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Characterisation of the Microbial Community in Indoor Environments: a Chemical-Analytical Approach

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Lund 2005

Bogdanowi, Julce i maleństwu ...

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LIST OF PAPERS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals:

I. **A. Sebastian and L. Larsson.** Characterisation of the microbial community in indoor environments: a chemical-analytical approach. *Applied and Environmental Microbiology* 2003 Jun; 69(6): 3103-9.

II. **A. Sebastian, A. Fox, W. Harley and L. Larsson.** Evaluation of muramic acid ester O-methyl acetate derivative for the determination of peptidoglycan in environmental samples by ion-trap GC-MSMS. *Journal of Environmental Monitoring* 2004 Apr; 6(4): 300-4. Epub 2004 Feb 26.

III. **A. Sebastian, B. Szponar and L. Larsson.** Characterisation of the microbial community in indoor environments: an update and critical evaluation. *International Journal of Indoor Air Quality and Climate*. 2005 May; 15(Suppl 9): 20-26.

IV. **A. Sebastian, C. Pehrson and L. Larsson.** Tobacco smoke introduces large amounts of endotoxin into indoor air. Manuscript.

V. **A. Fox, W. Harley, C. Feigley, D. Salzberg, A. Sebastian and L. Larsson**. Large particles are responsible for elevated bacterial marker levels in school air upon occupation. *Journal of Environmental Monitoring* 2005 May; 7(5):450-6. Epub 2005 Mar 29.

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ABBREVIATIONS

3-OH FAs	3-hydroxy fatty acids
BSTFA	*N,O*-bis-(trimethylsilyl) trifluoroacetamide
EI	electron ionisation
ETS	environmental tobacco smoke
FAs	fatty acids
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GC-MS/MS	gas chromatography-tandem mass spectrometry
IAQ	indoor air quality
IFNγ	interferon γ
IgE	immunoglobulin E
IL	interleukin
LAL	*Limulus* amebocyte lysate
LPS	lipopolysaccharide
m/z	mass-to-charge ratio
MMA	methyl ester O-methyl acetate
MVOCs	microbial volatile organic compounds
PCR	polymerase chain reaction
PRS	propylsulfonic acid
SPE	solid phase extraction
TLRs	Toll-like receptors
TMS	trimethylsilyl
TNFα	tumour necrosis factor α
VOCs	volatile organic compounds

INTRODUCTION

The relationship between the indoor environment and our health is very complex. Since many of us spend up to 90% of our time indoors, the indoor environment may strongly influence our wellbeing and effectiveness. In recent years, the prevalence of allergic diseases has increased substantially in countries with western lifestyle and it has been suggested that changes in the indoor environment may be one of causative agents. Certain contaminants associated with new building technologies and building materials may trigger allergic reactions leading e.g. to hypersensitivity pneumonitis, allergic rhinitis, and asthma, as well as general non-specific symptoms such as sneezing, watery eyes, coughing, shortness of breath, dizziness, eczema. Children, elderly people, and people with breathing problems, allergies, and lung diseases are particularly susceptible. Sensitive persons may react quickly and strongly to changes in the indoor environment. Both the type and the severity of symptoms may vary from person to person, even within the same building. It is therefore difficult to establish guidelines for maintenance of appropriate indoor air quality and threshold levels for given contaminants.

Indoor air quality – dampness and microorganisms

It is well known that dampness of buildings is associated with discomfort and health problems. Moisture in the building structure and high humidity indoors can affect building materials and result in microbial growth and emission of various irritant substances that, alone or in combinations, could act as causative agents for disease. High prevalence of respiratory infections among children in homes with building dampness and mould growth has been reported repeatedly (Jaakkola et al. 1993; Yang et al. 1997; Ross et al. 2000; Bornehag et al. 2005). Positive associations have been found between indoor concentration of moulds in classrooms and asthmatic symptoms among pupils (Smedje et al. 1997). Inhalation of air-borne microorganisms indoors has been also linked with the worsening of symptoms of asthma and allergic rhinitis (Bjornsson et al. 1995; Ross et al. 2000).

Microbial particles contain bioactive compounds including toxins. Exposure to various volatile and semivolatile organic compounds produced by bacteria and moulds that grow in damp environments has been implicated in a variety of biologic and health effects. Endotoxin, peptidoglycan, fungal cell-wall constituents such as (1-3)-β-D-glucans, mycotoxins, and microbial volatile organic compounds (MVOCs) are among the suspected causative agents.

Endotoxin originates from gram-negative bacteria and initially came to attention because of its potential to cause fever. More recently it has been established that at high exposure levels e.g. in agriculture (Donham 2000; Viet et al. 2001) and

related industries such as an animal feed production (Buchan et al. 2002), in cotton mills (Sigsgaard et al. 1992), and among fiberglass workers (Milton et al. 1996), endotoxin is strongly and consistently associated with airway inflammation and airflow obstruction. Endotoxin has been suggested to be positively associated with asthma development (Schwartz 2001) and worsening of asthma at domestic exposure (Ross et al. 2000). On the other hand, microbes may also be necessary for the development of a sound immunological system. Exposure to endotoxin in early age appears to be protective against allergy development among children (von Mutius et al. 2000; Braun-Fahrlander et al. 2002). Children with farming background have low prevalence of asthma and allergy (von Ehrenstein et al. 2000; Downs et al. 2001; Remes et al. 2005). Thus it appears like endotoxin may act both as a disease promoter or a health protector (hygiene hypothesis) depending upon time course, dose, and route of exposure.

Peptidoglycan (murein) is a vital part of cell wall of virtually all bacteria, especially of gram-positive bacteria. The murein structure is built up by chains of alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid which are β-1,4-linked. Although the biological potency of murein is lower as compared with endotoxin, elevated levels of muramic acid in inhaled air have been associated with wheezing. It may also serve as a marker of bacterial exposure (van Strien et al. 2004). Similarly to endotoxin, peptidoglycan is recognisable by the innate immune system (Dziarski 2003).

(1-3)-β-D-glucans are structural cell wall constituents of most fungi and have been considered as surrogate compounds to monitor environmental exposure to fungi. Associations between (1-3)-β-D-glucan exposure, airway inflammation and symptoms have been found. However, specific symptoms and potential underlying inflammatory mechanisms associated with exposure could not be identified (Douwes 2005).

Mycotoxins are toxic secondary metabolites produced by different genera of fungi. They have relatively low molecular weight and consist of non-volatile compounds with diverse chemical structures. Mycotoxins generally appear in the stage of mycelium and their production depends on food sources and other environmental factors. Filamentous fungi, especially those of *Fusarium*, *Trichoderma* and *Stachybotrys* genera, produce trichotecene mycotoxins. Since they are relatively non-volatile, inhalation exposure is mostly limited to the inhalation of airborne fungal particles.

Microorganisms can also produce volatile organic compounds (VOCs). Some microbial VOCs or MVOCs (such as alcohols, aldehydes, and ketones) are products of primary metabolism and are produced throughout an organism's life.

Others, which tend to be more complex, have characteristic mouldy, musty, or pungent odours. They are produced through secondary metabolism, in *Penicillium* and *Aspergillus*, around the time of sporulation, when mycotoxins also tend to be produced (Fiedler et al. 2001). Children living in dwellings with elevated MVOCs levels had a higher prevalence of asthma, hay fever, wheezing, and irritations of the eyes (Elke et al. 1999). MVOCs found in mouldy buildings are partly bound to air-borne inhalable dust particles (Wady and Larsson 2005).

Mechanisms of exposure to indoor microorganisms

The size of inhaled bioaerosol particles, including mould spores, bacteria, microbial debris, and contaminated dust, determines their deposition place in the respiratory tract. Spores of *Aspergillus* and *Penicillium* that use the air pathway for dispersion are in the respirable range of 1–2 μm. Moulds such as *Stachybotrys chartarum* and *Memnoniella echinata* are wet and slimy during sporulation, but once dry, the spores can get airborne to inhalable size (5–7 μm) (Sorenson et al. 1987). Bacteria such as *Streptomyces californicus* growing in damp indoor environments are about 1 μm in diameter and can reach the lower airways upon inhalation (Jussila et al. 2001). Neither normal clearance mechanisms such as mucociliary and alveolar clearance nor the immune system are able to remove such particles deposited in lungs.

Exposure to microorganisms and their products can irritate eyes and respiratory tract mucous membranes and lead to inflammation via immune response. Such immune responses are important in normal host defences, but chronic or excessive release of inflammatory mediators can cause damage to the lung and other adverse effects (Jussila et al. 2003).The epithelial cells recognize microbial particles using surface receptors such as CD14 and Toll-like receptors (TLRs). TLRs mediate recognition of a wide range of microbial products including lipopolysaccharides, peptidoglycan, lipoproteins, flagellin, and bacterial DNA. Signalling through TLRs leads to the increased production of inflammatory mediators such as cytokines (e.g., tumour necrosis factor α (TNFα) and interleukin-6 (IL-6)).

Allergic diseases have been closely related to allergen-specific immunoglobulin E (IgE) (Th2-associated) immune responses, which are characterized by high levels of interleukins (IL) such as IL-4, IL-5, IL-9 and IL-13. These cytokines take part in the recruitment and activation of eosinophils and mast cells, which leads to the development of chronic allergic inflammatory disorders, like airway hyperresponsiveness, reversible airway obstruction, and airway inflammation. It has been shown that challenging human peripheral blood mononuclear cells with citrinin, gliotoxin, and patulin (mycotoxins produced by fungi that may grow in indoor environments) caused strong inhibition of interferon γ (IFNγ)-producing T-helper 1 (Th1) cells leading to T-cell polarization toward the Th2 phenotype,

which may increase the risk for the development of allergies (Wichmann et al. 2002). Also, exposure to *Aspergillus* reduced the amounts of Th1 cells in the peripheral blood of children and significantly lowered the content of Th1 cytokines (IFNγ, TNFα, IL-2), which may be risk factor for the development of allergic diseases (Muller et al. 2002).

Exposure assessment methods for microorganisms indoors

Monitoring of indoor microbial populations to assess the indoor air quality (IAQ) requires methods that provide accurate and representative exposure estimates for bioaerosols. Traditionally, detection and quantification of microorganisms have been conducted using light microscopy and/or culture-based methods. Culturing is suitable for detection of culturable infectious agents and allow species identification, however, it is widely agreed that only a small fraction (0.1-10%) of the total microbial flora in an indoor environment is culturable (White 1983). Culture is also laborious and requires time for the organisms to grow into recognizable colonies, often taking days to weeks depending on the microorganism. Methods used for determination and quantification of non-viable microorganisms include among others microscopy, the *Limulus* amebocyte lysate (LAL) test, and polymerase chain reaction (PCR) techniques. Light microscopy is quick and direct but with low specificity and is at best semi-quantitative, unless accompanied by specialized staining. Although the LAL test is extremely sensitive to endotoxin and can detect glucans, it measures bioactivity rather than absolute amounts and its reproducibility and specificity has been questioned (Chun et al. 2000). Nucleic acid-based methods including PCR are very specific and by using broad-range (universal) probes and primers sets, based on 16S rDNA (Nadkarni et al. 2002) and 18S rRNA (Zhou et al. 2000), most bacteria and fungi present in a sample can be identified. To date, such universal probes and primers have not been widely used to characterize the microbiology of indoor environments. Instead, they are generally designed for the detection of a given genus (genus-specific primers) or a single species (species-specific primers) in such environments (Buttner et al. 2001; Haugland et al. 2002; Rintala et al. 2002). The presence of environmental background such as dust may inhibit the PCR reaction thus resulting in false negatives.

Microbial chemical markers

The answer to these difficulties may be the analysis of microbial chemical markers. Microorganisms synthesise a large variety of unique monomeric compounds not found anywhere else in nature that can serve as markers. Ideally, a microbial chemical marker should comprise a compound or groups of compounds unique to a certain microorganism or group of microorganisms that can be used for their identification or characterisation (Morgan et al. 1989; Larsson 1994). They are either an integral part of the cells or extracellular

metabolites. Analysis of microbial chemical markers can be used as an alternative or complement to various biological assays. Gas chromatography-mass spectrometry (GC-MS) is a very useful analytical method for the determination of specific microbial markers directly in complex clinical or environmental samples, without need for cultivation. Accuracy of such markers varies from being species-specific (Jantzen et al. 1993; Sonesson et al. 1993; Alugupalli et al. 1995) to fungi- or bacteria-specific. Different strategies for the analysis of chemical markers have been used for various purposes such as clinical diagnosis (Larsson et al. 1987; Larsson et al. 1994; Fox et al. 1996) or environmental monitoring (Gehron et al. 1984; Sonesson et al. 1988; Krahmer et al. 1998). GC-MS holds great promise as a method for examining a potential relationship between the inhalation of organic dust and the development of asthma and other respiratory tract diseases. GC - tandem MS (GC-MS/MS) improves considerably the specificity in determination of the studied compounds.

In present work GC-MS/MS, using ion-trap technology, was applied to determine the 3-hydroxy fatty acids (3-OH FAs) (marker of endotoxin – gram-negative bacteria), certain branched-chain non-hydroxylated fatty acids (gram-positive bacteria), muramic acid (peptidoglycan) and ergosterol (fungal biomass) (Figure 1) in house dust. This method has previously been shown to provide high detection selectivity allowing accurate determination of markers even when present at nanograms levels in chemically complex matrices such as e.g. tissue (Fox et al. 1996; Szponar et al. 2003; Ferrando et al. 2005), bioaerosols (Szponar and Larsson 2001; Gorny et al. 2004), organic dust (Saraf and Larsson 1996; Saraf et al. 1997; Park et al. 2004) and building materials (Szponar and Larsson 2000). The analyses are preceded by chemical marker liberation from larger macromolecular structures (methanolysis, saponification), various extraction steps, purification using a solid phase chromatography, and chemical derivatisation to make the markers less polar and thus more suitable for GC. Accurate detection of the markers is accomplished by using MS/MS, which allows monitoring of product ions of fragment produced at a preceding ionisation to achieve optimal sensitivity and specificity.

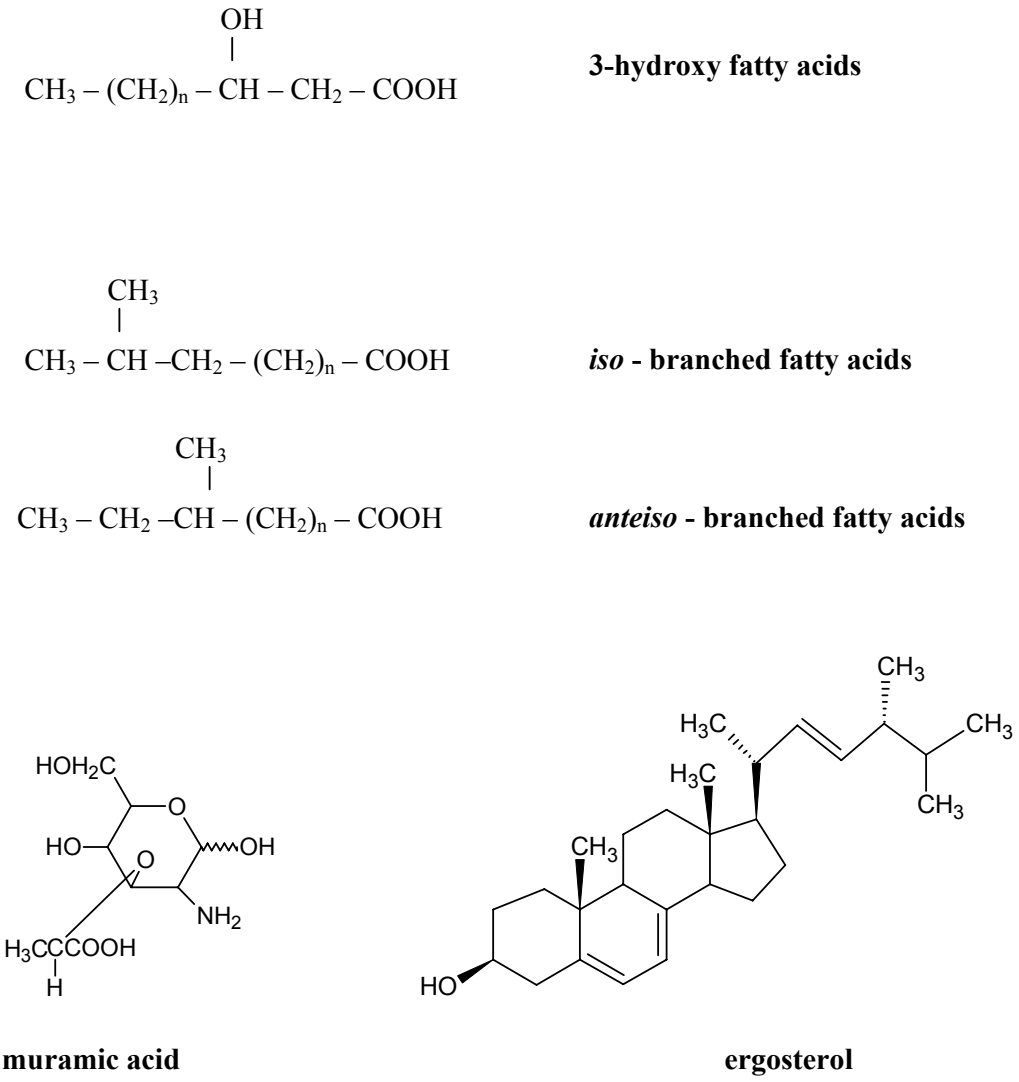


Figure 1. Chemical markers used in the study

Endotoxins (***LPS***) are major constituents of the outer membrane of gram-negative bacteria. LPS consist of a hydrophobic domain known as lipid A, which is chemically distinct from all other lipids in biological membranes, a non-repeating "core" oligosaccharide, and a distal polysaccharide (or O-antigen) (Figure 2). A backbone of Lipid A, the toxic component of the LPS molecule, carries in general 4 moles of unique 3-OH FAs, two of which are amide linked and two that are ester linked (Saraf and Larsson 1996; Saraf et al. 1997; Saraf et al. 1999; Szponar and Larsson 2001). The fatty acid composition of LPS varies between different gram-negative bacteria and can be unique for certain species (Rietschel 1976; Sonesson et al. 1989; Alugupalli et al. 1994). Because of their

distinctiveness these FAs were suggested as chemical markers of LPS by Rietschel (Rietschel 1976).

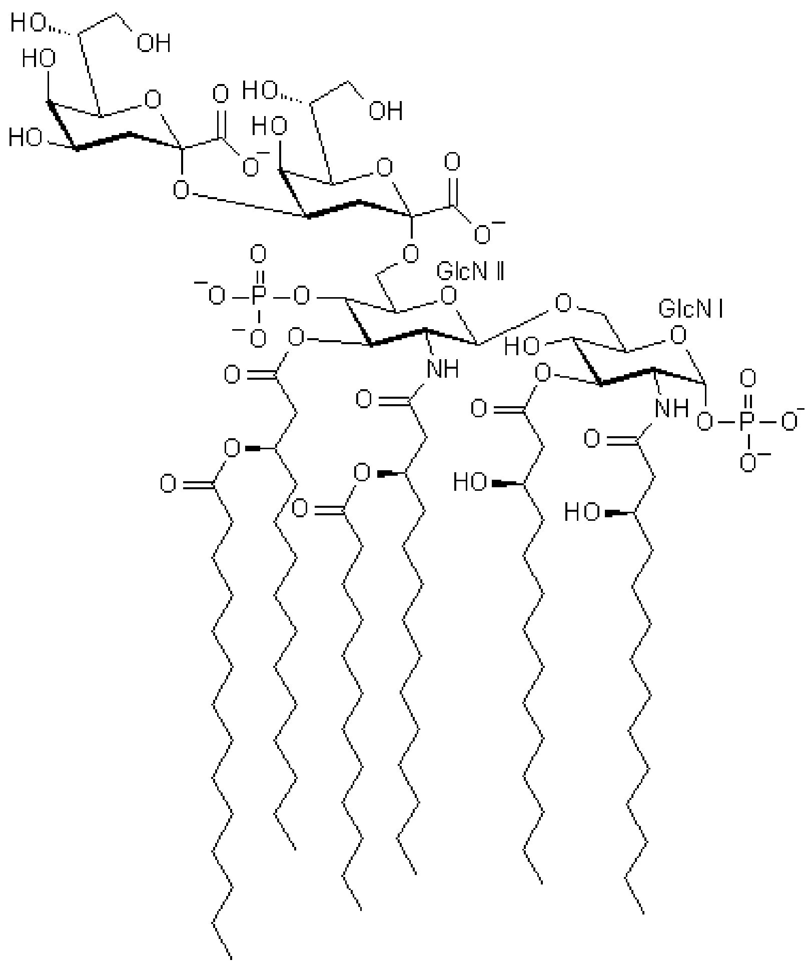


Figure 2. The basic structure of lipopolysaccharide from E. coli with lipid A. Drawn after The Lipid Library, W. Christie.

Branched-chain fatty acids of *iso* and *anteiso* forms are found in many bacteria as the major acyl constituents of membrane phospholipids. Membrane FAs can be divided into two major groups: the straight-chain and the branched-chain (including *iso*-, *anteiso*-, and ω-alicyclic-) fatty acids. The latter give very complex patterns and are therefore of great value in bacterial systematics (Kaneda 1991; Zelles 1997). The occurrence of branched-chain fatty acids as major constituents of bacteria was first reported for *Bacillus subtilis* (Kaneda 1967). In Bergey's Manual of Systematic Bacteriology (Schleifer and Seidl

1985; Sneath et al. 1986; Staley et al. 1989; Williams and Sharpe 1989) the group of gram-positive bacteria with the fatty acids that are branched contains the largest number of genera. These particular chemical markers may have limited specificity but give additional information about the bacterial flora and are very easy to prepare.

Muramic acid is a marker for peptidoglycan found in largest amounts in gram-positive bacteria (40-80 % of cell wall) and in smaller amounts in gram-negative bacteria (2-10 % of cell wall). In gram-positive bacteria peptidoglycan comprises the essential component of the cell wall, with covalently bound other polysaccharides and proteins. In gram-negative bacteria peptidoglycan is situated under an outer membrane that contains the LPS. It surrounds the cytoplasmic membrane in bacteria and maintains their shape. Peptidoglycan is a polymer formed by (1–4)-linked *N*-acetylglucosamine and *N*-acetylmuramic acid cross-linked by peptides (Figure 3). It does not exist elsewhere in nature and is thus not synthesized by mammalian cells (Schleifer and Seidl 1985). Sometimes, peptidoglycan is referred to as "gram-positive bacterial endotoxin" (Verhoef and Kalter 1985). Muramic acid is a unique amino sugar present in all peptidoglycan and has been used as a chemical marker of peptidoglycan in various environments (Sonesson et al. 1988; Fox et al. 1995; Fox et al. 1996; Bal and Larsson 2000).

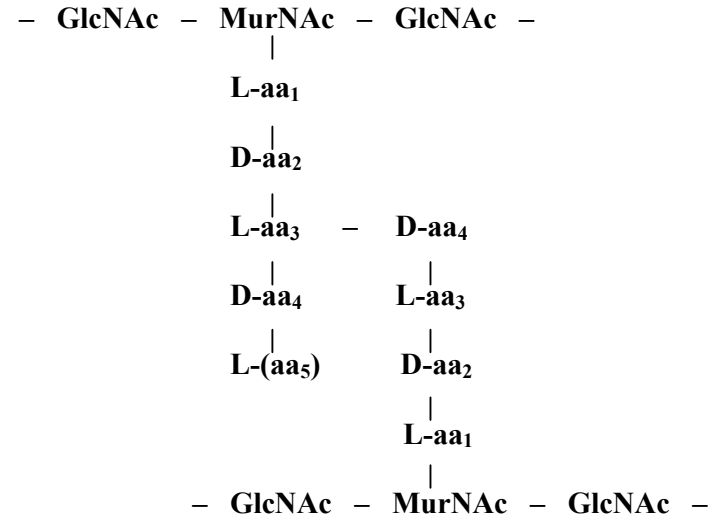


Figure 3. Basic structure of peptidoglycan. GlcNAc: *N*-acetylglucosamine; MurNAc: *N*-acetylmuramic acid; aa: amino acid; L and D: configuration of amino acids.

Ergosterol is the primary sterol in the cell membranes of filamentous fungi, yeast cell walls and a minor component of higher plants. Membrane sterols give structure, modulation of membrane fluidity, and possibly control of some physiologic events. Ergosterol is a component of membranes in mycelia, vegetative cells and spores (Newell 1992). Because a strong correlation between ergosterol content and fungal dry mass, it has been widely used to estimate the fungal biomass in various environments (Gessner and Chauvet 1993; Saraf et al. 1997; Pasanen et al. 1999; Newell et al. 2000). However, the amount of ergosterol in fungal tissue is not constant. The concentration depends upon species, developmental stage, age of the culture and growth conditions (Pasanen et al. 1999; Charcosset and Chauvet 2001; Reeslev et al. 2003; Mille-Lindblom et al. 2004).

AIMS OF THE STUDY

The aims of the present study were:

- To develop an integrated analytical methodology to characterise the microbial flora of indoor environments including both culturable and non-culturable microorganisms and cellular debris.

- To introduce branched-chain non-hydroxylated fatty acids as an additional marker of gram-positive bacteria.

- To improve the methodology for determination of muramic acid.

- To investigate the impact of cigarette smoking on endotoxin of air-borne and settled house dust particles.

- To study the effect of occupancy on airborne dust marker patterns in school classrooms.

MATERIALS AND METHODS

Samples

Settled house dust samples were used in three methodological studies (**Paper I, II** and **III**) and in a study on tobacco smoking-generated endotoxin (Paper **IV**). The dust samples were collected on cellulose ALK filters by using a vacuum cleaner equipped with a filter holder. According to the manufacturer, these filters retain 74% of particles 0.3-0.5 μm, 81% of particles 0.5-1.0 μm, and 95% of particles 1-10 μm. 2-4 m² areas (floors, shelves, bed) were vacuumed during 5-10 min. The dust samples were sieved (particle diameter <400 μm) and 1-5-mg portions of the fine dust fractions were subjected to GC-MS analysis. In addition, house dust samples were collected by allowing air-borne dust particles to sediment, during 4-5 weeks, on plexiglass plates (0.5 x 0.5 m) that were spatially well-distributed in a studied room and positioned at different distances (0.2-2.5 m) from the floor; these samples were collected from the plates using a rubber scraper and directly subjected to GC-MS analysis (**Paper III**).

Airborne dust samples were collected by pumping 18-40 L/min of air for 5-72 h through Teflon filters (0.2 and 0.5 μm pore size, 37 and 47 mm diameter) chosen because of their inertness to the chemicals used in sample preparation. Air-borne dust samples were used in a methodological study on muramic acid (**Paper II**), in a study on tobacco smoking-related endotoxin **(Paper IV**), and in a study on bacterial markers in school classes (**Paper V).** The filters were positioned in or close to the breathing zone (1.5-2 m above the floor) during the samplings.

Bacteria (**Paper III**). Seventeen species of *Actinobacteria* comprising both clinical and environmental isolates were studied for 3-OH FA composition.

Internal standards

The following internal standards were used to assist in the identification and quantification of the chemical markers in the studied bacterial and dust samples (Table 1).

^{13}C-labeled 3-OH C $_{16:0}$, C $_{16:0}$, and ***muramic acid***, in methanolysates of ^{13}C-labeled cyanobacteria, were used as internal standards for 3-OH FAs, non-hydroxylated FAs, and muramic acid, respectively (**Paper I, II** and **III**). 30 μl of the methanolysate (corresponding to 30 μg of cyanobacteria) were added to each sample.

^{13}C-labeled 3-OH $C_{13:0}$, in methanolysates of ^{13}C-labeled *Pectinatus cerevisiiphilus* (3-OH $C_{13:0}$ is rarely found in organic dust), was introduced as an internal standard for 3-OH FAs in **Paper III**.

Non-labeled 3-OH $C_{13:0}$ methyl ester was also used (**Paper IV**). 50 ng of the standard were added to each sample.

^{13}C-labeled muramic acid, in hydrolysates of ^{13}C-labeled cyanobacteria, was used as an internal standard in **Paper II** and **V**. 34 ng of the standard were added to each sample.

Dehydrocholesterol (100 ng) was used as an internal standard for ergosterol (**Paper I** and **III**).

Table 1. Studied markers and internal standards

Marker	Internal standard	Paper
3-hydroxy fatty acids	cyanobacterial ^{13}C-labeled 3-OH $C_{16:0}$	I, III and V
	^{13}C-labeled 3-OH $C_{13:0}$ in *Pectinatus cerevisiiphilus*	III
	non- labeled 3-OH $C_{13:0}$	IV
non-hydroxylated fatty acids	cyanobacterial ^{13}C-labeled $C_{16:0}$	I
muramic acid	cyanobacterial ^{13}C-labeled muramic acid	I, II, III and V
ergosterol	dehydrocholesterol	I and III

Sample preparation (Figure 4)

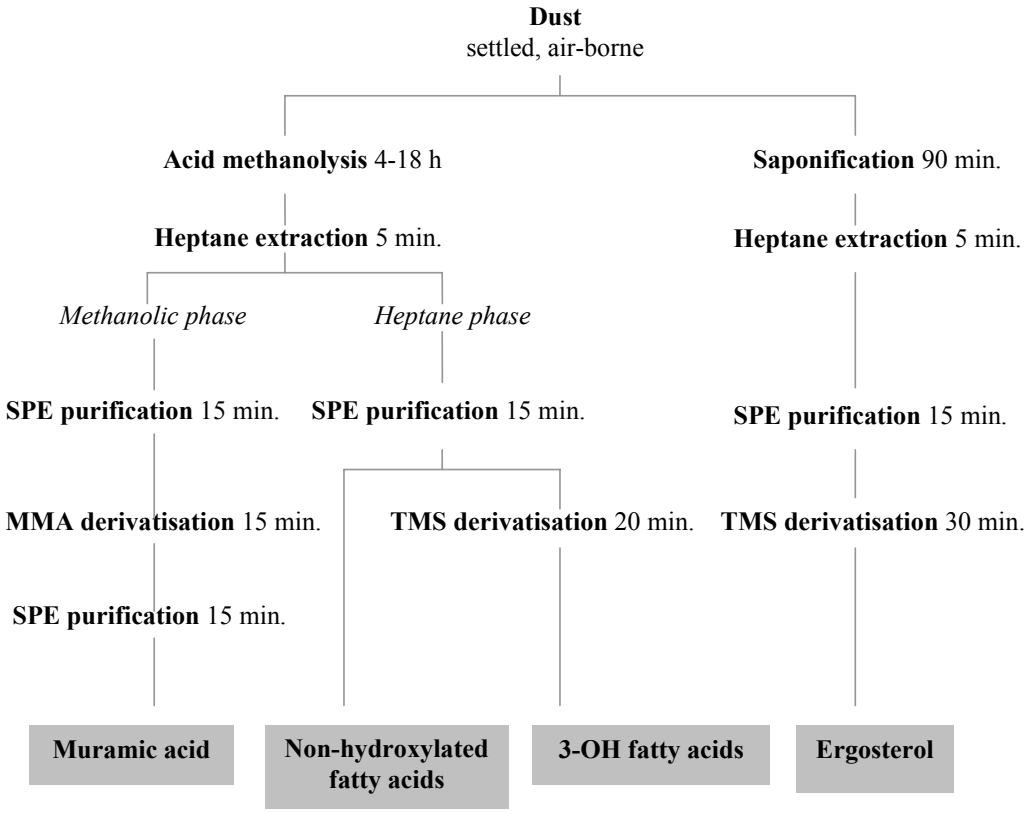


Figure 4. Integrated procedure developed for chemical marker analysis.

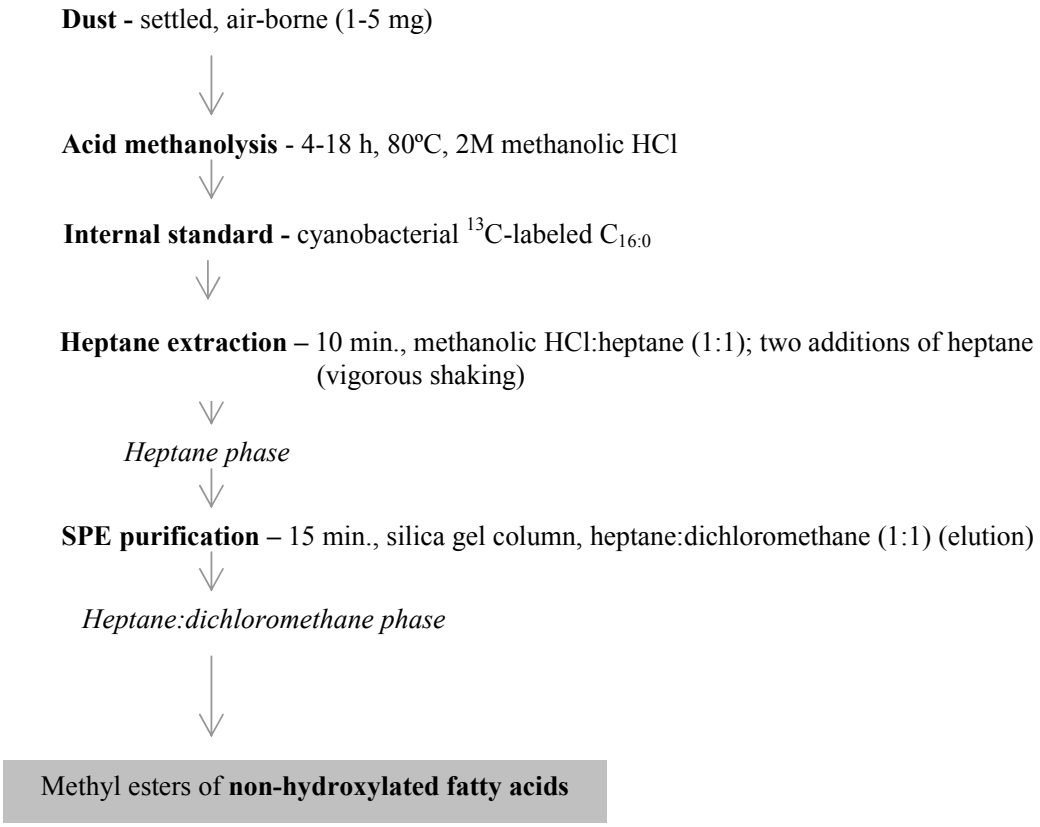
Fatty acids and muramic acid

Samples, in Teflon-lined glass test tubes, were heated overnight in 1 ml of 2M methanolic HCl at 85^{o}C. This procedure results in release of the chemical markers from larger bacterial structures; at the same time, the FAs (Figure 5) and muramic acid (Figure 6) are methylated. The internal standard was added to each sample and the mixture was extracted either with 1.5 ml of water:heptane (**Paper I**) or (twice) with 1ml of heptane (**Paper II**). The heptane layer (upper), containing hydrophobic compounds, was used for analysis of FAs whereas the lower layer, containing components of more hydrophilic nature, was used for analysis of muramic acid.

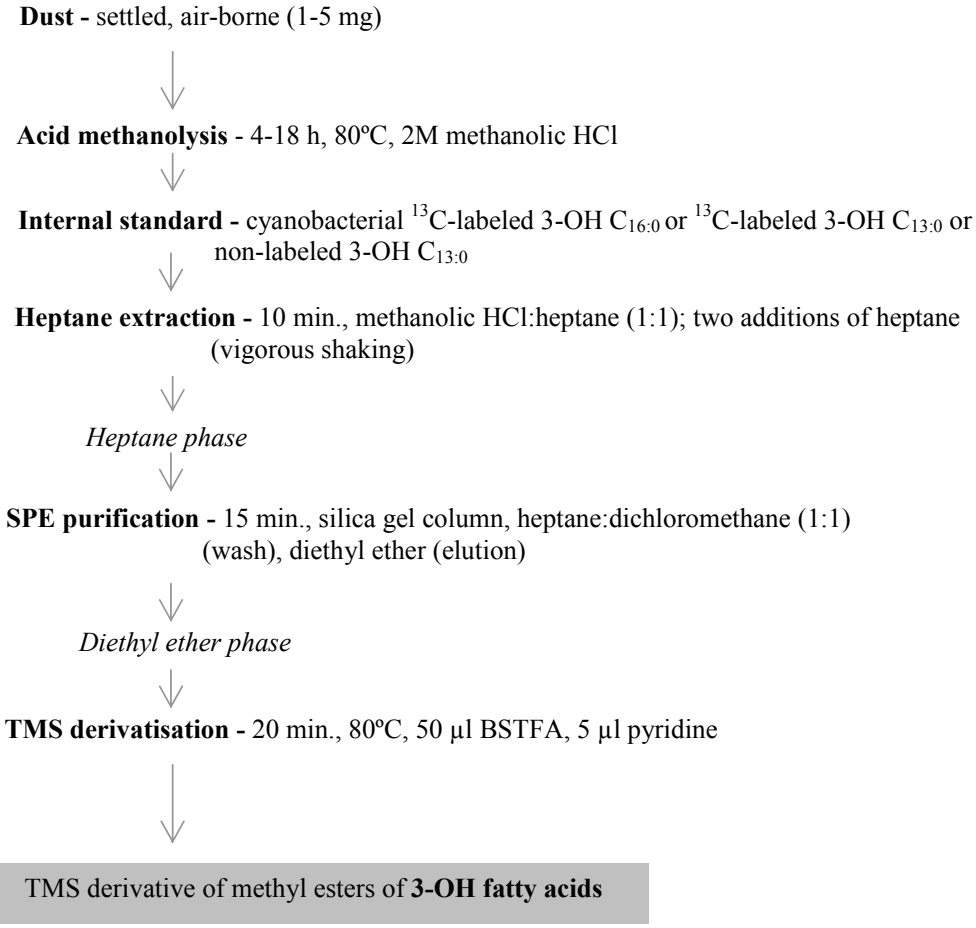
Fatty acids

(**Paper I**) The heptane layer was transferred to a separate test tube, evaporated to dryness at room temperature under a stream of nitrogen, redissolved in 1 ml of heptane:dichloromethane, and applicated onto a disposable silica gel column (100 mg). Heptane:dichloromethane (2 ml) was added to the column to elute the

(less polar) non-hydroxylated FA esters. The eluate was collected in a separate test tube, evaporated and redissolved in heptane for analysis.



Then, diethyl ether (2 ml) was added to the silica gel column to elute the more polar hydroxy FA esters; also this eluate was evaporated at room temperature. Trimethylsilyl (TMS) derivatives of the hydroxy FA esters were formed by adding N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (50 µl), attaching silyl groups to the compound, and pyridine (5 µl) acting as a catalyst in the reaction, followed by heating for 20 min. at 80^{o}C (Figure 5). Heptane was then added and the preparations were analysed. In addition, some strains of *Actinobacteria* (**Paper III**) with high contents of 3-OH FAs were also subjected to chiral separation for studying the absolute configurations of the acids. Chiral (S)-phenylethylamide methoxy derivatives of the 3-OH FAs were prepared according to the method described by Gradowska and Larsson (Gradowska 1994).


Dust - settled, air-borne (1-5 mg)
Acid methanolysis - 4-18 h, 80ºC, 2M methanolic HCl
Internal standard - cyanobacterial ^{13}C-labeled 3-OH $C_{16:0}$ or ^{13}C-labeled 3-OH $C_{13:0}$ or non-labeled 3-OH $C_{13:0}$
Heptane extraction - 10 min., methanolic HCl:heptane (1:1); two additions of heptane (vigorous shaking)
Heptane phase
SPE purification - 15 min., silica gel column, heptane:dichloromethane (1:1) (wash), diethyl ether (elution)
Diethyl ether phase
TMS derivatisation - 20 min., 80ºC, 50 µl BSTFA, 5 µl pyridine
TMS derivative of methyl esters of **3-OH fatty acids**

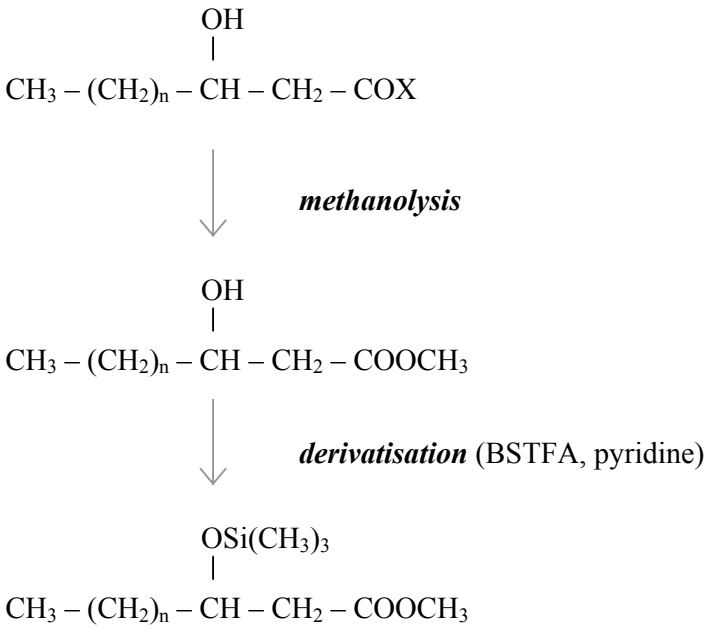
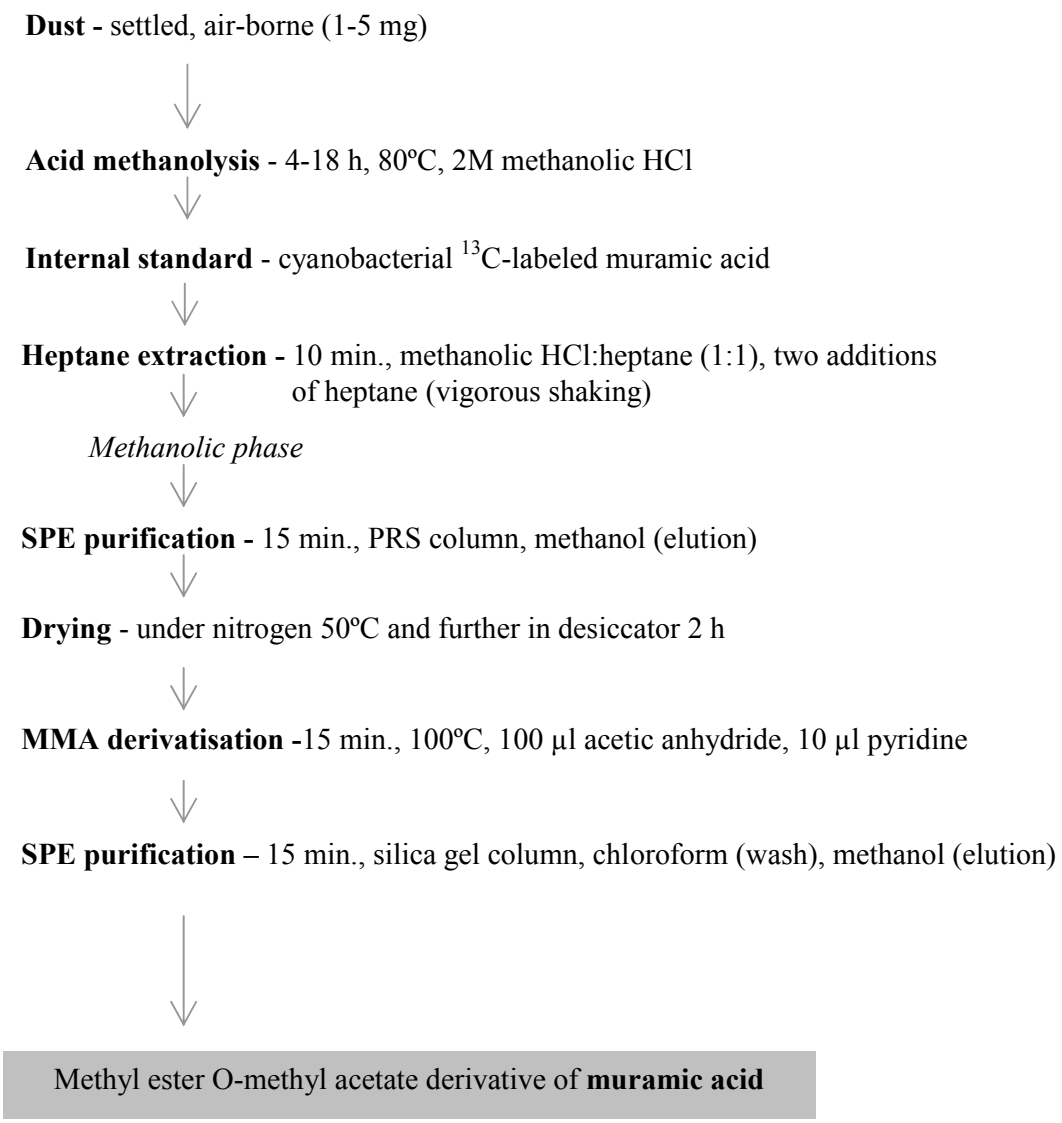


Figure 5. Methylation and TMS derivatisation of 3-OH fatty acids.

Muramic acid
Two different derivatives of muramic acid - the methyl ester O-methyl acetate (MMA) derivative and the alditol acetate derivative - were prepared as described below.

Methyl ester O-methyl acetate (MMA) derivative **(Paper I** and **II)**
Paper I: Methanol (1 ml) was added to the lower methanolic phase and the mixture was applied onto a propylsulfonic (PRS) acid column followed by 1 ml of methanol. This procedure removes possible traces of lipid material not separated during the extraction and was also found to remove dust particles. The entire elute was collected in a test tube, evaporated to dryness under nitrogen, and further dried under vacuum in a desiccator (2 h). Acetylation was accomplished by heating the preparations in a mixture of acetic anhydride (100 µl) and pyridine (100 µl) at 60°C for 1 h (Figure 6). Then, the formed MMA derivatives were purified as follows. The reaction mixture was evaporated, dissolved in 2 ml of dichloromethane, and subsequently washed with weak aqueous HCl solution and water. The samples were then evaporated to dryness, dissolved in 200 µl of chloroform, and applied onto a small disposable silica gel column (25 mg). 200 µl first of chloroform and then methanol were added to elute the sample. The combined chloroform-methanol phases were evaporated, dissolved in chloroform, and analysed.
Paper II: In a further development of the method, following the same initial steps as described above, acetylation was accomplished by heating the

preparation in a mixture of acetic anhydride (100 µl) and pyridine (10 µl) at 100°C for 15 min. (Figure 6). The reaction mixture was evaporated, dissolved in 0.5 ml of chloroform, and purified using a disposable silica gel column. The sample was applied, washed with 1 ml of chloroform, and eluted with 0.5 ml of methanol. The methanolic phase was evaporated, reconstituted in chloroform, and analysed.



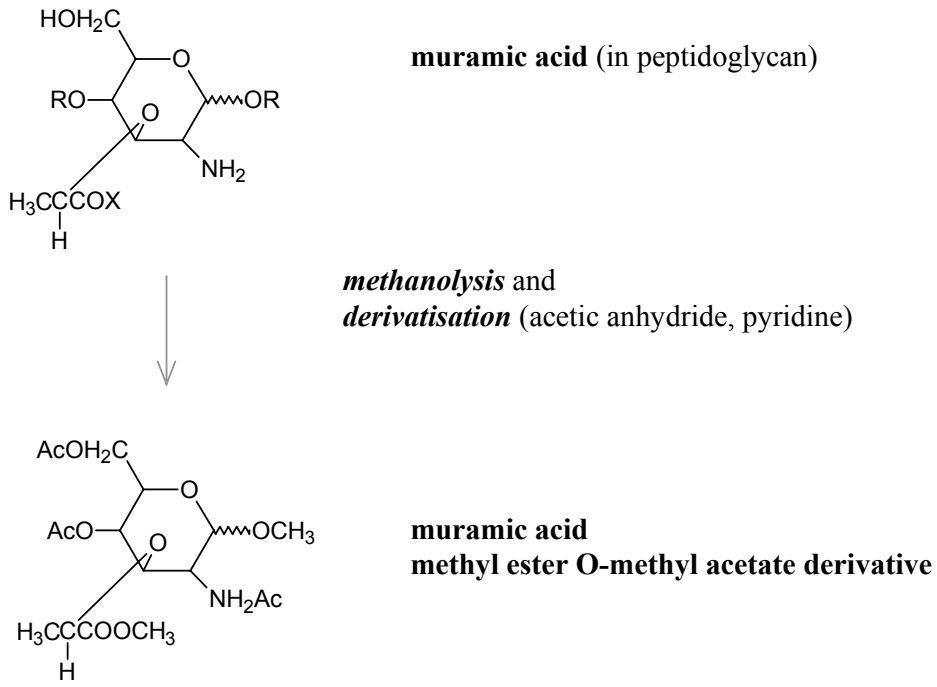
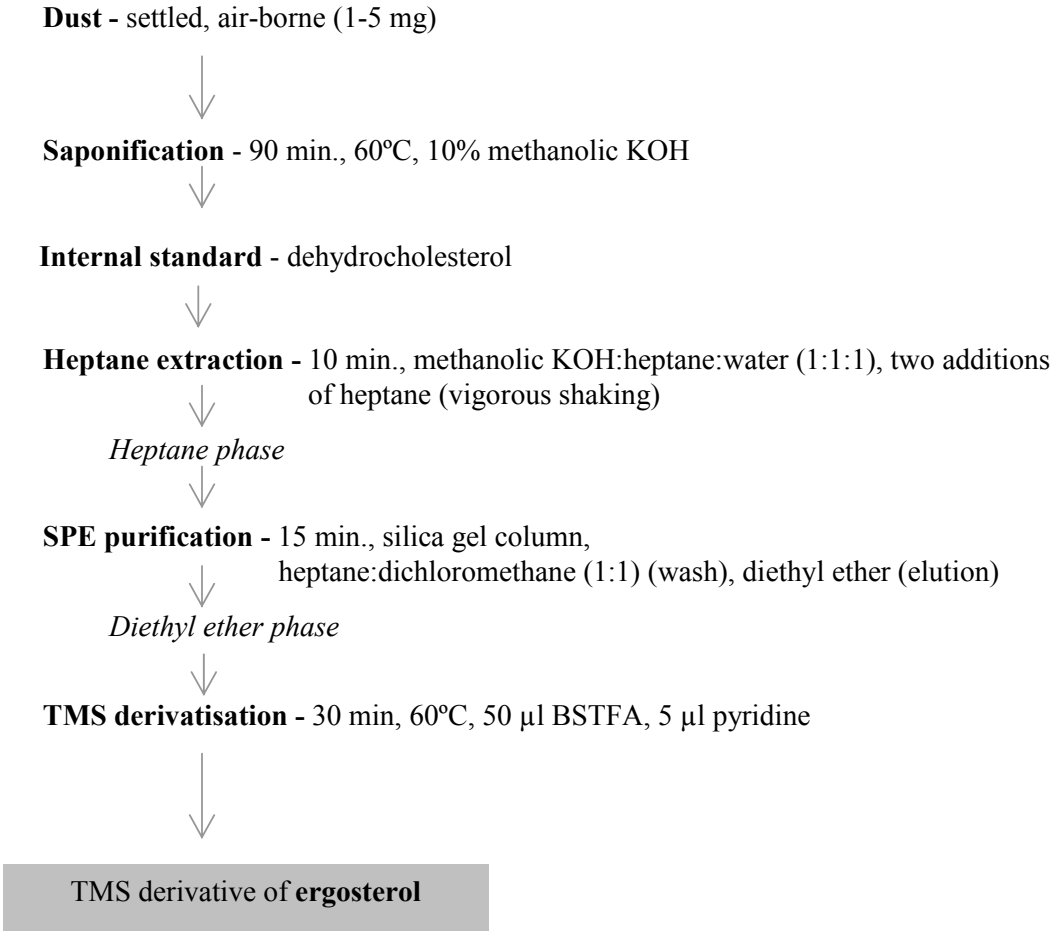


Figure 6. Muramic acid methyl ester O-methyl acetate derivative.

Alditol acetate derivative (**Paper II**)
These derivatives were prepared as described previously (Fox et al. 1995; Krahmer et al. 1998). In brief, filter samples were hydrolysed in 1.5 ml of 2N sulfuric acid for 3 h at 100ºC. ^{13}C-labeled muramic acid was used as the internal standard. Samples were neutralized by mixing with 3 ml N,N-dioctylmethylamine:chloroform. The aqueous phase was passed through a C-18 column and reduced with sodium borohydride. To remove generated borate, evaporation was performed after addition of methanol:acetic acid (200:1). The alditols were acetylated at 100ºC overnight. Acetic anhydride was decomposed with water, chloroform added and after mixing the aqueous phase discarded. Ammonium hydroxide was added and the mixture passed through a Chem Elut column; the chloroform phase was collected. Samples were evaporated and resuspended in chloroform for analysis.

Ergosterol
Samples, in Teflon-lined glass test tubes, were heated in 3 ml of 10% methanolic KOH at 60^{o}C for 90 min. to release ester-bound ergosterol. Then, internal standard was added. Samples were partitioned with 2 ml of heptane:water (1:1), the heptane layer was recovered together with a second ml of heptane added to the reaction mixture. The combined heptane phases containing hydrophobic compounds were evaporated to dryness under a stream

of nitrogen at room temperature. The dried samples were subsequently dissolved in 1 ml of heptane:dichloromethane (1:1) and purified using a disposable silica gel column as described above for the 3-OH FA esters to remove non-polar lipids. The diethyl ether eluate was evaporated and TMS derivatisation was performed by heating in BSTFA (50 µl) and pyridine (5 µl) at 60^{o}C for 30 min. (Figure 7). Heptane was added to each preparation which was then stored overnight at room temperature prior to analysis.



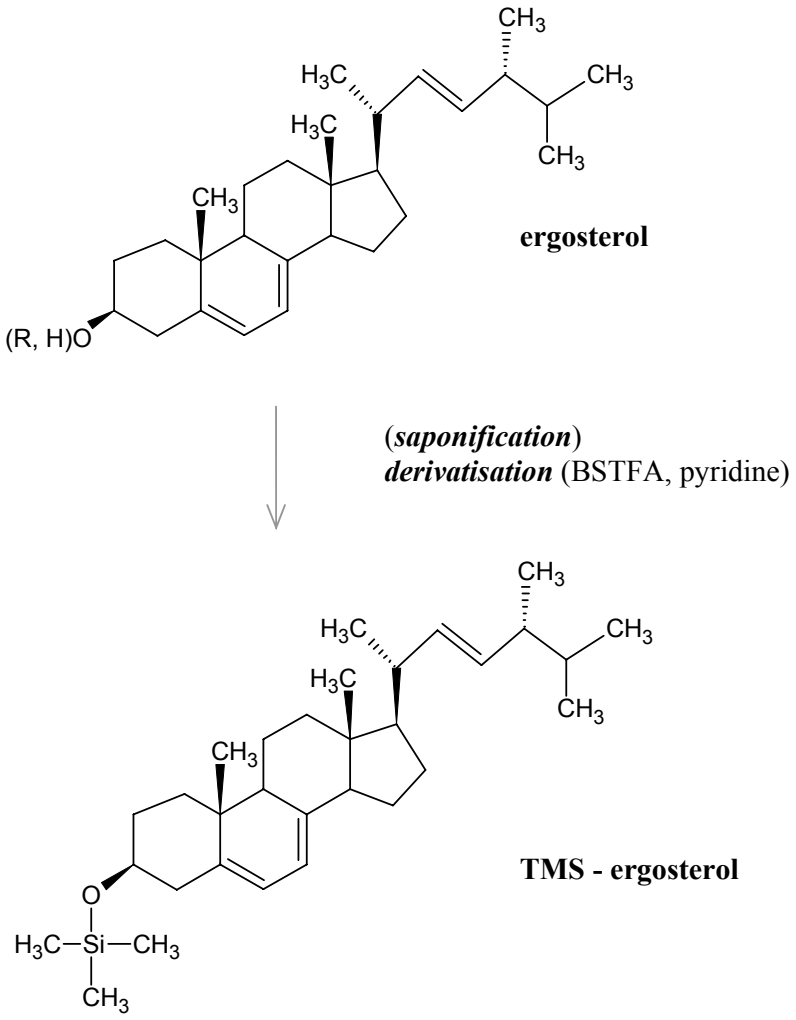


Figure 7. TMS derivatisation of ergosterol.

GC-MS/MS

Equipment

A Saturn 2000 ion-trap GC-MS instrument (Varian) (Figure 8) equipped with a fused-silica capillary column (CP-Sil 8 CB low bleed, 0.25 µm film thickness, 30 m, 0.25 mm i. d.) was used in most of the experiments. Volumes of 1-2 µl of the samples were injected in the splitless mode with helium head column pressure of 69 kPa using a Combi Pal autosampler. The temperature of the column was programmed from 90-280ºC when analysing the fatty acids and muramic acid and from 170-290ºC when analysing ergosterol; the temperature of the injector was 280˚C and that of transfer line (between GC and MS system) was 290˚C. The ion trap temperature varied between 180˚C and 220˚C. All analyses were made in the electron impact (EI) mode.

The chiral (S)-phenylethylamide methoxy 3-OH FA derivatives were analysed by using a column temperature that was programmed from 90-280ºC operating the MS in the selected ion storage mode.

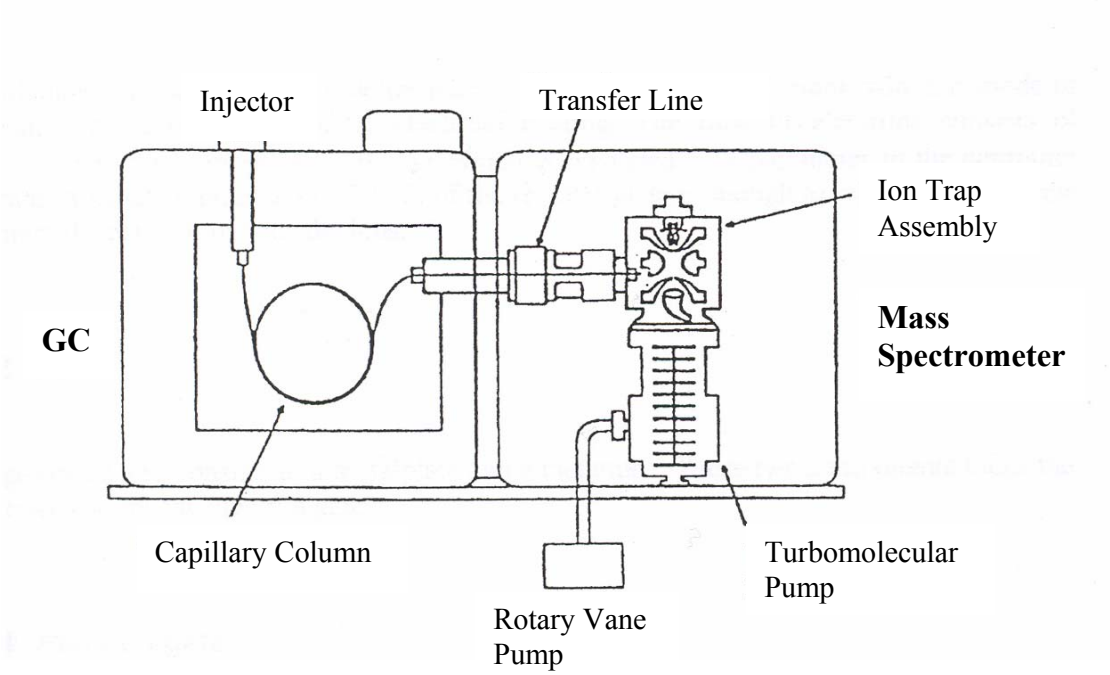


Figure 8. Schematic diagram of ion trap GC-MS.

For analysis of the alditol acetate derivative an ion trap tandem mass spectrometer (GCQ, Finnigan) was employed (Fox et al. 1995; Krahmer et al. 1998). Ionisation was performed in EI mode followed by collision induced dissociation and multiple reaction monitoring. The GC was equipped with a non-polar DB-5MS column.

Monitored ions

Mass spectra of the methyl ester/TMS 3-OH FA derivatives showed abundant ions of *m/z* (M-15), due to loss of a CH_3 group, and *m/z* 175, due to cleavage of C3-C4 linkage. The derivatised acids were measured by monitoring *m/z* 131 (a product ion of *m/z* 175) in GC-MS/MS. The amount (moles) of LPS in each sample was calculated by dividing the number of moles of the 3-OH $C_{10\text{-}16}$ acids by four.

EI spectra of the studied non-hydroxylated branched-chain FA methyl esters showed abundant ions of *m/z* (M-43). The acids were measured by monitoring *m/z* 101 (a product of *m/z* 213 for $C_{15:0}$ fatty acids and a product of *m/z* 241 for $C_{17:0}$ fatty acids).

The fragmentation of the muramic acid derivative precursor ion *m/z* 187 led to high intensity of the product ion *m/z* 145, which was therefore monitored (**Paper**

I). An alternative precursor ion of *m/z* 213 fragmenting to product ion of *m/z* 127 was used in **Paper II**.
The EI mass spectrum of the ergosterol TMS derivative was dominated by ions of *m/z* 363 (M-105, loss of the trimethylsilanol group and one methyl group). The derivative was measured by monitoring product ion of *m/z* 157.

Table 2. Precursor and product ions monitored for chemical markers and internal standards.

Marker	Ion-Trap GC-MS/MS				Paper
	ions fragmented		ions monitored		
	marker	internal standard	marker	internal standard	
3-hydroxy fatty acids	175 *m/z*	178 *m/z*	131 *m/z*	134 *m/z*	I, III and V
	175 *m/z*	175 *m/z*	131 *m/z*	131 *m/z*	III and IV
non-hydroxylated fatty acids	213 *m/z* , 241 *m/z*		101 *m/z*	286 *m/z*	I
muramic acid	187 *m/z*	192 *m/z*	145 *m/z*	150 *m/z*	I and II
	213 *m/z*	221 *m/z*	127 *m/z*	132 *m/z*	II and III
ergosterol	363 *m/z*		157 *m/z*	351+352 *m/z*	I and III

Limulus amebocyte lysate (LAL) assay

In addition to the GC-MS analyses mentioned above, the settled dust samples (approximately 5 mg each) were subjected to analysis for endotoxin activity by using the chromogenic *Limulus* amebocyte lysate (LAL) endpoint assay as described (Saraf et al. 1997).

Additional analyses (Paper V)

To characterize particle sizes over time the average particle concentrations in fifteen size ranges (from 0.3 to >20 μm) were recorded in each studied classroom using an optical particle counter (Grimm Technologies Inc.). The average CO_2 concentration, temperature, and humidity were also recorded.

DISCUSSION OF RESULTS

Microorganisms are ubiquitous in our environment, including indoor air, and do not necessarily constitute a health hazard. The concentration at which contamination becomes a threat to health is unknown, and may actually vary greatly with each individual. Intrusion of water into a building clearly contributes to microbial growth and is associated with adverse health effects. However, there is a lack of epidemiological data to clearly link disease states with the growth of microorganisms in damp buildings. One reason could be the lack of standardized methods to quantify and characterize the microbial flora in indoor environments.

Our investigation has resulted in the development of an integrated chemical-analytical approach to characterise microbial communities in indoor environments. This approach provides information on both viable and non-viable microorganisms in a sample e.g. of air-borne and settled dust or building material (Szponar and Larsson 2001). Chemical marker analysis using GC-MS/MS of 3-OH FAs (endotoxin markers), certain branched-chain FAs (markers of gram-positive bacteria), muramic acid (peptidoglycan marker) and ergosterol (fungal biomass marker) represents a unique approach to the characterization of the microbiology of indoor environments and forms a firm basis for studying relations between our exposure to microorganisms indoors to well-being and health.

Developments in methodology

As mentioned, an integrated procedure was developed for determination of four different types of markers. ***Ergosterol*** used as a fungal biomass marker was analysed as TMS derivative as described (Axelsson et al. 1995) where the parent ion in EI (*m/z* 363) was further fragmented in MS/MS to the monitored product ion of *m/z* 157. ***3-OH FAs*****,** used as LPS markers, were also determined as described (Saraf et al. 1999) as TMS/methyl ester derivatives where product ion *m/z* 131 (following fragmentation of the parent ion *m/z* 175) of acids of 10 – 16 carbon chain lengths was monitored.

Branched-chain non-hydroxylated fatty acids of 15 and 17 carbon chain lengths were introduced as bacterial markers in the present thesis. In the integrated procedure developed these acids appear in the same extract as the 3-OH FA methyl esters, and are separated from the latter by silica gel column chromatography. The mass spectra of straight-chain and *iso*- or *anteiso*-branched C_{15} and C_{17} FAs were very similar, the high-mass regions giving distinctive ions of *m/z* 213 for the $C_{15:0}$ and *m/z* 241 for the $C_{17:0}$ fatty acids (M-43). These ions were therefore selected for fragmentation in MS/MS, which resulted in product ions of *m/z* 101 used for quantification (loss of $CH(CH_3)$-

CH_2-$COOCH_3$) (Zirrolli and Murphy 1993; Gross 1994) of both groups of acids. Not fragmented ion of *m/z* 286 was used for quantification of internal standard.

Muramic acid**,** a peptidoglycan marker, was studied extensively in this thesis mainly for optimizing the MMA derivatisation procedure. In **Paper I,** muramic acid was established as a part of the integrated strategy for chemical marker analysis. In **Paper II,** additional improvements in chemical preparation and GC-MS/MS analysis were made. We refrained from adding water to the methanolysate since it improved the yield of MA and considerably shortened the time required for the evaporation. A decreased amount of pyridine (10 instead of a 100 µl), an increased temperature (from 60 to 100°C), and a shorter reaction time (15 instead of 30 min.) improved the performance of the acetylation and reduced unwanted browning coloration. In addition, we eliminated the post-derivatisation washing steps (with 0.05M HCl solution and distilled water) since they did not affect the results, and we collected separately the MMA derivative in the methanolic elute from the silica gel column instead of collecting the chloroform and methanol elutes together.

The mass spectrum of the MMA derivative exhibits abundant ions of *m/z* 187, *m/z* 213, *m/z* 302, *m/z* 374 and *m/z* 406 (Bal and Larsson 2000), which were all tried as parent ions in MS/MS. Distinctive product ions were only produced by ions of *m/z* 187 (product ion *m/z* 145, **Paper I**) and of *m/z* 213 (product ion *m/z* 127, **Paper II**). The intensity of the latter was approximately 30% higher than of the former resulting in higher detection sensitivity. The MS/MS spectra were dominated by abundant ions of *m/z* 127 (non-labeled muramic acid) and *m/z* 132 (^{13}C-labeled muramic acid). Product ion formation was optimal at an energy where the parent ions disappeared almost completely. MS/MS chromatograms with corresponding mass spectra are shown in Figure 9. Muramic acid appears as a distinct single peak with low background and can be detected in diluted preparations down to 25 pg (injected amount) of the compound.

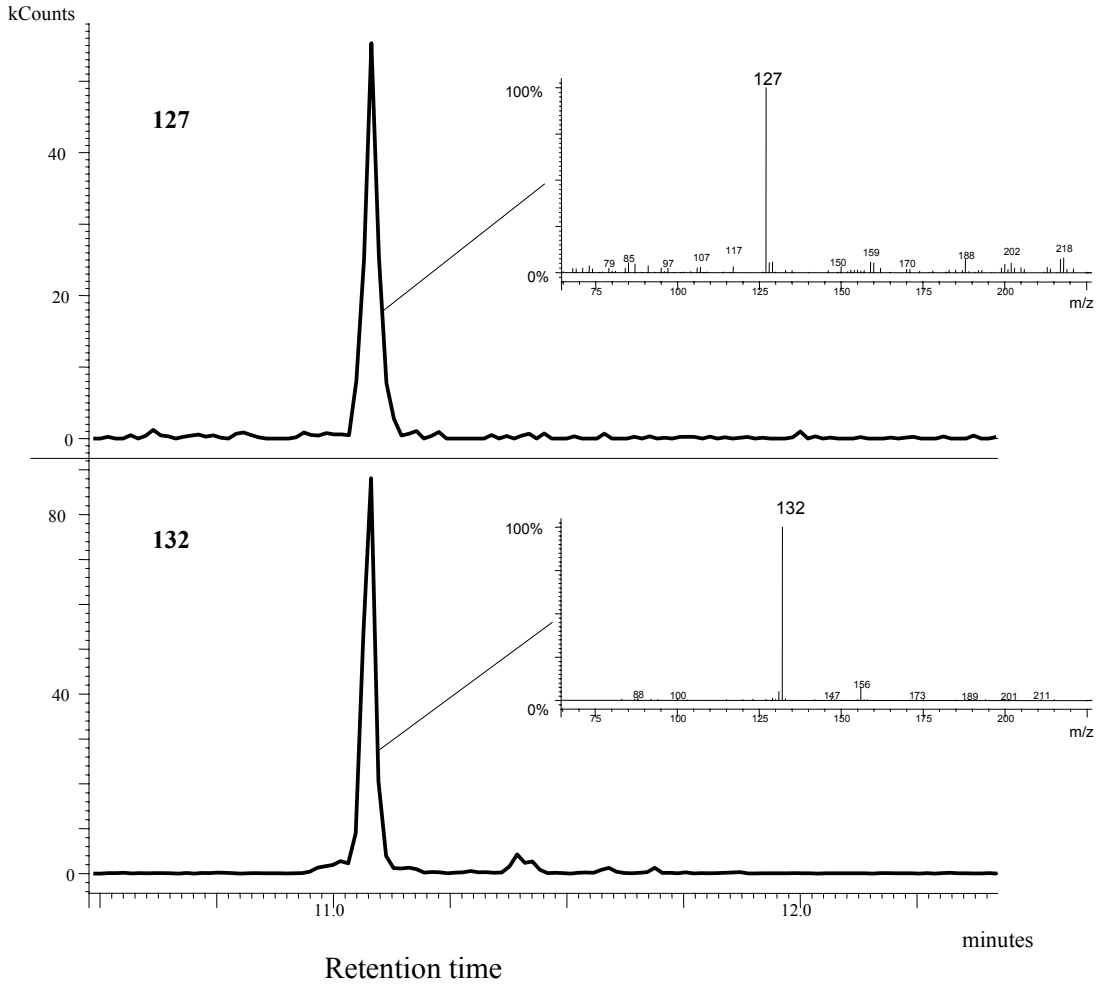


Figure 9. GC-MS/MS chromatograms and MS/MS spectra of muramic acid (MMA derivative) in a settled house dust sample (upper) and in cyanobacteria (lower).

The suggested fragmentation pattern leading to ion *m/z* 213 as elucidated previously (Bal and Larsson 2000) may further lead to *m/z* 127 due to loss of $CH_3CHCOOCH_3$ (Figure 10).

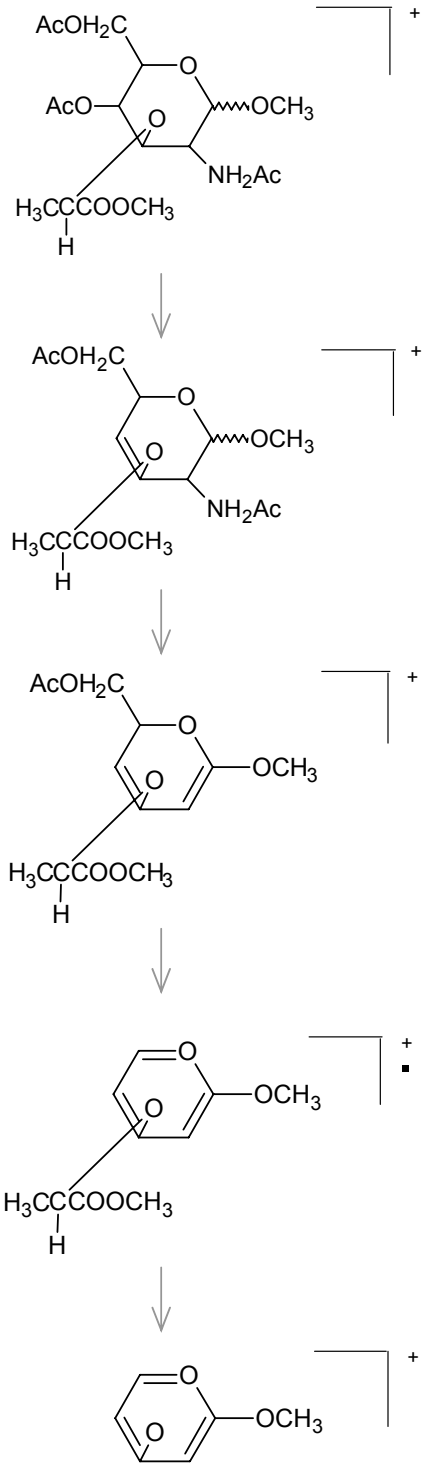


Figure 10. Suggested fragmentation pattern of derivatised bacterial muramic acid.

The MMA derivative offers several advantages. First, it allows 3-OH FAs (markers of endotoxin) and muramic acid (marker of peptidoglycan) to be simultaneously determined in a studied sample since in both cases the first step is methanolysis followed by extractions for separating the 3-OH FA and muramic acid methyl esters (**Paper I**). Second, the derivative is quick to prepare. Sample preparation can be made in a single day. Indeed, we have found that the methanolysis time can be shortened to 4 h without impairing the results allowing derivatisation to be completed in a single working day, although overnight methanolysis is more in accordance with our laboratory routines.

The main disadvantage of the MMA derivative is that, since it is difficult or impossible to prepare it from the pure muramic acid standard (Bal and Larsson 2000), the internal standard must be calibrated against pure muramic acid to achieve quantitative results. We therefore conducted research to relate results of the MMA derivative with results of the alditol acetate derivative. Preparing the latter derivative is much more time-consuming than the MMA derivative but the alditol acetate method has been applied so frequently for determination of muramic acid in both environmental and clinical samples that it should be regarded as a gold standard to which any alternative method should be compared. A calibration graph was constructed ($R^2 = 0.841$) where the ratios of the peak areas of *m/z* 127 and *m/z* 132/mg dust on the filter halves (MMA derivative) were plotted against ng muramic acid/mg dust as calculated by using the alditol acetate method on the parallel filter halves coming from the school study (**Paper V**). These samples were from occupied and unoccupied school rooms where dust was collected by pumping 90 - 230 m^3 air through the filters. Slight differences in detection limits of the two different derivatives were found which may be due to differences in the yield of the derivative, in sample clean-up methods, or in the performance of the two GC columns or GC-MS instruments. It should be noted that the ion-trap instrument (used to analyse the MMA derivative) utilized internal ionisation whereas the ion-trap instrument (used to analyse the alditol acetate derivative) utilized external ionisation.

Internal standards

In quantitative GC-MS it is essential to use proper internal standard(s). The internal standards should be very similar chemically to the given analyte and, of course, not be present in the studied type of samples. Previous work at our laboratory had made use of the pure marker chemicals as internal standards. One major achievement with this thesis work is the introduction of isotopically labeled internal standards, which are easily identified by use of mass spectrometry. In addition, in most cases the labeled internal standards have been part of labeled bacteria, which is advantageous since then the entire sample preparation, thus including the hydrolysis/methanolysis step, is controlled for.

Cyanobacterial ^{13}C-labeled 3-OH $C_{16:0}$, $C_{16:0}$ and muramic acid were introduced as internal standards for 3-OH FAs, non-hydroxylated FAs and muramic acid, respectively (**Paper I, II).** The labeled cyanobacterial cells were found to contain 3-OH $C_{14:0}$ and 3-OH $C_{16:0}$ in an approximately 1:2 proportion, hence 3-OH $C_{16:0}$ (3.3 ng/µg dry mass) was chosen as an internal standard. For non-hydroxylated FAs, $C_{16:0}$ was chosen as the internal standard since this acid is found in suitable amounts (19.1 ng/µg dry mass) in the cyanobacteria and its retention time does not overlap those of the monitored acids. The cyanobacteria contained 4 ng/µg of muramic acid as determined by the alditol acetate method described above. For convenience, probably also resulting in improved reproducibility, methanolysates of the cyanobacterial cells rather than dry, intact cells were added to the sample methanolysates (**Paper I)**. It was possible to adjust the amount of cyanobacterial methanolysate added to each sample to allow for simultaneous, convenient identification and calculation of all three markers.

It was found, however, that the *m/z* 133 ions of the cyanobacterial ^{13}C-labeled 3-OH FAs to a small but noticeable extent also are produced by the sample 3-OH FAs. This led us to investigate alternative internal standards for 3-OH FAs, and ***^{13}C-labeled 3-OH $C_{13:0}$***, in labeled *Pectinatus cerevisiiphilus*, was introduced (**Paper III)**. Indeed, the dominating 3-OH FA in this *Pectinatus* species is 3-OH $C_{13:0}$ (Helander et al. 1994; Helander and Haikara 1995), an acid rarely found in environmental samples and therefore, particularly if labeled, constituting an almost ideal internal standard. The mass spectrum of the non-labeled methyl ester/TMS derivative of this acid exhibited abundant ion of *m/z* 175 (Saraf and Larsson 1996). *P. cerevisiiphilus* was cultivated in medium containing labeled substrates with the aim of producing ^{13}C-labeled 3-OH $C_{13:0}$. We found that our preparation, although not fully incorporating ^{13}C from the culture medium, was labeled up to at least C_3 producing the fragment of *m/z* 178 (corresponding to *m/z* 175 for the non-labeled acids) with no signs of *m/z* 175. The retention times of labeled and non-labeled FAs were virtually identical.

Additional analytical aspects

GC-MS/MS is recommended for achieving optimal analytical performance. In this technique, ions formed initially in the mass spectrometer's ion source are subjected to further fragmentation, and the fragment (daughter) ions are monitored. This leads to a very high degree of detection specificity (Saraf and Larsson 1996; Saraf et al. 1999). However, ion-trap GC-MS-instruments have limited dynamic range and may yield mass spectra that depend upon the concentration of the injected analyte. In the present thesis the temperature of ion trap was also found to strongly affect the detector signal and peak shape. This effect was most evident for the muramic acid MMA derivative (Figure 11).

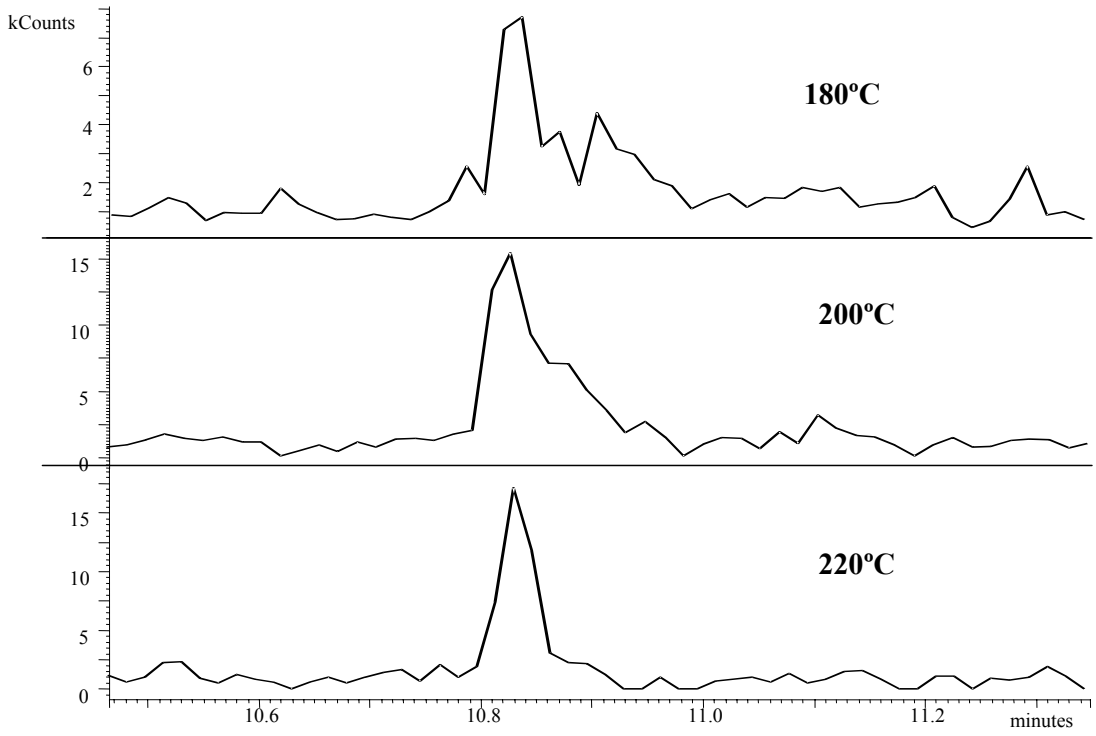


Figure 11. Influence of ion trap temperature on detector signal and peak shape of muramic acid MMA derivative.

Optimal peak shape and enhanced detection sensitivity was achieved at 220˚C for the muramic acid and ergosterol derivatives whereas 180˚C was optimal for the FA derivatives. The mean values and standard deviations of markers/mg dust sample were as follows: LPS 0.026 nmol ± 0.002 (S.D.), *iso*-$C_{15:0}$ 0.125 nmol ± 0.015 (S.D.), *anteiso*-$C_{15:0}$ 0.187 nmol ± 0.018 (S.D.), $C_{15:0}$ 1.588 nmol ± 0.072 (S.D.), *iso*- $C_{17:0}$ 0.128 nmol ± 0.014 (S.D.), *anteiso*- $C_{17:0}$ 0.093 nmol ± 0.009 (S.D.), $C_{17:0}$ 0.695 nmol ± 0.054 (S.D.), muramic acid 22.124 ng ± 0.881(S.D.), and ergosterol 2.115 ng ± 0.154 (S.D.) (**Paper I**).

Markers profiles of different indoor environments

The described integrated chemical marker methodology provides data about the bacterial and fungal biomass as well as patterns of the gram-negative and gram-positive bacterial population regardless whether the microbes are culturable or non-culturable or present as cellular debris. A comparative study comprising five house dust samples collected from a bedroom shelf, a bed, a sitting room floor, a water-damaged garage (shelf) and a basement (shelf) in a single household was performed in the present thesis. All dust samples contained detectable amounts of 3-OH FAs, branched-chain FAs, muramic acid and ergosterol. The biggest differences were found between bed dust and water damaged garage dust. Examples of chromatograms of dust samples collected from the same villa are shown in Figures 12 and 13. The garage dust sample contained much more ergosterol, muramic acid and 3-OH FAs than the bed dust sample, but had notably less of the non-hydroxylated FAs. This suggests that the garage microflora was dominated by fungi and gram-negative bacteria. The bed dust sample contained larger relative amounts of the straight-chain $C_{15:0}$ and $C_{17:0}$ acids compared to the branched-chain acids than the garage dust sample (Figure 12), and 3-OH $C_{16:0}$ dominated more over the other 3-OH FAs in bed dust than in garage dust.

These results clearly indicate differences in bacterial populations from different locations. The observed marker levels in the presented study are in general agreement with those found in other studies (Saraf et al. 1997; Saraf et al. 1999; Fox et al. 2003; Nilsson et al. 2004; Wady et al. 2004). Previous work has revealed that the relative amounts of muramic acid and 3-OH FAs in air-borne dust are different in damp houses as compared with non-damp houses (Nilsson et al. 2004), that the 3-OH FA patterns of settled dust in aircraft cabins, homes, and offices are distinct (Hines et al. 2003), and that the marker compositions of settled dust in class rooms of schools in different geographical regions differ (Wady et al. 2004). Air concentrations of ergosterol and 3-OH FAs were found to increase in a building upon water intrusion, the concentrations then slowly decreased during drying (Larsson and Fredborn Larsson 2001). Clearly, applying the novel integrated procedure rather than the more limited approach used in the studies mentioned above would increase the amount of obtained information.

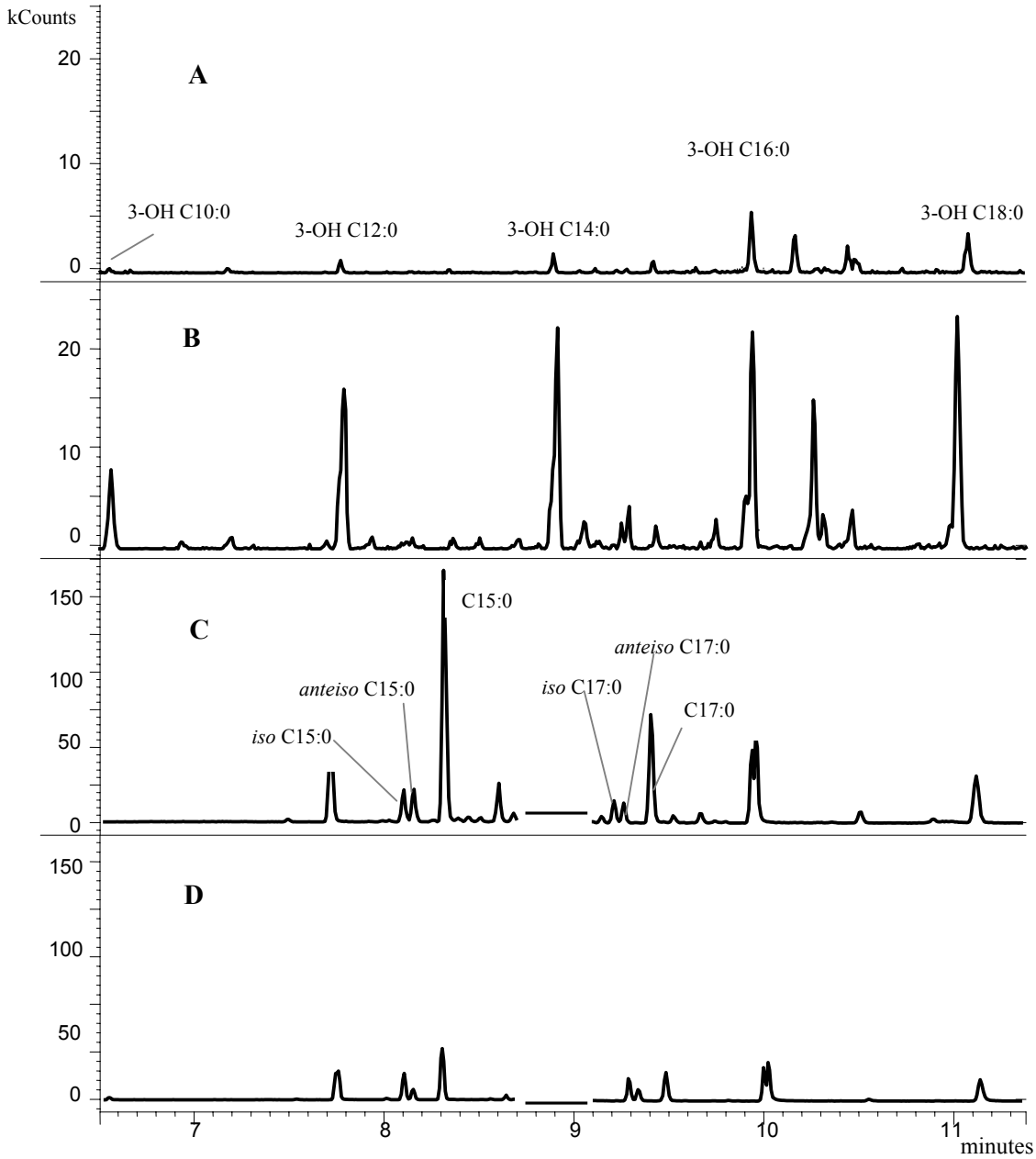


Figure 12. 3-Hydroxy fatty acid profiles of dust samples from a bed (A) and a garage (B); non-hydroxylated fatty acid profiles of dust samples from the bed (C) and the garage (D).

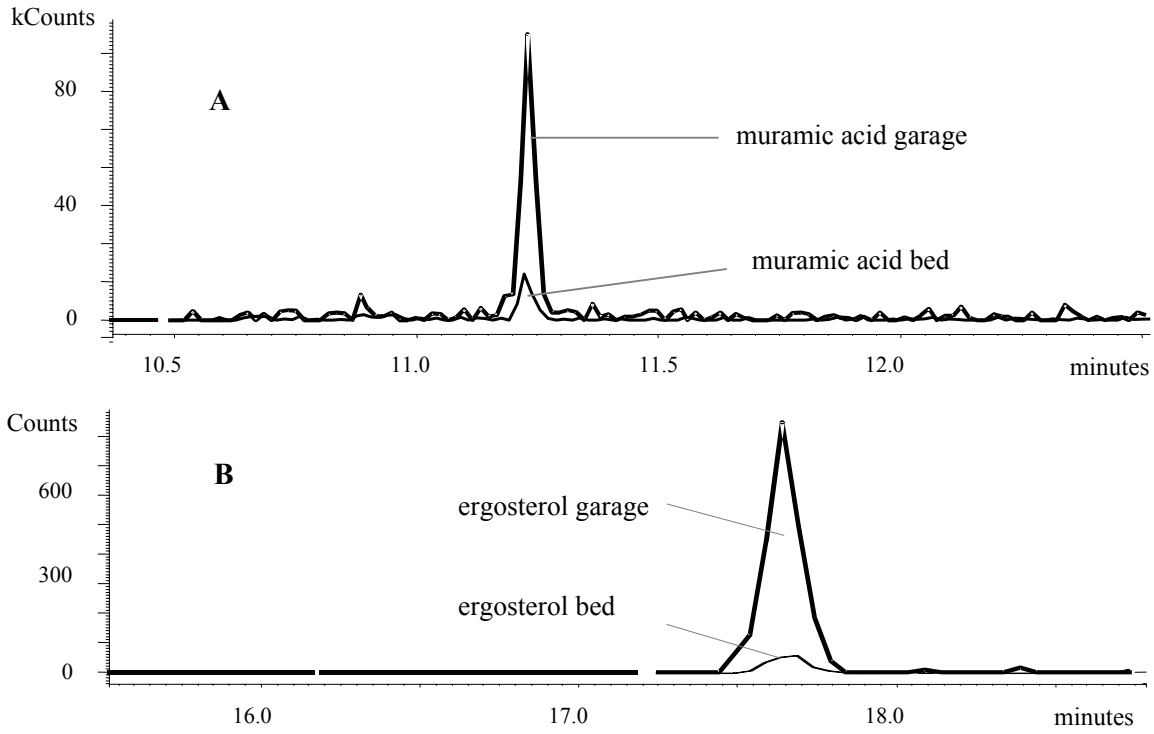


Figure 13. Muramic acid profiles of dust samples from a bed and a garage (A) and ergosterol profiles of dust samples from the bed and the garage (B).

Levels of ergosterol in dust have been found to correlate with cultivable fungi (Saraf et al. 1997) and 3-OH FA levels with endotoxin as determined by *Limulus* methods, particularly when only 3-OH FAs of 10, 12, and 14 (and sometimes also 16) carbon chain lengths are considered (Saraf et al. 1997; Saraf et al. 1999). Thus, the correlation between LPS and endotoxin bioactivity decreases when the longer 3-OH FAs (of 16 and - particularly - 18 carbon chain lengths) are included in LPS calculation. These 3-OH FAs may to an appreciable extent originate from *Actinobacteria* rather than from gram-negative bacterial LPS. All of 17 studied actinobacterial strains contained 3-OH FAs that ranged from 14 to 22 carbon chain lengths (**Paper III**). Most of the strains contained 3-OH FAs with 16 or more carbon atoms; the shortest 3-OH FA detected, 3-OH $C_{14:0}$, was found only in five strains, and in comparatively small amounts. Additionally, chiral separation of 3-OH FAs of *Rhodococcus rhodochrous*, *Gordonia terrae*, *Nocardiopsis dassonvillei*, and *Streptomyces* sp. revealed that all of them were of (R)-configuration. These results suggest that only the shorter 3-OH FAs should be used as LPS markers and that the longer 3-OH FAs rather may represent indicators of *Actinobacteria*.

Impact of tobacco smoke on marker patterns

Presence of tobacco smoke in a studied room was found to have a marked effect on the 3-OH FA pattern. The air concentrations of 3-OH $C_{14:0}$ were significantly higher, by 4 – 63 times ($p = 0.0008$), in rooms of smokers in comparison with "paired" rooms of non-smokers (Figure 14). In the absence of tobacco smoke, analysis of airborne dust revealed only very small amounts of 3-OH FAs. These results are in agreement with previous work performed at our laboratory (Larsson et al. 2004) demonstrating the presence of large amounts of 3-OH $C_{14:0}$, of R-configuration (used as an LPS marker (Rietschel 1976)), in tobacco and cigarette smoke in laboratory experiments. In this thesis study it was also found that the correlation between numbers of cigarettes smoked and amounts of air-borne 3-OH $C_{14:0}$ was significant. However, we did not find any differences in endotoxin levels in settled dust from the floor, and from surfaces above the floor, in rooms of smokers' v non-smokers'. This work shows that cigarette smoke can be a major source of endotoxin in indoor air. Since tobacco endotoxin appears not to adsorb well on settled house dust, clearly, air-borne - and not settled - house dust should be analyzed when monitoring tobacco-generated endotoxin and 3-OH FA analysis is a direct and very useful method.

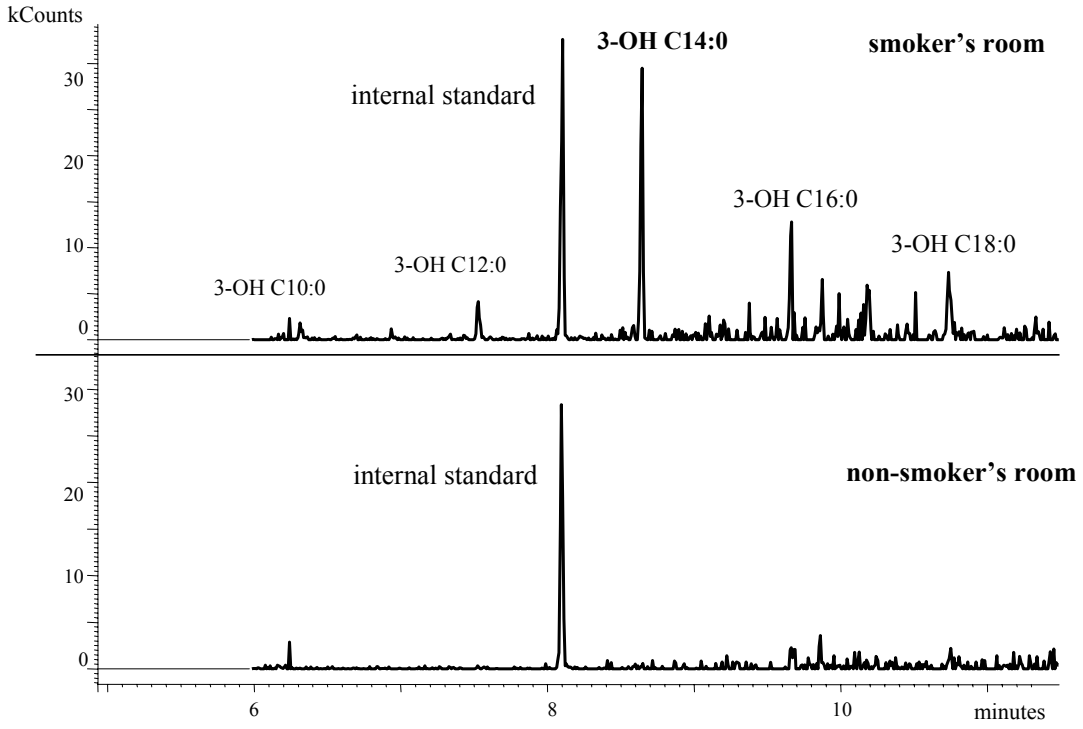


Figure 14. Patterns of 3-hydroxy fatty acids (endotoxin markers) of air-borne dust in a room of a smoker and a non-smoker. 3-OH $C_{14:0}$ strongly dominates the pattern in the smoker's room, followed by 3-OH $C_{16:0,}$ 3-OH $C_{18:0,}$ 3-OH $C_{12:0}$ and 3-OH $C_{10:0}$; only minute amounts of 3-OH FAs were detected in the room of the non-smoker.

This study confirms that the endotoxic activity of tobacco smoke as demonstrated by Hasday et al. (Hasday et al. 1999) *de facto* stems from bacterial LPS. Exposure to environmental tobacco smoke (ETS) ("passive smoking") has been associated with the development of a range of respiratory diseases (Vineis et al. 2005). It is known that exposure to ETS can trigger asthma attacks at susceptible individuals (Simoni et al. 2003; Tatum and Shapiro 2005). These results are important since they may partly explain why e.g. non-smoking individuals that are exposed to high concentrations of endotoxin frequently develop symptoms similar to smokers.

Elevated amounts of muramic acid in occupied school rooms

In schools, where young children are present in crowded groups, exposure to airborne bacteria may result in serious health problems including infections, allergies, and respiratory irritation. Several studies have shown elevated levels of culturable microbes in the air of school rooms (Liu et al. 2000; Scheff et al. 2000; Meklin et al. 2002). However, the sources of culturable and non-culturable bacteria (almost 90% of total load of bacteria) in school room air have not been well characterized. Here, the effects of occupancy on airborne contaminants in classrooms were examined in three schools. In all schools, the average LPS concentration in dust (pmol LPS/mg dust) was one-fourth higher in occupied rooms than in unoccupied rooms. As percentages of total airborne LPS in dust, the average levels of $C_{12:0}$ and $C_{14:0}$ 3-OH FAs were consistently higher in occupied than in unoccupied classrooms, and the average levels of 3-OH $C_{16:0}$ were consistently lower in the occupied classrooms (results statistically significant). The concentration of muramic acid in airborne dust (pmol muramic acid/mg dust) in occupied rooms, on average, was seven times higher than the concentration in unoccupied rooms. This suggests that airborne dust sampled from occupied rooms was distinct from airborne dust when rooms were unoccupied, and may have been predominantly from different sources. By contrast, concentrations of LPS in dust were only slightly changed by occupancy. This suggests a substantial increase in the gram-positive bacterial population during occupancy, but only slight increases in the gram-negative bacteria. These results are in general agreement with results published previously by us (Fox et al. 2003).

The mass concentrations of particles with a diameter > 2 μm were found to be several orders of magnitude higher in occupied rooms than in unoccupied rooms and accounted for most of the airborne particulate mass. Individual bacterial cells generally vary from 0.2 to 2 μm in diameter. Thus, dust particles with diameters greater than 0.8 μm, which were found at high concentrations in the 12 rooms when children were present, may well contain bacteria, bacterial aggregates, or bacteria attached to flaking skin or other tissue shed in occupied rooms. Unlike concentrations of larger particles, those in the lower size ranges

(diameter < 0.65 μm) were higher during unoccupied periods. As anticipated, CO_2 concentrations were lowest in unoccupied rooms. The higher bacterial marker concentrations in occupied classrooms strongly suggest that increased airborne dust concentration and elevated bacteria load, like higher CO_2 levels, is caused by the presence of children. These results are in agreement with those of a more limited study by Jansen (Janssen et al. 1997) focusing on dust concentration.

Sampling

It is not yet known which type of house dust samples that are preferred in terms of providing health-relevant chemical marker data. Sampling of air-borne dust by using pumping is usually very time-consuming (and noisy); electrostatic sampling may represent a viable alternative (Nilsson et al. 2004). However, in any case this type of air sampling is less suitable for application in epidemiological studies that may well include up to a hundred or more samples. Floor dust (usually collected by a vacuum cleaner) may contain material that has never been airborne. Interestingly, however, while Dales et al. (Dales et al. 1999) could not associate levels of ergosterol in air-borne dust in bedrooms with respiratory symptoms, Dharmage et al. (Dharmage et al. 2001) was able to identify ergosterol in floor dust as a risk factor for wheeze among adults. Indeed, various house dust sampling methods may have different implications and it may be necessary to collect both airborne and settled dust samples. Sometimes semi-quantitative dampness/mould exposure estimates based on visual and olfactory observations may predict building-related respiratory symptoms and diseases (Park et al. 2004). Significant monitoring of microbial populations to assess IAQ and health risks is also held back by lack of accurate exposure estimates.

In this thesis work we aimed to standardize a sampling method for dust using natural sedimentation on horizontally placed plexiglass plates (0.5 x 0.5 m) (**Paper III**). The amounts of the dust (2.7 – 90.4 mg) that, after five weeks of sedimentation, were found on plates situated at different distances above the floor were in all cases sufficient for analysis. The dust concentrations of ergosterol, muramic acid and LPS varied between 0.22 – 8.88 ng/mg, 6.90 – 18.67 ng/mg, and 0.0183 – 0.0668 nmol/mg, respectively. No significant differences were found between the dust concentrations of any of the markers in relation to how the plates were distributed in a studied room. There were, however, clear differences in marker compositions between dusts collected in a similar fashion in different rooms indicating heterogeneity in microbial composition. Thus, this type of controlled dust sedimentation represents a reproducible and cost-effective way of sampling that does not depend upon the spatial distribution of the plates in a given room and does not involve

disturbance of the indoor environment as opposed to dust collection using high capacity pumps. A similar dust collecting device was also used by Wurtz et al. (Wurtz et al. 2005); however, the health relevance of chemical marker data of dusts collected this way has not been evaluated.

CONCLUSIONS

- The developed integrated strategy for analysing 3-hydroxy fatty acids, branched-chain fatty acids, muramic acid and ergosterol by GC-MS/MS is useful for characterising bacteriological and fungal profiles of environmental samples including both culturable and non-culturable microbes.

- The integrated method allows microbial communities in different environments to be distinguished.

- The methyl ester O-methyl acetate derivative of muramic acid is practical, quick to prepare, and useful for application in complex matrices such as organic dust.

- Cigarette smoke is a major source of air-borne endotoxin in indoor environments. Cigarette smoke endotoxin does not adsorb on settled dust.

- Dust concentrations of muramic acid are much higher in occupied than unoccupied rooms suggesting that gram-positive bacteria in indoor environments largely stem from room occupancy.

Populärvetenskaplig sammanfattning på svenska

Bakterier har funnits på jorden långt (miljontals år) innan det fanns människor. Människans evolution har alltså pågått under ett ständigt tryck av bakterier, och mycket tyder på att dessa naturligt förekommande bakterier - och andra mikroorganismer - påverkar oss mer, och delvis på ett annat sätt, än vi tidigare trott. Vi vet alla att infektioner kan spridas genom inandning av luftburna levande mikroorganismer. Även döda mikrober innehåller ämnen som kan vara giftiga för människan. Men bakterier innehåller dessutom ämnen som kan stimulera vårt immunförsvar, vilket kan verka hälsofrämjande. Det är till och med så att ett och samma bakteriella ämne kan vara både skadligt och nyttigt för oss, troligen beroende på när i livet vi utsätts för dem och i vilka mängder. Vi vet inte särskilt mycket om hur mikroorganismerna i miljön – särskilt inomhusmiljön - påverkar vår hälsa. Detta beror till en del på att det idag inte existerar några bra och allmänt vedertagna metoder för mätning av luftburna mikroorganismer inomhus. Denna avhandling handlar just om utveckling av reproducerbara mätmetoder för bakterier och svampar och är den senaste i en rad avhandlingar och rapporter från vår forskargrupp. Genom att mäta specifika mikrobiella markörsubstanser såsom 3-hydroxyfettsyror (markörer för endotoxin), muraminsyra (markör för peptidoglykan) samt ergosterol (markör för svamp) är det möjligt att karakterisera vår exponering mot mikroorganismer i miljön. Med denna bestämningsmetod påvisas både döda och levande mikrober; i inomhusmiljöer dominerar vanligtvis de förra. Analyserna sker med en analytisk-kemisk metodik som kallas gaskromatografi-masspektrometri. I avhandlingen utvecklades en integrerad metod för bestämning av ovan nämnda markörsubstanser, och när denna metod tillämpades visades att den mikrobiella sammansättningen av små dammpartiklar tydligt skiljer sig åt beroende på den (inomhus)miljö varifrån provet togs. Särskilt visade det sig att luftkoncentrationen av endotoxin var flera tiotals högre i studentrum bebodda av rökare än i likadana studentrum bebodda av icke-rökare; dessa resultat är i dessa dagar intressanta bl a för design av - till exempel - restaurangers rökrum. Ett annat slående resultat var att den mikrobiella sammansättning i skolsalar skilde sig markant åt beroende på om salarna var tomma eller om elever var närvarande: Bakterietätheten i ”bebodda” skolsalar var flera gånger högre än i tomma skolsalar. Dessa resultat kan komma att ge en fingervisning om rekommenderad elevtäthet i skolsalar. Sammanlagt kan sägas att denna metodinriktade avhandling utgör ett avstamp för kommande epidemiologiska studier rörande hälsokonsekvenser av vår exponering mot mikroorganismer, särskilt i inomhusmiljöer.

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