, our results should be interpreted to mean that composting at low oxygen levels is possible, but it is not recommended in practice.

Influence of temperature

Temperature plays a major role in the composting process. It is partly the result of microbial activity, but at the same time, temperature also affects the microbiological community. Its impact on other vital reactions in the process, and the interaction between temperature and various other parameters makes it difficult to distinguish cause and effect (Epstein, 1997). In this study, the effects of different temperature regimes on the changes in microbial community structure were investigated in one trial with a maximum temperature at 40°C, also referred to as the mesophilic trial, and two thermophilic trials with peak temperatures at 55 and 67°C, respectively (paper III). Signature fatty acid analyses were used to study the overall changes in microbial biomass and community structure.

The mesophilic trial at 40°C differed substantially from the two thermophilic trials, since the peak in microbial biomass was reached much later, between days 17 and 24, when almost 60% of the organic matter had been degraded (paper III). At 55°C, the peak in microbial biomass occurred simultaneously with the peak in decomposition activity around day 5, when about 40% of the initial carbon compounds had been decomposed. At 67°C, the peak in microbial biomass was also reached early, but in contrast to the 55°C trial, only 20% of the organic matter had been decomposed. Although the rate of degradation of organic matter was similar in the beginning of all three trials (Eklind *et al.*, unpublished), it seems that the incorporation of carbon into microbial biomass was slower under mesophilic conditions. On the other hand, at the later stages, the microbial biomass yield was obviously higher in the 40°C trial than at 55 or 67°C (paper III). This can partly be explained by the fact that in general, microorganisms show decreased biomass yield with increased temperature, at least in the range above the temperature for maximal growth rate (Coultate & Sundaram, 1975; Pirt, 1975). The fact that the PLFA composition in the trial at 40°C differed from those at the higher temperatures, implies that selection of different microbial communities may also have contributed to the differences in growth yields, and therefore to higher microbial biomass at 40°C.

The contribution of several mono-unsaturated fatty acids (16:1ω9, ω7 and ω5; 17:1ω9, ω8 and ω6; 18:1ω7 and ω5) was larger in the 40°C trial than at higher temperatures, whereas the opposite pertained for several of the branched fatty acids (i14:0; a15:0; i16:0; i17:0, a17:0; i18:0; i19:0). This indicates that the ratio between populations of gram-negative bacteria versus those of gram-positive bacteria was higher in the mesophilic than in the thermophilic trials (paper III). Furthermore, multivariate analyses of relative PLFA concentrations (mol%)

revealed that the position of the three fatty acids 10Me18:0, 10Me17:0 and 10Me16:0 implied a dominance of Actinobacteria in the later stages, particularly in the 40°C trial. The importance of Actinobacteria for the composting process is discussed in more detail later. In general, the growth of Actinobacteria in the later compost phases is partly responsible for the increase in microbial biomass, in particular in the thermophilic trials after the period of peak temperatures.

In summary, the effects of peak temperatures on the microbial growth and succession during composting in our laboratory reactor were particularly evident at the lowest temperature. Since the compost material in the reactor is quite homogenous, the trial at 40°C simulates to a certain extent the processes in the outer parts of large-scale composts where thermophilic temperatures are often not reached. Apart from lacking the sanitizing effect of temperatures above 40°C (Epstein, 1997), this study revealed that composting at mesophilic temperature is dominated by the growth of Gram-negative bacteria and an enhanced growth of Actinobacteria in the later compost stages. In contrast, the trials at 55 and 67°C delineate the processes in different inner parts of large-scale composts, and the typical shift from Gram-negative bacteria to a dominance of Gram-positive and/or thermophiles was observed in both trials. Although there were only slight differences in the fatty acid composition between the two thermophilic trials, a major difference was that the decomposition of organic matter was fastest at 55°C.

The full-scale composting facility

Source-separated organic household waste, collected in paper bags, was transported from several municipalities to the treatment plant of Isätra, Uppland, Sweden (Fig. 8). Garden waste was used as a bulking agent and mixed with the organic household waste (1:3). The mixture was placed into force-aerated concrete boxes (21m x 6.50m) covered with semi-permeable membranes, which release CO_2 and small amounts of the water vapour that is formed during the process, whereas any odorous compounds are dissolved in the condensate of the water vapour that is retained inside the membranes. All boxes were equipped with countersinks with pipes in the floor to force aeration. After the initial four-week treatment under the membrane, the material was transferred to open concrete boxes with forced aeration, where decomposition of the material continued for an additional four-week treatment.

Fig. 8. The full-scale composting plant in Isätra, Uppland (photo by J. Gustafsson, Vafab, Sweden).

For the final maturing stage, the material was placed in open-air windrows without forced aeration. During this stage, a front loader mixed the material once a month. Except for the first sampling at week 6, all other samplings were done in these open-air windrows without forced aeration (Fig. 9). The sampling procedure and the analyses of physicochemical parameters, like temperature, pH, dry and organic matter content as well as maturity index, are described in detail in paper IV. Some important results of the analyses were:

- Thermophilic conditions were established in week 6, whereas mesophilic conditions predominated at week 57 (Fig. 10).
- pH increased gradually from 5.4 to 8.5, with the largest increase between week 6 and 9.
- Dry matter content fluctuated, with the lowest value of 36% in week 9 and the highest value of 63% in week 24.
- A gradual decrease of organic matter content was monitored from 63 to 48% of dry matter.
- Compost maturity (measured with the SOLVITA® test) improved from index 1 (description of "raw compost") at week 21 to index 5-6 (compost in the "curing stage") at week 57, respectively.

Some of the changes in pH and dry matter content were correlated with changes in composting management. For example, the water content increased substantially when the material was transferred from forced aerated boxes to the open-air windrows without aeration (between week 6 and 9) and the pH also increased probably due to an increased loss of ammonia.

Fig. 9. Sampling of material in the curing phase at the composting plant in Isätra, Uppland (photo by I. Sundh).

The long period of high temperatures ensures a sanitization of the material, but at the same time, such high temperatures probably depressed the reaction rate (Haug, 1993). A slow decomposition rate might be an explanation for the low maturity index after 21 weeks of composting when the material was still characterized as "raw compost". Anyhow, from week 21 and onwards, a decrease in temperature

was obvious (Fig. 10), and lower temperatures characterize the curing phase of the process where a new mesophilic microbial community can be established. By week 57, the compost was defined as in the "curing stage", which means that the compost did not obtain the highest maturity index and could not yet be defined as "finished compost".

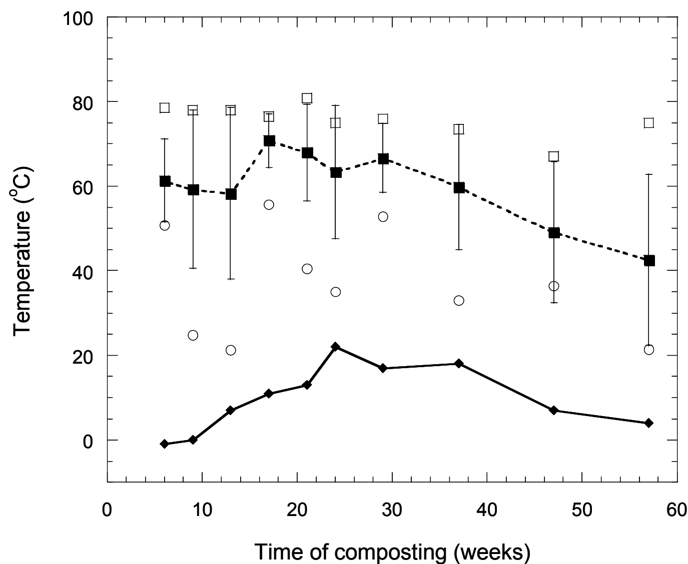

Fig. 10. Temperature profile during full-scale composting of organic household waste (diamonds: air temperature; filled squares: mean temperature in the material; open squares: highest measured temperature; open circles: lowest measured temperature; error bars = ± SD.).

The time-course of the PLFA concentration during the process shows similarities with the reactor studies: a relatively early peak followed by a noticeable decrease, and a slight increase in PLFA concentrations in the later stage (paper IV). The peak concentration of 1250 nmol g^{-1} dry weight was comparable to that in the reactor system (Sundh & Rönn 2002; papers I-III) and to other studies in full- and pilot-scale facilities (Hellmann *et al.* 1997; Herrmann & Shann 1997; Klamer & Bååth 1998). Thus, the maximal microbial biomass during the thermophilic phase was similar although different organic materials were composted and different techniques were used. The changes in fatty acid composition from straight-chain saturated and unsaturated towards branched-saturated and cyclopropane fatty acids points to the typical shift from Gram-negative bacteria and fungi to a community with more Gram-positive bacteria and/or thermophiles.

Furthermore, the decrease in microbial biomass during a period of continued high temperatures can be explained by the fact that temperatures exceeded the range (75°C to 85°C) within which thermophilic microorganisms can still maintain their biochemical functions (Haug, 1993). However, when the temperature started to decrease in the material, the microbial biomass increased slightly, indicating the re-establishment of a mesophilic microbial community represented by unsaturated fatty acids (paper IV). This increase in microbial biomass towards the later

compost stages was even more obvious when PLFA concentrations were related to organic matter, because inorganic material accumulated during the degradation process.

Development of Actinobacteria in composts

The presence and development of Actinobacteria populations and their potential use in characterizing the final compost product were investigated in both the pilot and full-scale composting processes (papers III and IV). In both studies, the changes in the size of Actinobacteria populations were determined by analysis of phospholipid fatty acids typical of Actinobacteria. Additionally, qualitative changes in this group of bacteria were followed by performing PCR-DGGE of 16S rRNA genes with Actinobacteria specific primers.

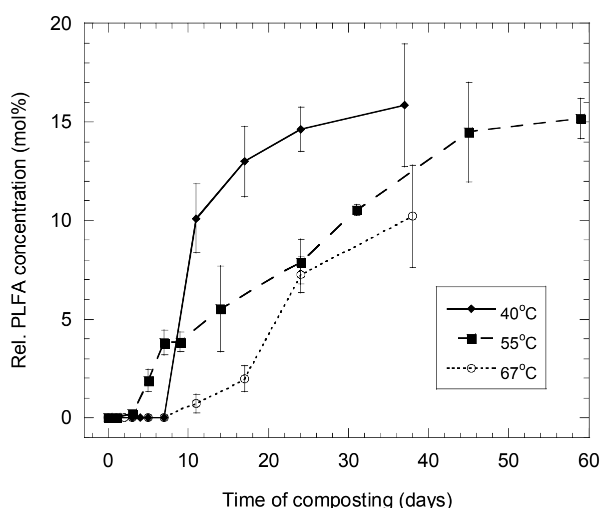

Fig. 11. Total relative concentrations of PLFAs typical for Actinobacteria (sum of 10Me16:0, 10Me17:0 and 10Me18:0) during composting in a laboratory reactor with peak temperatures at 40, 55 and 67°C (error bars = ± SD.).

The growth of Actinobacteria seemed to be favoured at moderate temperatures in the reactor system, since the contribution of Actinobacteria increased faster in the 40°C trial than in the trials where peak temperatures of 55 and 67°C were reached (Fig. 11). This indicates that populations of Actinobacteria are often reduced during the stage of elevated temperatures, a phenomenon that has been noted previously (Klamer & Bååth, 1998; Ryckeboer *et al.*, 2003a). However, in both the mesophilic and thermophilic trials, the 10-methyl fatty acids reached between 10 and 15mol% towards the end of the processes. Interestingly, the simpler, whole cell fatty acid method EL revealed results in the range between 7 and 12mol%

towards the end of all three temperature trials, which was in accordance with the PLFA results (paper III).

The Actinobacteria is a large group of relatively diverse organisms, which implies a large variation in the fatty acid composition. Indeed, the presence of 10-methyl branched fatty acids is not common to all Actinobacteria (Kroppenstedt, 1992). Thus, accounting only for 10-methyl branched fatty acids underestimates the Actinobacteria biomass in environmental samples, at least in theory. A conservative estimate of the contribution of Actinobacteria can be made by assuming that all Actinobacteria contain 1/3 10-methyl branched fatty acids in their structural lipids. Our results then suggest that up to half of the microbial biomass towards the end of the reactor trials consisted of Actinobacteria (paper III). In other pilot-scale systems, the use of quinone profiling, a fingerprinting method based on the analysis of respiratory quinones in the fraction of neutral lipids (Fig. 3), have also revealed that the importance of Actinobacteria increases in the later composting period (Hiraishi *et al.*, 2003; Tang *et al.*, 2004). Hiraishi *et al.* (2003) showed, for example, that 60% of the total population in old compost samples were Actinobacteria. This underlines the importance of this group of organisms towards the end of the composting process, when relatively complex, recalcitrant compounds dominate the remaining substrate. It is known that Actinobacteria have the ability to degrade many polymers in waste material, including hemicelluloses, celluloses, lignin, pectin and chitin (Goodfellow & Williams, 1983).

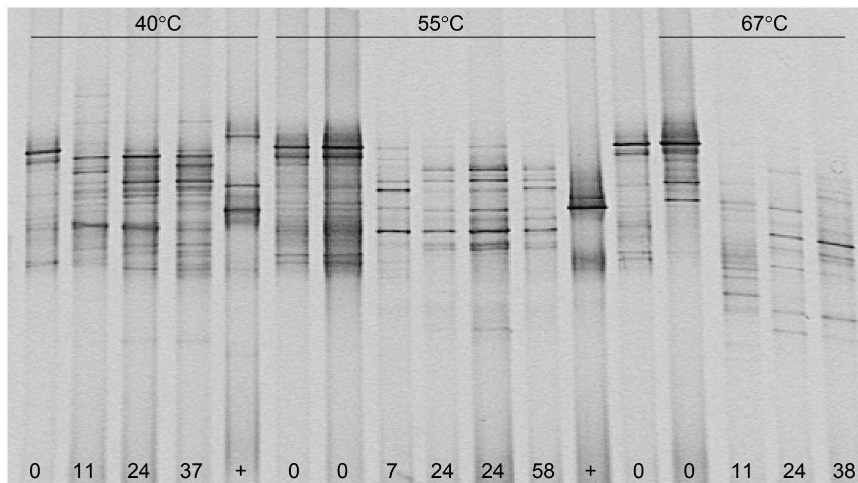

Fig. 12. DGGE separation of 16S rDNA after PCR with Actinobacteria-specific primers.

The presence of different species of Actinobacteria in the reactor runs was revealed by the PCR-DGGE and sequencing analyses. Among the three different temperature trials, slight differences were seen in the changes of Actinobacteria populations with time (Fig. 12). Interestingly, the three genera *Corynebacterium*, *Rhodococcus* and *Streptomyces* were only found in the original organic material. They were not detected in any later samples at any temperature, implying that they

were largely eliminated during the warmest phases. This result is in contrast to another study that reported the reappearance of *Rhodococcus* and *Streptomyces* in the cooling and maturing phase (Ryckeboer *et al.*, 2003a). Moreover, species of *Saccharomonospora* seemed to be characteristic for the later stages of all three trials (paper III). This genus contains moderately thermophilic actinomycetes and has been isolated from hot composts (Dees & Ghiorse, 2001). Other species of thermo-tolerant Actinobacteria were found in late samples from the thermophilic trials and affiliated to *Thermobifida fusca*, *Thermobispora bispora*, *Actinomadura sp.* and *Symbiobacterium thermophilum* (paper III).

Members of the genera *Corynebacterium, Thermobifida* and *Saccaropolyspora* were found in the samples from the full-scale study when temperatures around 60°C prevailed in the compost material (week 6 to 13; paper IV). Besides *Saccaropolyspora rectivirgula* and *Thermobifida fusca,* other genera of thermo-tolerant Actinobacteria (e.g. *Saccharomonospora* and *Streptosporangium*) were present in the hottest period (up to 80°C) of the process (week 17 to 29). Genera affiliated to *Streptomyces, Arthrobacter, Actinomadura, Thermocrispum* and *Microbacterium* were found in the material during the curing stage, accompanied by decreasing temperatures.

The changes in the concentration of 10-methyl branched fatty acids (i.e. the changes in Actinobacterial biomass) followed the changes in the total PLFA concentration (paper IV). The contribution of actinobacterial fatty acids to total PLFAs stayed at a constant level of about 3%. Thus, unlike the reactor study (Fig. 11), no obvious increase in the growth of Actinobacteria was noted in the full-scale composting process. This phenomenon is intriguing, although the explanation is not clear. Environmental factors, e.g. temperature, oxygen and substrate, might not have favoured the growth of Actinobacteria. The effect of high temperatures over a relatively long period may have eliminated many species of Actinobacteria. Although the molecular analyses revealed the presence of thermo-tolerant species even during the periods of highest temperature (paper IV), our molecular approach does not give any information about the abundance of these Actinobacteria species. Another explanation for the conflicting results of the PLFA and molecular methods might be that the Actinobacteria species found in the compost material have predominately iso- and anteiso- branched fatty acids in their phospholipids, but very low contents of the 10-methyl branched fatty acids. It has been reported that members of the species *Actinomadura* sp. and *Thermocrispum municipale* contain 10-methyl branched fatty acids (Kroppenstedt, 1992; Kornwendisch *et al.*, 1995), but their presence in the other species is uncertain. This emphasises the heterogeneity among the different genera and species of the Actinobacteria.

Peak temperature had obvious effects on the development of Actinobacteria in the reactor system (paper III), but the extent to which temperature influenced their development in the full-scale compost is uncertain. Oxygen supply and substrate composition also had clear effects (paper II), and it can be speculated that variation in these parameters as well as among the sub-samples might have affected the development of Actinobacteria in the full-scale study. Additionally,

small Actinobacteria populations can perhaps be explained by the result of the maturity test. The compost was still in the "curing stage" at week 57, which indicates that maybe a low abundance of Actinobacteria implies that the compost is not yet mature. Anyhow, this discussion underlines the importance of applying different methods to expand the scope for assessing microbial community structure in composts. This is not only a matter of methods, but also of the samples used for the investigations. Therefore, in order to resolve this issue, the study in the full-scale facility should be continued with analyses of several "mature" samples from different composting plants, as has been done in earlier studies on compost stability (Hue & Liu, 1995) and sanitary quality (Christensen *et al.*, 2002).

Concluding remarks

Advanced fingerprinting methods for the analysis of microbial biomass, community structure and diversity have been used in this thesis to gain a better understanding of microbial succession during composting. Important findings can be summarised as follows:

- Relatively simple methods that extract total microbial fatty acids directly from the compost material yield similar fatty acid profiles in the later composting stages as PLFA analysis. Thus, the simpler extraction methods are suitable tools to describe the succession in microbial community structure and show potential to be used as indicators of compost maturity.
- In the reactor system, a fairly similar process, with regard to both the growth of microbial biomass and the decomposition of organic matter, was achieved at different oxygen contents in the compost atmosphere, although the process was delayed at lower contents.
- Although starch and fat represented only about 10% and 15% respectively of the total carbon source in the reactor substrate, both fractions were important substrates for the growth of thermophilic microorganisms during composting.
- The effects of temperature on microbial growth and succession are particularly distinct in the lower, mesophilic temperature range. In the reactor system, for example, the growth of Actinobacteria was favoured when the maximum temperature was 40ºC, compared to the trials with thermophilic peak temperatures.
- Actinobacteria populations constituted a large part of the community during the later stages of the reactor experiments, when only relatively complex, recalcitrant compounds remained.
- The molecular analyses showed that Actinobacteria populations in the compost reactor shifted from being dominated by members of *Corynebacterium*, *Rhodococcus* and *Streptomyces* at the onset, to genera of thermo-tolerant Actinobacteria in the thermophilic and curing phase, e.g. *Saccharomonospora, Thermobifida* and *Thermobispora*.

- In the full-scale study, members of *Corynebacterium* were present at the early stage, and thermo-tolerant Actinobacteria (e.g. *Streptosporangium* and *Saccharopolyspora*) were found throughout the long thermophilic phase. A community including both thermo-tolerant and mesophilic Actinobacteria was found during the stages of decreasing temperature.
- The maximum microbial biomass during the thermophilic phase is similar irrespective of the kind of organic material that is composted and the scale of the system.
- The combination of signature lipid and nucleic acid-based analyses greatly expands the specificity and the scope for assessing the microbial community composition in composts.

A general conclusion drawn from this research is that whole cell fatty acid methods describing microbial community structure can give information that is related to aspects of maturity. With methodological development and automation, the EL method shows promise as a relatively simple and fast method of assessing compost maturity. However, further investigations of these interrelationships are needed in samples considered as mature from several different full-scale composting facilities. These investigations would complement the studies of Actinobacteria development in the reactor and full-scale processes of this thesis, and assess the potential to use assays of Actinobacteria populations for determining compost maturity. Another approach for future research could be the use of advanced molecular analyses, e.g. real-time PCR, which allows quantification of extracted and amplified DNA and thus give an estimate of the abundance of sequenced organisms. The extraction of RNA instead of DNA would make it possible to target the active organisms and thus help to answer the intriguing question of how structural diversity is linked to corresponding functions. This kind of information can connect specific microorganisms in the compost to specific processes, for example, in the curing phase to the decomposition of complex, recalcitrant compounds and to the production of mature compost.

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