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Microbial Communities in Drinking Water Systems Analysed by Lipid Biomarkers

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Microbial communities in drinking water systems analysed by lipid biomarkers

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ACADEMIC DISSERTATION

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ABSTRACT

Microbial growth in drinking water systems evokes quality, health, aesthetic, and technical problems. The conventional analyses of microbes in drinking water systems include determination of microbial biomass, activity or specific microbes. Unfortunately, culturing techniques reveal only 0.01-10% of the microbes actually present in drinking water systems. Lipid biomarkers *e.g.* phospholipid fatty acids (PLFAs) and lipopolysaccharide hydroxy fatty acids (LPS OH-FAs) provide quantitative insight to the complex microbial communities and their amounts in environmental samples without the need for microbial culturing. PLFAs can also be used to monitor differences in the physiological state of microbes, such as the stationary growth phase of microbes. Microbes change their fatty acid composition with changing environmental conditions. However, thus far only limited use of lipid biomarkers for microbial community studies in drinking water systems has been made.

In this work, the applicability of lipid biomarkers to characterise microbial communities in drinking water systems was studied both with water and biofilms samples. Soil and sediment samples were used as environmental reference material. In the analyses, lipids were first extracted, fractionated, PLFAs were methyl esterfied, and analysed by gas chromatography- mass spectrometry (GC-MS) using selected ion monitoring. 2- and 3-hydroxy fatty acid methyl esters were prepared by mild acid hydrolysis directly from the extraction residue of lipids without further purification or derivatisation, and analysed by GC-MS using selected ion monitoring. The ions monitored were m/z 90 and M-59 for 2-OH-FAs and m/z 103 for 3-OH-FAs.

Water and biofilm samples revealed a wide range of (from 21 to 26) PLFAs. Most of the PLFAs were typical for microbial biomass or gram-negative bacteria (16:0, 18:0, 16:1ω7, 18:1ω7). In the biofilms, 3 or 16 LPS 3-OH-FAs were found, whereas in drinking and warm waters 3 LPS 3-OH-FAs were detected. The most abundant 3-OH-FAs were typical of those present in gram-negative bacteria (*i.e.* 3-OH-10:0, -12:0, -14:0, -16:0, -18:0). Any LPS 2-OH-FAs were never detected in either biofilms or water samples.

There were differences in the microbial community structures between biofilms and waters, and drinking water and warm water. In the laboratory experiment, addition of 1, 2 or 5 μg l^{-1} phosphate phosphorus to water increased the proportion of gram-negative bacteria and changed their community structure as judged by the PLFAs and 3-OH-FAs, respectively. There also were differences in microbial communities between two full-scale distribution systems. The microbial community was more complex in those biofilms which had a development time of 6 weeks compared to those growing for 23 or 40 weeks in the full-scale distribution system A. In full-scale distribution systems, microbial biomass, as assessed by the quantitative amount of PLFAs, increased with increasing water residence time.

These laboratory and full-scale studies demonstrated that lipid biomarkers are a sensitive method to analyse microbial communities and viable biomass in drinking water systems.

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TIIVISTELMÄ (ABSTRACT IN FINNISH)

Mikrobien kasvu talousvesijärjestelmissä aiheuttaa laatu-, terveys-, esteettisiä ja teknisiä ongelmia. Perinteisesti talousvesijärjestelmien mikrobeja on analysoitu määrittämällä mikrobibiomassaa, aktiivisuutta tai jotain tiettyä mikrobia. Viljelymenetelmillä saadaan esiin vain 0.01-10% mikrobeista, jotka todellisuudessa esiintyvät talousvesijärjestelmissä. Lipidibiomarkkereilla, kuten fosfolipidien rasvahapoilla (PLFAs) ja lipopolysakkaridien hydroksihapoilla (LPS OH-FAs) saadaan kvantitatiivista tietoa monimutkaisista mikrobiyhteisöistä ja niiden biomassasta ympäristönäytteissä ilman mikrobien viljelemistä. PLFA:ta voidaan käyttää myös mikrobien fysiologisen tilan erojen havaitsemiseen, kuten mikrobien stationäärikasvuvaihe. Mikrobit muuttavat rasvahappokoostumustaan muuttuvissa ympäristöolosuhteissa. Tähän asti lipidibiomarkkereita on kuitenkin käytetty rajoitetusti mikrobiyhteisöjen tutkimiseen talousvesijärjestelmissä.

Tässä työssä lipidibiomarkkerien käyttökelpoisuutta talousvesijärjestelmien mikrobiyhteisöjen kuvaajina tutkittiin sekä vesi- että biofilminäytteillä. Maa- ja sedimenttinäytteitä käytettiin ympäristövertailunäytteinä. Analyysissä lipidit ensin uutettiin, fraktioitiin, PLFA:t metyyliesteröitiin ja analysoitiin kaasukromatografia-massaspektometrialla käyttäen selektiivistä ionien monitorointia. 2- ja 3-hydroksirasvahappojen metyyliesterit valmistettiin miedolla happamalla hydrolyysillä suoraan lipidien uuttojäänteestä ilman jatkopuhdistuksia tai kemiallisten johdosten valmistusta ja analysoitiin kaasukromatografia-massaspektometrialla käyttäen selektiivistä ionien monitorointia. 2-hydroksirasvahappojen analyysissä seurattiin ioneja m/z 90 ja M-59 ja 3-hydroksirasvahappojen ionia m/z 103.

Vesi- ja biofilminäytteistä löydettiin laaja määrä (21-26 kpl) fosfolipidien rasvahappoja. Suurin osa PLFA:sta oli tyypillisiä mikrobibiomassalle tai gram-negatiiviselle bakteereille (16:0, 18:0, 16:1ω7, 18:1ω7). Biofilmeissä havaittiin 3 tai 16 LPS 3-OH-rasvahappoa, kun taas kylmästä talousvedestä ja lämpimästä vedestä havaittiin kolme LPS 3-hydroksirasvahappoa. Suurin osa 3-OH-rasvahapoista oli tyypillisiä gram-negatiivisille bakteereille (3-OH-10:0, -12:0, -14:0, -16:0, -18:0). LPS 2-OH-rasvahappoja ei havaittu biofilmi- eikä vesinäytteissä.

Biofilmien ja vesien välillä sekä kylmän ja lämpimän talousveden mikrobiyhteisöissä havaittiin eroja. Laboratoriokokeessa 1, 2 tai 5 µg l^{-1} fosfaattifosforin lisäys lisäsi gram-negatiivisten bakteerien osuutta ja muutti niiden sisäistä yhteisörakennetta PLFA:n ja LPS 3-OH- rasvahappojen perusteella. Myös kahden täyden mittakaavan talousvesiverkostojen mikrobiyhteisöt erosivat. Mikrobiyhteisö oli monimuotoisempi biofilmeissä, jotka olivat kehittyneet 6 viikkoa verrattuna 23 tai 40 viikkoa kehittyneisiin biofilmeihin täyden mittakaavan talousvesiverkostossa A. Täyden mittakaavan talousvesiverkostoissa mikrobibiomassa arvioituna PLFA:n kvantitatiivisesta määrästä lisääntyi, kun veden ikä kasvoi.

Laboratorio- ja täyden mittakaavan tutkimukset osoittivat, että lipidibiomarkkerit ovat herkkä menetelmä mikrobiyhteisöjen ja elävän biomassan määrittämiseen talousvesijärjestelmissä.

To my Mum

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Minna Keinänen

Kuopio, October 2003 Minna Keinänen

ABBREVIATIONS

AOC	assimilable organic carbon
AODC	acridine orange direct count
ATP	adenosine triphosphate
CFU	colony forming units
$CHCl_3$	chloroform
CTC	5-cyano-2, 3-ditolyl tetrazolium chloride
DGGE	denaturating gradient gel electrophoresis
DNA	deoxyribonucleic acid
EPS	extracellular polymeric substances
FAME	fatty acid methyl ester
FISH	fluorescent *in situ* hybridisation
GC	gas chromatography
HGR	maximum number of heterotrophic bacteria during the 21 days incubation of water
INT	2-(*p*-iodo-phenyl)-3-(*p*-nitrophenyl)-*s*-phenyl tetrazolium chloride
LPS	lipopolysaccharide
m/z	mass/charge
MAP	microbially available phosphorus
MeOH	methanol
MIDI	microbial identification with whole cell fatty acids
MS	mass spectrometry
OH-FA	hydroxy fatty acid
PE	polyethene
PLFA	phospholipid fatty acid
PVC	polyvinyl chloride
RNA	ribonucleic acid
SIM	selected ion monitoring
TOC	total organic carbon

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following four original articles, which are referred to in the text by the Roman numerals.

I **Keinänen M. M.**, Korhonen L. K., Martikainen P. J., Vartiainen T., Miettinen I. T., Lehtola M. J., Nenonen K., Pajunen H., and Kontro M. H. 2003. Gas chromatographic-mass spectrometric detection of 2- and 3-hydroxy fatty acids as methyl esters from soil, sediment and biofilm. Journal of Chromatography B 783:443-451.

II **Keinänen M. M.**, Korhonen L. K., Lehtola M. J., Miettinen I. T., Martikainen P. J., Vartiainen T., and Suutari M. H. 2002. The microbial community structure of drinking water biofilms can be affected by phosphorus availability. Applied and Environmental Microbiology 68(1):434-439.

III **Keinänen M. M**., Martikainen P. J., Korhonen L. K., and Suutari M.H. 2003. Microbial community structure in biofilms and water of a drinking water distribution system determined by lipid biomarkers. Water Science and Technology 47(1):143-147.

IV **Keinänen M.M.**, Martikainen P.J., Kontro M.H. 200X. Microbial community structure and bomass in developing drinking water biofilms, submitted.

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1 INTRODUCTION

Bacterial growth in aquatic systems *e.g.* in drinking water distribution systems, is often associated to biofilms (Laurent et al., 1993; van der Wende et al., 1989). Biofilms are a microbial life form where microbes aggregate and growth on a surface (for a review, see Flemming, 2002; O'Toole et al., 2000; Watnick and Kolter, 2000). Most micro-organisms can form biofilms, in fact most of micro-organisms on Earth are living in biofilms (for a review, see Flemming, 2002; Watnick and Kolter, 2000). Biofilms occur at solid-liquid, solid-air, and liquid-air interfaces (for a review, see Flemming, 2002). Biofilms colonise soils, sediments, mineral and plant surfaces. They are involved in biogeochemical pathways of basic elements, self-purification processes in Nature, in drinking water treatment, and in wastewater treatment (for a review, see Flemming, 2002; Tolker-Nielsen and Molin, 2002). In natural environments, biofilms are complex, highly differentiated multicultural communities (for a review, see Tolker-Nielsen and Molin, 2002; Watnick and Kolter, 2000).

The microbes present in drinking water systems are generally characterised by analysing the biomass or the activity of micro-organisms. Another way would be to analyse lipid biomarkers *e.g.* phospholipid fatty acids (PLFAs) and hydroxy fatty acids (OH-FAs), which have provided quantitative and qualitative insight into the complex microbial world present in environmental samples (for a review, see White et al., 1996; Zelles, 1999). However, there are only a few studies, which have examined lipid biomarkers from drinking water systems.

In drinking water systems, microbial life is dependent on many only partly understood factors, such as differences in water treatment practices, disinfection, temperature, pipe materials, nutrients, hydraulic conditions.

In this study, microbial communities in drinking water systems were analysed using lipid biomarkers *i.e.* phospholipid fatty acids and lipopolysaccharide 2- and 3-hydroxy fatty acids. Soil and sediment samples were used as reference materials.

2 REVIEW OF THE LITERATURE

2.1 Biofilms

2.1.1 Formation

During biofilm formation, microbes first migrate towards an interface, become loosely attached to the surface, then migrate over its surface to form a microcolony, grow and finally produce exopolysaccharides to form a three dimensional structure containing 95% water (for a review, see O'Toole et al., 2000; Stoodley et al., 2002; Watnick and Kolter, 2000). Occasionally, biofilm-associated microbes become detached from the surface. Biofilm formation is dependent on many factors, such as genotypic and physico-chemical factors, mechanical processes, temporal changes in the biotic and abiotic environments, import and export of materials, initial colonisation, and interactions between microbes (for a review, see Wimpenny et al., 2000). The formation process of biofilms is relatively rapid. Biofilms in drinking water system have achieved a stationary phase in their growth within 3 weeks to 4 months (Block et al., 1993; Volk and LeChevallier, 1999; Zacheus et al., 2000). The stationary phase with negligible microbial growth is due to the densely settled area surrounded by exopolysaccharides or growth and death of microbes, and attachment and detachment are in balance (for a review, see Watnick and Kolter, 2000).

Different genes are transcribed in the planktonic and biofilm-associated phases of the bacterial life cycle (for a review, see O'Toole et al., 2000; Stoodley et al., 2002; Watnick and Kolter, 2000). For example, the transcription of 38% of the genes was different during the biofilm development, compared to planktonic phase of *Escherichia coli* (Prigent-Combaret et al., 1999). Natural conjugative plasmids express factors that induce planktonic bacteria to form and enter biofilm communities (Ghigo, 2001). On the other hand, some genes are expressed in response to the specific surface on which microbes have chosen to settle (for a review, see O'Toole et al., 2000; Watnick and Kolter, 2000). In biofilms, bacteria communicate with each other using intercellular signalling using molecules, such as bacterial metabolites, acylhomoserine lactones, secreted proteins and genetic material. These signals might alter the distribution of microbes in the biofilms, change protein expression, introduce new genetic traits into neighbouring cells or lure and incorporate other microbes into the structure (for a review, see Stoodley et al., 2002; Watnick and Kolter, 2000).

2.1.2 Structure

From 1980's into the 1990's, biofilms were considered simply as having a flat two dimensional structure, with a relatively constant thickness. There has been a major change in respective of the structure of these communities. The current image of microbial biofilms in aquatic systems is a mushroom or tulip model with the stalk narrower than the upper surface parts, the whole being penetrated by channels allowing the transportation of water, nutrients and metabolites (Fig. 1). The structures resemble a heterogeneous mosaic. Biofilm structures have been studied with many techniques *e.g.* microscopy such as traditional light, transmission electron, scanning electron, atomic force and confocal laser scanning microscopy, microelectrodes, molecular methods such as 16S or 23S rRNA directed probes and fluorescent *in situ* hybridisation (FISH), even mathematical, computer-based modelling (for a review, see Wimpenny et al., 2000).

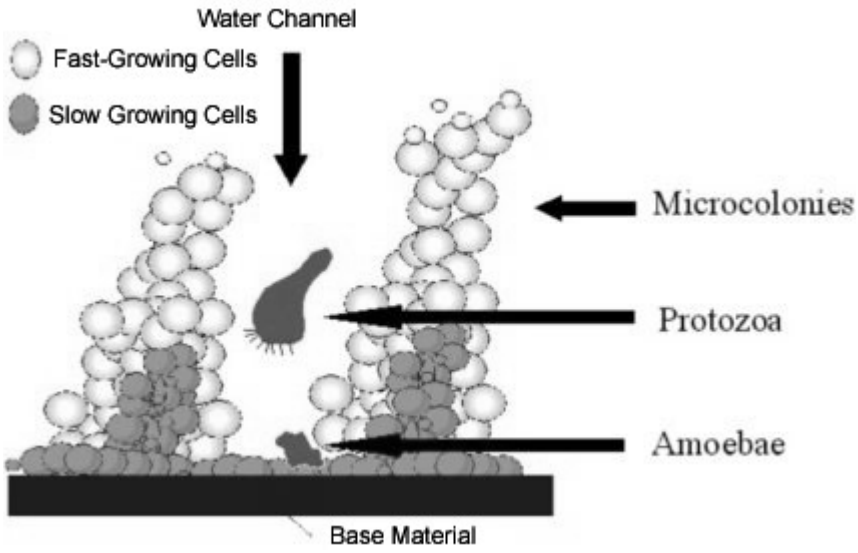

Fig 1. Open architecture structure of biofilm in a drinking water system (printed with kind permission from C.W. Keevil).

2.1.3 Ecological advantages of biofilms

Biofilms offer many ecological advantages for microbes. Biofilms act as a protective barrier against toxic substances such as antibiotics, disinfection chemicals and detergents, enable high cell densities, accumulate nutrients and pollutants, and retain water (for a review, see Costerton et al., 1987; Flemming, 2002). Extracellular polymeric substances provide mechanical stability, permit the development of microconsortia, concentration gradients, the retention of extracellular enzymes, the convective mass transport through channels, easy horizontal gene transfer, a matrix for exchange of

signalling molecules and light transmission into the deeper layers of the biofilm structure (for a review, see Flemming, 2002).

2.1.4 Biofouling

Biofouling is the term for undesired development of microbial layers on surfaces. Usually biofouling is caused by heterotrophic organisms, which convert dissolved organic matter into biomass (for a review, see Flemming, 2002). In many industries, *e.g.* food processing, power generation, pulp and paper industry, chemical process industry and drinking water systems, biofilms create problems for hygiene and cleaning, as well as being responsible for energy losses, blockages in systems and corrosion (for a review, see Mattila-Sandholm and Wirtanen, 1992; Videla, 2001). In drinking water systems, various microbes, including actinomycetes, cyanobacteria and fungi, produce earthy tastes and odours in the water (for a review, see Wood et al., 2001). Biofilms generally contaminate dental unit waterlines (for a review, see Barbeau et al., 1996; Walker et al., 2000), and are contributors to many persistent and chronical bacterial infections contracted from medical devices (for a review, see Costerton et al., 1987; Costerton et al., 1999; Donlan and Costerton, 2002; O'Toole et al., 2000).

2.2 Microbes in drinking water systems

The strict quality requirements demand the absence of classical pathogens, such as *Vibrio cholera*, *Salmonella typhi* and *Shigella* spp. In recent years, emerging pathogens have evoken novel challenges for drinking water systems. The emerging pathogens originate from fecal sources, such as *Campylobacter jejuni*, pathogenic strains of *Escherichia coli*, *Yersinia enterocolitica*, rotavirus, Norwalk-like viruses (calicivirus), small round-structured viruses, astrovirus, *Giardia lamblia*, *Cryptosporidium parvum* and microsporidia (for a review, see Szewzyk et al., 2000). In Northern America, coliform bacteria were present in 1.4% of 115 000 samples from 31 water systems, whereas in Finland 19% of 47 drinking water samples from 6 water supplies were positive for coliforms (Lahti, 1993; LeChevallier et al., 1996). In Korea, infectious viruses have been detected in 65% of drinking water samples (Lee and Kim, 2002). In Finland, fourteen waterborne epidemics occurred during the period 1998-1999. Thirteen of these epidemics were associated with undisinfected groundwaters, which were contaminated because of floods and surface runoffs. Norwalk-like viruses caused eight and *Campylobacter* three of the outbreaks (Miettinen et al., 2001).

Environmental bacteria that are able to grow in distribution systems, such as *Legionella* spp., *Aeromonas* spp., *Mycobacterium* spp. and *Pseudomonas aeruginosa* are now recognised as potentially important pathogens (*e.g.*, for a review, see Szewzyk et al., 2000). *Legionella pneumophila* was isolated from 30% of water samples taken from the distribution systems of buildings in Finland (Zacheus and Martikainen, 1994). When samples of hospital water systems were taken in USA, Canada and Great Britain, 12 to 70% were colonised with *Legionella* (Lin et al., 1998). *Mycobacterium spp.* was isolated from 5 to 32% samples of drinking water and from 17 to 100% of biofilms (Falkinham III et al., 2001; von Reyn et al., 1993). In drinking water and biofilms, *Acinetobacter*, *Algaligenes*, *Arthrobacter*/*Corynebacterium*, *Bacillus*, *Flavobacterium*, *Methylobacterium*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, and *Sphingomonas* have been the predominant genera detected (Koskinen et al., 2000; Lahti, 1993; LeChevallier et al., 1987; Payment et al., 1988; Penna et al., 2002; Percival et al., 1999; Rogers et al., 1994). Nitrifying bacteria, especially ammonia-oxidising *Nitrosomonas* and nitrite-oxidising *Nitrospira* species are common in those distributions systems where chloramine is used as the disinfection chemical (Lipponen et al., 2002; Regan et al., 2002; Regan et al., 2003; Wolfe et al., 1990). Filamentous fungi and microfungi are also frequently been present in distribution systems (Lahti, 1993; Nagy and Olson, 1982).

2.3 Factors affecting the microbial growth in drinking water systems

Micro-organisms face a great diversity of habitats with very different physicochemical and nutritional conditions during the treatment, storage and distribution of drinking water.

2.3.1 Nutrients

Organic substances in drinking water originate from the raw water and from materials such as pipe material, lubricants and sealing. In well-functioning waterworks, the distributed drinking water is generally low in organic carbon content. Therefore, microbial growth in distribution systems is often carbon limited (Chandy and Angles, 2001; Frias et al., 2001; van der Kooij, 1992; LeChevallier et al., 1991; Szewzyk et al., 2000). Heterotrophic microbial growth in drinking water has been limited when microbially available assimilable organic carbon (AOC) concentrations have been less than 10 µg acetate-C eg. l^{-1} (van der Kooij, 1992). In the boreal climate, raw water sources have a high content of organic matter originating from humic substances of peatlands and forests (Kortelainen, 1993). In this kind of drinking water, microbial growth is regulated by the availability of phosphorus, not by the organic matter (Lehtola et al., 2002b; Miettinen et al., 1996b; Miettinen et al., 1997b; Sathasivan and

Ohgaki, 1999; Sathasivan et al., 1997). In these waters a very minor increase in the phosphorus concentration can greatly enhance microbial growth, both in water and biofilms (Lehtola et al., 2001; Lehtola et al., 2002a; Miettinen et al., 1997b; Sathasivan et al., 1997). Lehtola et al. (1999) developed a sensitive bioassay for the determination of microbially available phosphorus (MAP) in water. MAP concentrations in Finnish drinking waters are generally low, from 0.06 to 10.2 µg PO_4-P l^{-1} (Lehtola et al., 1999; Lehtola et al., 2002b). However, even such low concentrations correlated positively with microbial growth in drinking waters (Lehtola et al., 2002b). In contrast, addition of phosphate has been proposed as an anticorrosion treatment, decreasing the bacterial adhesion to corrosion products and bacterial concentration in water (Appenzeller et al., 2001; Appenzeller et al., 2002). In North America, the use of phosphate based corrosion inhibitors was found to be associated with lower coliform levels in drinking water (LeChevallier et al., 1996). However, the application of phosphate containing products, such as corrosion inhibitors and additives of plastic materials need to be considered cautiously in phosphate limited drinking waters (Szewzyk et al., 2000).

2.3.2 Water purification

Water treatment techniques aims to eliminating and inactivating potential pathogens, and removing as many compounds as possible from the raw water to fullfill the requirements of consumers and the authorities (for a review, see Szewzyk et al., 2000). In most countries, raw water purification consists of a variety of treatment processes, such as chemical coagulation, filtration and disinfection (Fig. 2).

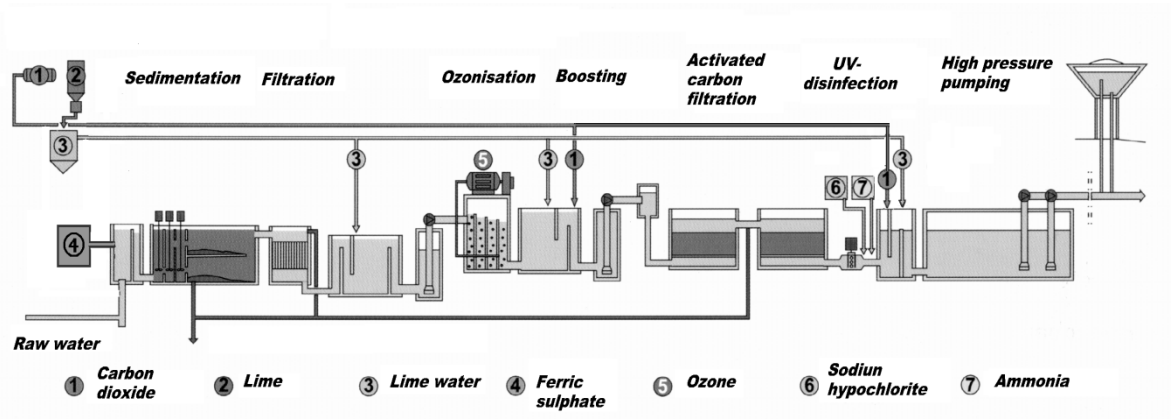

Fig. 2. Example of a water purification process (Pitkäkoski Water Treatment Plant, Helsinki) (printed with kind permission from Helsinki Water).

The aim of chemical coagulation is to produce particles of a size that can be removed by settlement, flotation or filtration. The effect of coagulation is dependent on pH, the type of coagulant and its dose, as well as the water characteristics. The typical coagulant chemicals are metal salts, such as iron and aluminium salts (Binnie et al., 2002; Tchobanoglous and Schroeder, 1987). Coagulation of Finnish drinking waters has resulted in decreased amounts of total organic carbon (TOC), AOC, total P, MAP, total bacteria, CFU, maximum number of heterotrophic bacteria during the 21 days incubation of water (HGR) but increased the HGR when there was addition of 20 µg l^{-1} phosphorus (Lehtola et al., 2002b).

In filtration, water passes through a granular bed of sand or some other suitable medium at low speed. Slow sand filters operate at low loading rates, whereas rapid gravity filters work at higher loading rates. The slow sand filters treat water by physical straining and biological action, whereas rapid gravity filters act by physical treatment only (Binnie et al., 2002; Tchobanoglous and Schroeder, 1987). The effectiveness of filtration is dependent on the size of the media, its particle size, the density of particles and the temperature (Binnie et al., 2002; Tchobanoglous and Schroeder, 1987; Urfer et al., 1997). The particle attachment to the filter media needs electrically neutral conditions, which can be achieved with coagulation (Binnie et al., 2002; Tchobanoglous and Schroeder, 1987). Filtration can also be used as a pre-treatment of raw water before coagulation, such as the production of artificially recharged groundwater from lake water using bankfiltration. Bankfiltration has shown to decrease TOC by 40-70%, chemical oxygen demand by 47%, non-purgable organic matter by 70%, AOC by 40%, total phosphorus to levels below the detection limit, and MAP by 67% (Lehtola et al., 2002b; Miettinen et al., 1994; Miettinen et al., 1996a; Miettinen et al., 1997a). In addition, the counts of heterotrophic bacteria, total bacteria, and bacterial enzymatic activities have decreased significantly during bankfiltration.

Disinfection of water is used to eliminate all pathogens that have passed through the various treatment processes and on the other hand, to guarantee microbially safe water through the distribution system to the point of use. The generally used disinfection methods are chlorination, ozonation and UV disinfection (Binnie et al., 2002; Tchobanoglous and Schroeder, 1987). In chlorination, carcinogenic organochlorine compounds can be formed (for a review, see Horth, 1989). Chloramines are better than free chlorine for many reasons, such as decreased coliform concentrations, decreased heterotrophic plate counts and disinfection by-products, and improved maintanence of a disinfection residue (Norton and LeChevallier, 1997). Disinfection resistance against free chlorine is increased by biofilm formation, the age of biofilm, encapsulation and nutrient effects. In contrast, disinfection efficiency of monochloramine has only been decreased because of biofilm formation (LeChevallier et al., 1988).

However, a chlorine residue of up to 0.9 mg l^{-1} has been found to be ineffective against resistant micro-organisms, such as sporulating bacteria and viruses (Payment, 1999). Ozonation has been shown to remove pathogenic microbes, taste and odor. On the other hand, as a strong oxidizing agent, ozone can decrease TOC, but it increases the AOC and MAP, which can promote microbial growth in distribution systems (Lehtola et al., 2002b; Zacheus et al., 2000). Biologically stable ground water can be distributed without disinfection if there is effective removal of nutrients during the water purification process (Hambsch, 1999; van der Kooij et al., 1999).

Biofilm development in a distribution system has promoted decay of chloramine (Chandy and Angles, 2001). Several mechanisms have been proposed to explain the resistance of microbes in biofilms against biocides. Extracellular polymers associated with biofilms prevent the penetration of biocides, protecting the microbes in the biofilm. Altered rates of bacterial growth dictate the response to antimicrobial agents. The microenvironment of the biofilm adversely affects the activity of antimicrobial substances. Induction of stress responses and development of biofilm-specific biocide resistant phenotype may contribute to biocide resistance (for a review, see Dunne, 2002; Mah and O'Toole, 2001; Morton et al., 1998). Bacterial responses to biocides are dependent on the nature of the biocide and the type of organism involved (Flemming, 2002).

2.3.3 Distribution systems

The chemistry and composition of drinking water vary enormously in different distribution systems. The concentration of disinfection residues diminishes, whereas other factors such as temperature, nutrient levels, ion composition, oxygen concentration, pipe material and hydraulic conditions may change in drinking water distribution systems (Block et al., 1993; van der Kooij et al., 1995; Niquette et al., 2000; Pedersen, 1990; Percival et al., 1999; Rogers et al., 1994; Szewzyk et al., 2000; Volk and LeChevallier, 1999; Zacheus et al., 2000). Temperature is thought to be the most important factor controlling microbial growth in drinking water, and it affects directly or indirectly a wide array of chemical and physical properties (LeChevallier et al., 1996). The coliform bacteria were detected in higher frequencies in 31 full-scale drinking water systems in North America, when the water temperature was over 15°C, as well as when the disinfectant residue was low, the AOC level was high, the corrosion rate was high, the rainfall in the area was high, the distribution systems contained many storage tanks, and the systems were not flushed annually (LeChevallier et al., 1996). Thus, an integrated approach, including design of surface materials, monitoring and analysis of deposits, and

biofilm management and engineering is needed, if one is to achieve a successful and a sustainable antifouling strategy (for a review, see Flemming, 2002).

2.4 Methods to analyse microbes in drinking water systems

The methods used to analyse microbes in drinking water systems are summarised in Table 1.

The viable count procedure is the most commonly used method to measure microbes in drinking water. The detached biofilm cells are plated onto a solid microbial medium, incubated and counted (for a review see, Donlan and Costerton, 2002). These cultivation methods have been applied in drinking water systems to judge related health risks, not the total cell numbers. Indicator organisms are used to indicate the possible presence of pathogens, as they originate from the same fecal contamination sources as pathogens. Heterotrophic plate counts have become the standard technique used in microbiological water quality testing. Unfortunately, culturable microbes represent only a small proportion (0.01-10%) of the total microbial cells in drinking water systems, and standard methods greatly underestimate the number and diversity of the micro-organisms present (for a review, see Amann et al., 1995; Szewzyk et al., 2000). However, in drinking water biofilms, all of the microbes were culturable after 13 days, and later as many as 43-65% were culturable, which might indicate that the steady state for all bacteria was not reached in 70 days of biofilm formation (Lehtola et al., 2002a). The culturability of microbes has been shown to decrease with succession, due to selection of organisms that allocate a greater proportion of energy resources into maintenance relative to growth (Garland et al., 2001).

Nucleic acid stains, such as DAPI (4’,6’-diamidino-2-phenylindole), acridine orange and SYTO9 stain the DNA and RNA of all cells regardless of their viability and can be used to estimate the total microbial biomass (Donlan and Costerton, 2002; Hobbie et al., 1977; Schwartz et al., 1998). The content of adenosine triphosphate (ATP) can be used to measure metabolically active biomass (Boe-Hansen et al., 2002; Hallam et al., 2001; van der Kooij et al., 1995; Lehtola et al., 2002a). The exoproteolytic activity of bacteria in drinking water biofilm has been shown to be proportional to the bacterial biomass (Laurent and Servais, 1995). The incorporation of the amino acid, leucine, has been used to estimate the rate of protein synthesis as a measure of the bacterial growth (Boe-Hansen et al., 2002; Butterfield et al., 2002). New molecular tools in combination with advanced microscopic techniques have also provided information on the activity and physiological status of individual cells in the biofilms (for a review, see Tolker-Nielsen and Molin, 2000; Wimpenny et al., 2000).

Tetrazolium salts, such as 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC), and 2-(*p*-iodo-phenyl)-3-(*p*-nitrophenyl)-*s*-phenyl tetrazolium chloride (INT), have been used to measure dehydrogenase activity, which is a component of microbial respiration (Fonseca et al., 2001; Kalmbach et al., 1997; Rodriguez et al., 1992; Schaule et al., 1993; Schwartz et al., 1998; Servais et al., 1992). *In situ* hybridisation allows the detection of specific nucleic acid sequences in eucaryotic and prokaryotic cells via the binding of oligonucleotide probes to their complementary target sequences (Kalmbach et al., 1997; Manz et al., 1993; Schwartz et al., 1998; Szewzyk et al., 2000). Denaturating gradient gel electrophoresis (DGGE) of 16S RNA has been used to provide a qualitative analysis of microbial community structure (Fonseca et al., 2001; for a review, see Muyzer and Smalla, 1998). The BIOLOG system detects metabolism of sole carbon sources, and has been found to be suitable for characterising microbial communities in biofilters (Moll and Summers, 1999; Moll et al., 1998). The phospholipid fatty acids (Chang et al., 2001; Fonseca et al., 2001; Herb et al., 1995; Jain et al., 1997; Moll and Summers, 1999; Moll et al., 1998; Moll et al., 1999; Smith et al., 2000) and MIDI-FAME techniques (Glucksman et al., 2000; Massol-Deyá et al., 1995; Moll et al., 1999; Norton and LeChevallier, 2000) in drinking water systems have been used to study microbes in ground water, in biofilters, in corroded surfaces in water resorvoirs, and impact of chlorine exposure.

Table 1. Methods that have been used to analyse microbes in drinking water systems.

Method	Parameter	Study subject	References
culturing	culturable biomass	widely used	Percival et al., 2000; Szewzyk et al., 2000
microscopy with nucleic acid stains	total biomass	widely used	Donlan and Costerton, 2002; Hobbie et al., 1977; Schwartz et al., 1998
ATP	metabolically active biomass	drinking water, biofilms	Boe-Hansen et al., 2002; Hallam et al., 2001; van der Kooij et al., 1995; Lehtola et al., 2002a
potential exoproteolytic activity	bacterial biomass	drinking water biofilms	Laurent and Servais, 1995
leucine incorporation	bacterial growth	drinking water, biofilms	Boe-Hansen et al., 2002; Butterfield et al., 2002
dehydrogenase activity (CTC, INT)	actively respiring bacteria	drinking water, biofilms	Kalmbach et al., 1997; Schaule et al., 1993; Schwartz et al., 1998
		ground water	Rodriquez et al., 1992
		biofilters	Fonseca et al., 2001; Servais et al., 1992
in situ hybridization (16S and 23S RNA)	phylogenic diversity	drinking water, biofilms	Kalmbach et al., 1997; Manz et al., 1993; Schwartz et al., 1998
DGGE	microbial community	biofilters	Fonseca et al., 2001
DNA fingerprinting	microbial community	biofilters	Moll et al., 1998
BIOLOG	metabolic capability, microbial community	biofilters	Moll and Summers, 1999; Moll et al., 1998
PLFA	viable microbial community and biomass, physiological state	corroded surfaces in water resorvoirs	Herb et al., 1995
		biofilters	Fonseca et al., 2001; Moll and Summers, 1999; Moll et al., 1998; Moll et al., 1999
		ground water	Chang et al., 2001; Jain et al., 1997
		biofilms in laboratory and full-scale	Smith et al., 2000; I-IV
		drinking water	II, III
MIDI	microbial community	ground water	Glucksman et al., 2000
		biofilters	Massol-Deya et al., 1995; Moll et al., 1999
		drinking water, biofilms	Norton and LeChevallier, 2000

Appreviations: ATP, adenosine triphosphate; BIOLOG, metabolism of sole carbon sources; CTC, 5-cyano-2, 3-ditolyl tetrazolium chloride; DGGE, denaturating gradient gel electrophoresis; INT, 2-(*p*-iodo-phenyl)-3-(*p*-nitrophenyl)-*s*-phenyl tetrazolium chloride; PLFA, phospholipid fatty acids; MIDI microbial identification with fatty acids

2.5 Lipid biomarkers

2.5.1 Microbial lipids

Lipids are classified as compounds which are weakly soluble in water, but readily soluble in organic solvents (Christie, 1989; Ratledge and Wilkinson, 1988a). Lipids act as storage materials in microbes, are responsible for the structure of cell membranes mainly as phospholipids, participate in the organisation of bacterial cell envelopes, and are associated with photosynthetic processes. Microbes synthesise many of these lipid cell components rapidly in respond to changes in their environment (Ratledge and Wilkinson, 1988a).

The structures of the common lipids are divided into two categories; those containing long-chain fatty acids or their immediate derivatives, and structures derived from isoprene units and known as terpenoid lipids. Lipids can also be classified as neutral or polar lipids. Neutral lipids include lipids in which the hydrophilic function has relatively little impact on the overall molecular characteristics. Neutral lipids include simple hydrocarbons, carotenes, triacylglycerols, wax esters, sterol esters, fatty acids, polyprenols and sterols. Polar lipids contain a polar head group, which has a major influence on the solubility characteristics. Polar lipids include phospholipids, glycolipids, sulpholipids, some sphingolipids, oxygenated carotenoids and chlorophylls (Ratledge and Wilkinson, 1988a). Phospholipids are glycerophospholipids or sphingophospholipids. In glycerophospholipids, an apolar phosphatidyl group is attached via ester-linked fatty acids in the sn-1 and sn-2 positions (Fig. 3). Esterification with several mono- and polyhydroxy compounds gives rise to a family of phosphodiesters which are structurally important microbial lipids. Sphingophospolipids are phosphoric esters based on ceramide 1-phosphatases (Ratledge and Wilkinson, 1988b).

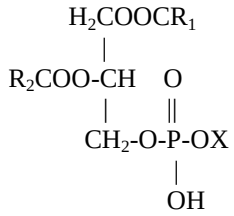

Fig. 3. Basic chemical structure of glycerophospholipids base on phosphatic acid. R_1CO and R_2CO are fatty acyl groups. Group x contains a variaty of compounds such as inositol, serine or ethanolamine.

Lipopolysaccharides (LPS) are structures of the cell wall in gram-negative bacteria (Smith, 1988; Wilkinson, 1988). The lipopolysaccharide consists of a tripartitate structure, in which an endotoxic O-specific, antigenically dominant polymeric side chain is attached to the amphiphilic lipid A via a common oligosaccharide containing hydroxy fatty acids (Wilkinson, 1988).

2.5.2 Fatty acids

Fatty acids are organic substances, which are linked to variety of molecules, most commonly to glycerol (Ratledge and Wilkinson, 1988b). Fatty acids are synthesised in nature via condensation of malonyl-coenzyme A units by a fatty acid synthetase complex (Christie, 1989). The carbon chain lengths of PL fatty acids in microbes commonly range between 14 and 20. Fatty acids may be saturated, unsaturated, straight-chain, branched or contain alicyclic rings. Furthermore, the fatty acids can contain a second oxygen-containing functional group besides the carboxyl group, such as hydroxyl group in hydroxy fatty acids (Ratledge and Wilkinson, 1988b). The hydroxyl group can be linked to the first carbon atom next to the carboxyl group (2-OH- or α-OH-FAs), or the second carbon atom (3-OH- or β-OH-FAs) or to the third carbon carbon (4-OH- or ω-OH-FAs). Organisms having extractable lipids rich in branched-chain acids are expected to produce lipopolysaccharide fatty acids with the same characteristics (Wilkinson, 1988). Fatty acids can be named with systematic or trivial names, or with a shorthand designation, e.g. decanoic acid, capric acid, 10:0 (Ratledge and Wilkinson, 1988b). Examples for chemical structures of fatty acids and 2- and 3-hydroxy fatty acids are presented in Fig. 4. Fatty acids have been widely used to characterise monocultural microbial species (for a review see “Microbial lipids, volume 1, edited by Ratledge C. and Wilkinson S.G.”).

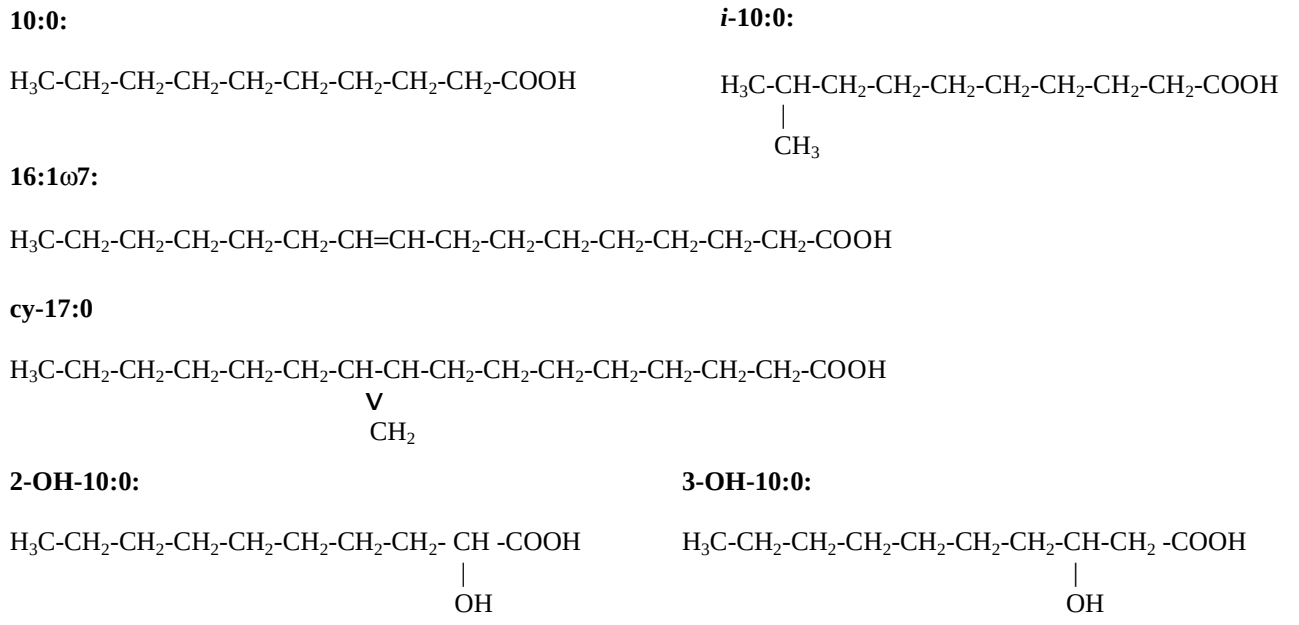

Fig. 4. Examples for chemical structures of fatty acids and 2- and 3-hydroxy fatty acids.

2.5.3 Analyses of lipid biomarkers

Phospholipid fatty acids

Analysis of phospholipid fatty acids has provided a quantitative insight into complex microbial communities in environment without the need for isolation. PLFAs are present in all living cells, but not in *Archaea*. However, *Archaea* do likely not make any significant contributions to microbial communities in drinking water systems (Manz et al., 1993). Different microbial groups have specific PLFAs, such as gram-negative bacteria contain monounsaturated or cyclopropane fatty acids (Wilkinson, 1988), gram-positive bacteria have *iso*-, *anteiso*- or otherwise branched-chain fatty acids (for a review, see Kaneda, 1991; O´Leary and Wilkinson, 1988), actinomycetes and some sulphate-reducing bacteria have a methyl group in the tenth carbon atom from the carboxyl end of molecule (Brennan, 1988; Wilkinson, 1988), and yeasts and fungi contain polyunsaturated fatty acids (Lösel, 1988; Rattray, 1988). The microbial groups might also contain same PLFAs, which in turn triggers the interpretation of their profiles (Zelles, 1997). Phospholipid fatty acids can also be used to estimate viable biomass, as their proportions in a cell are rather constant, they are not present in storage lipids and are rapidly degraded following cell death (King et al., 1977; White et al., 1979). The biomass can be analysed by performing a quantitative estimation of the PLFAs or the lipid phosphate content (Frostegård et al., 1991; White et al., 1979). Differences in physiological status of microbes can be monitored by comparison of certain fatty acids. The ratio of saturated to unsaturated PLFAs, an increase in ratio of cyclopropyl fatty acids to their monoenoic precursors and an increase in the *trans*/*cis* ratio of the unsaturated PLFAs have been used as indicators for starvation, stress or stationary growth phase (for a review, see Suutari and Laakso, 1994; White et al., 1996).

Hydroxy fatty acids

The normal OH-FAs of gram-negative bacteria are straight-chain saturated fatty acids containing an even number of carbon atoms in the molecule such as OH-12:0, -14:0 and –16:0 (Smith, 1988; Wilkinson, 1988). The amount of LPS in a bacterial cell is relatively constant, so the community structure and biomass of the gram-negative bacteria can be analysed (Watson et al., 1977). However, 2- and 3-OH-FAs have also been found in gram-positive bacteria, fungi and plants in molecular structures other than LPS (Brennan, 1988; Lösel, 1988; Rattray, 1988; for a review, see Van Dyk et al., 1994; Zelles, 1997). Gram-negative bacteria, *Sphingomonas* spp. contain several 2-OH-FAs, with 2-OH-14:0 being the major hydroxy fatty acid in their sphingolipids (Balkwill et al., 1997). Yeast and

other fungi contain several OH-fatty acids in their phosphosphingolipids (Lösel, 1988; Rattray, 1988; Wells et al., 1996). *Mycobacteria* have straight chain, 2-methyl-branched and 2,4,6-trimethyl-branched 3-OH-FAs with 14 to 28 carbon atoms in their glycolipids (Alugupalli et al., 1994; Brennan, 1988).

Analysis of phospholipid fatty acids

One of the most widely used extraction and separation techniques for PLFAs was originally proposed by Bligh and Dyer (1959) and subsequently modified by White et al. (1979) and Frostegård et al. (1991). The method includes extraction with a mixture of chloroform, methanol and water or buffer (1 : 2 : 0.8 vol/vol/vol). Dilution of the solvent mixture with buffer and chloroform separates solvent phase into two phases (chloroform: methanol: water or buffer, 1 : 1 : 0.9 vol/vol/vol), with the lower phase containing all of the extracted lipids. The extraction has been most efficient when the solvents have been added in the order of increasing polarity *i.e.* chloroform- methanol- water compared to adding solvents in the reverse order (Lewis et al., 2000; Smedes and Askland, 1999). The solutions can be buffered with water, citrate, acetate, Tris or phosphate. Citrate buffer has the best extraction efficiency in organic soil, whereas in sandy soil it was found that citrate and phosphate buffers had better extraction efficiences than the other buffers studied (Frostegård et al., 1991). The extraction can be done by standing, shaking, sonication, homogenisation or in a pressurised hot solvent extractor (Bligh and Dyer, 1959; Frostegård et al., 1991; Macnaughton et al., 1997b; White et al., 1979). However, neither sonication nor homogenisation increased the amount of the extracted lipids (Frostegård et al., 1991; Lewis et al., 2000). Shaking for two hours is sufficient to extract the maximum amount of lipid material from soil samples. The lipids are further separated with silicic acid chromatography to neutral, glyco- and phospholipids using chloroform, acetone and methanol as solvents, respectively (Frostegård et al., 1991). Finally, the fatty acids are methyl esterificated with mild alkaline hydrolysis and analysed with gas-chromatography and mass spectrometry. In mass spectrometry, fatty acids can be detected either with total ion monitoring or selected ion monitoring.

More complex extraction techniques can be used to obtain more detailed information on fatty acid binding in the lipid molecule. One method includes solid phase extraction columns after mild alkaline hydrolysis to separate PLFAs into their sub-classes (Zelles, 1999; Zelles and Bai, 1993; Zelles et al., 1992).

Microbial identification with whole cell fatty acids

Microbial identification with whole cell fatty acids (MIDI) can be used to differentiate microbial communities (Haack et al., 1994; Zelles, 1999). However, the method cannot be used to estimate microbial biomass or changes in physiological status of microbes, because extraction of the fatty acids is not restricted to those originating from living microbes.

Analysis of hydroxy fatty acids

The hydroxy fatty acids are bonded to larger molecules via an ester or amide linkage (Alugupalli et al., 1994; Wilkinson, 1988). The OH-FAs are generally analysed from the extraction residue of lipids by method of Bligh and Dyer (1959). OH-FAs can be methyl esterified using acidic methanolysis or alkaline saponification followed by methylation (Larsson, 1983; Wollenweber and Rietschel, 1990). Alkaline saponification cleave ester linkages, whereas acidic methanolysis degrades both ester or amide linkages. OH-FA methyl esters can be further derivatised such as with pentafluorobenzoyl, trifluoroacetylation or trimethylsilylation (Balkwill et al., 1997; Larsson, 1983; Mielniczuk et al., 1992; Wollenweber and Rietschel, 1990). The derivatives can be purified with thin-layer chromatography and analysed with GC-MS (Balkwill et al., 1997).

2.5.4 Studies on environmental samples

Lipid biomarkers have been widely analysed from environmental samples. PLFA and OH-fatty acids were first applied to study the structure and biomass of microbial communities in aquatic environments such as sediments as well as microbes in soil (Bobbie and White, 1980; Federle et al., 1983; King et al., 1977; Odham et al., 1985; Parker et al., 1982; Tunlid et al., 1985; White et al., 1979). The effects of environmental changes on microbial communities have been studied widely since the first publications utilizing these methods.

Sediments

In sediments, changes in microbial community structure changes as determined by PLFAs have been described in geochemical processes (Coleman et al., 1993), drilling operation samples from different depths of sediments (Lehman et al., 1995; Ringelberg et al., 1997), in the trench of a volcano zone

(Guezennec and Fiala-Medioni, 1996), in examinations of hydrographic and chemical properties in different parts of Osaka bay (Rajendran et al., 1994), differences between eutrophic bays in Japan (Rajendran et al., 1992; Rajendran et al., 1997), differences between organic matter mixing zones (Shi et al., 2001), seasonal and spatial variations (Smooth and Findlay, 2001), eutrophication (Pinturier-Geiss et al., 2002), mercury pollution (Macalady et al., 2000), hydrocarbon contamination (Fang and Barcelona, 1998; Franzmann et al., 1996; Langworthy et al., 2002, and *in situ* bioremediation of hydrocarbon contaminated sediments (Ringelberg et al., 2001; White et al., 1998).

Soils

In soil environments, PLFAs and OH-FAs have been used to study microbial community changes in geochemical processes (Bull et al., 2000), botanical composition (Borga et al., 1994; Ibekwe and Kennedy, 1998; Priha et al., 1999), management practices (Bai et al., 2000; Bossio and Scow, 1998; Ibekwe et al., 2001; Peacock et al., 2001; Steenwerth et al., 2003; Yao et al., 2000; Zelles et al., 1992; Zelles et al., 1994; Zelles et al., 1995a; Zelles et al., 1995b), succession transects of forest (Merilä et al., 2002), spatial position of trees (Saetre and Bååth, 2000), soil fertility (Pennanen et al., 1999), climatic changes (Insam et al., 1999; Ronn et al., 2002; Steinberger et al., 1999; Zak et al., 1996), heating (Pietikäinen et al., 2000), pH changes (Bååth et al., 1992; Bååth et al., 1995; Frostegård et al., 1993a; Pennanen et al., 1998a; Pennanen et al., 1998b), and pollution with metals (Fritze et al., 2000; Frostegård et al., 1993b; Frostegård et al., 1996; Khan and Scullion, 2000; Pennanen et al., 1996).

Indoor air

In indoor air environments, PLFAs and 3-OH-FAs have been studied to define biomass, community composition and physiological status of airborne microbes (Macnaughton et al., 1997a; Macnaughton et al., 1999; Sebastian and Larsson, 2003). In particular, 3-OH-FAs have been used as markers for gram-negative bacterial lipopolysaccharides, which can act as inflammatory agents (Fox et al., 1993; Krahmer et al., 1998; Laitinen et al., 2001; Larsson and Larsson, 2001; Liu et al., 2000). Interestingly, endotoxin exposure via lipopolysaccharides from drinking water have been implicated as a potential health risk following hemodialysis and inhalation (for a review, see Anderson et al., 2002).

Water systems

Differences in microbial communities of activated sludge of wastewater treatment plants have occurred at various geographical locations and due to temporal changes over time (Forney et al., 2001). Certain pathogenic microbes such as *Mycobacterium* spp. or *Legionella pneumophila* can be detected from drinking water systems with lipid biomarkers (Alugupalli et al. 1992; Slosárek et al. 1996; Walker et al. 1993). However, there have been very few analyses of microcial communities in drinking water systems utilising lipid profiling (see section 2.4, “Methods to analyse microbes in drinking water systems”).

3 AIMS OF THE STUDY

The overall aim of the work was to study the applicability of lipid biomarkers in the analysis of microbial communities, biomass and physiological state in drinking water systems, and further to develop the methods for drinking water research. Soil and sediment samples were used as reference materials.

The specific goals were:

1. to simplify the analytical procedures for determining 2- and 3-hydroxy fatty acids directly from the extraction residue of lipids without further purification or derivatisation (I).

2 to analyse microbial communities in drinking water and biofilms developed under drinking water flow in a laboratory and full-scale drinking water systems using phospholipid fatty acids (PLFA) and lipopolysaccharide (LPS) 3-OH-fatty acids (3-OH-FAs) (I-IV).

3 to study the effects of phosphorus availability, aging of biofilms and water residence time on the microbial communities, biomass and physiological state present in drinking water biofilms (II, III, IV).

4 MATERIALS AND METHODS

4.1 Biofilms

4.1.1 Laboratory experiments (I, II)

The formation of biofilms in a laboratory-scale was studied in studies I-II. The drinking water used in these experiments was from Kuopio waterworks in Finland (61°51‘N 27°45’E), which processes bank-filtered lake water (Table 2). The biofilms were developed under the water flow of 0.5 ml min^{-1} in the dark at 21±2°C on a glass slide (41.6 cm^2) in glass chambers for four (I, II) or eleven weeks (II). Biofilms were detached from glass slides by 5 min sonication (40 kHz) (Finnsonic mO3, Lahti, Finland). Separate Na_2HPO_4 solution flows of 0, 1, 2 and 5 µg l^{-1} of phosphorus to the chambers were used to study the effects of phosphorus supplementation (II).

Table 2. Water purification processes at waterworks for biofilm and water samples from studies (I-IV).

	Chemical	**Filtration**		**Disinfection**		
	coagulation	**rapid sand**	**slow sand**	**hypochlorite**	**chloramine**	**ozone**
Biofilm						
Laboratory scale, Kuopio (I, II)	x	x				
Full scale						
A (IV)	x	x	x		x	
B (III, IV)	x	x			x	x
Water						
Kuopio (II)	x	x		x		
B (III)	x	x			x	x

4.1.2 Full-scale systems (III, IV)

The formation of biofilms in full-scale drinking water distribution systems was studied as a function of water residence time and biofilm formation time in studies III and IV. The biofilms were collected on a series of polyvinylchloride (PVC) tubes (inner diameter 10 mm, length 200 mm, area 62.8 cm^2) connected to two full-scale drinking water distribution systems A and B receiving drinking water from two separate waterworks (Table 2). Biofilms were collected with a water flow rate of 1 l min^{-1} between July 1997 and March 1998. Biofilms were detached from PVC tubes by shaking for 20 min with glass beads. The samples from water distribution system A were collected with water residence times of 10, 39, and 141 hours after the biofilm development of 6, 23 and 40 (no sample for the 141 hour time point) weeks (IV). Samples from distribution system B were collected with a water residence time of

22 hours after biofilm development periods of 11, 24 and 41 weeks, and with a water residence time of 62 hours after 11 weeks of biofilm growth (IV). Samples with the water residence time of 22 and 62 hours with a development period of 11 weeks were used in study III.

4.2 Water samples (II, III)

To study the effects of water temperature on microbial communities, drinking water (7°C) and warm water (45°C) samples of 30 liter volume were collected from the distribution system of Kuopio waterworks with a water retention time of 1-2 days (Table 2, II).

To study the effect of residence time, 20 liters water samples were taken one week after the biofilm sampling at the sampling sites of 22 and 62 hours from the same sites where biofilms were grown in distribution system B (Table 2, III).

4.3 Soil and sediment samples (I)

Soil and sediment samples were analysed as references for samples from drinking water distribution systems. The soil sample was collected (July 7, 1998) at a depth of 0-10 cm from an experimental field with an area of 2 m^2 at the Agricultural Research Center in Jokioinen, Finland (60°45'N 23°22'E). The soil type was clay, and it was sown with barley. The sediment sample (layer of 310-320 cm) was taken on April 15, 1997 from Lake Ahmasjärvi in Finland (64°39'N 26°27'E). The depth of the water column at the sampling point was 2.2 m.

4.4 Lipid biomarker analyses

The procedure for the lipid biomarker analyses was as presented in Fig. 5.

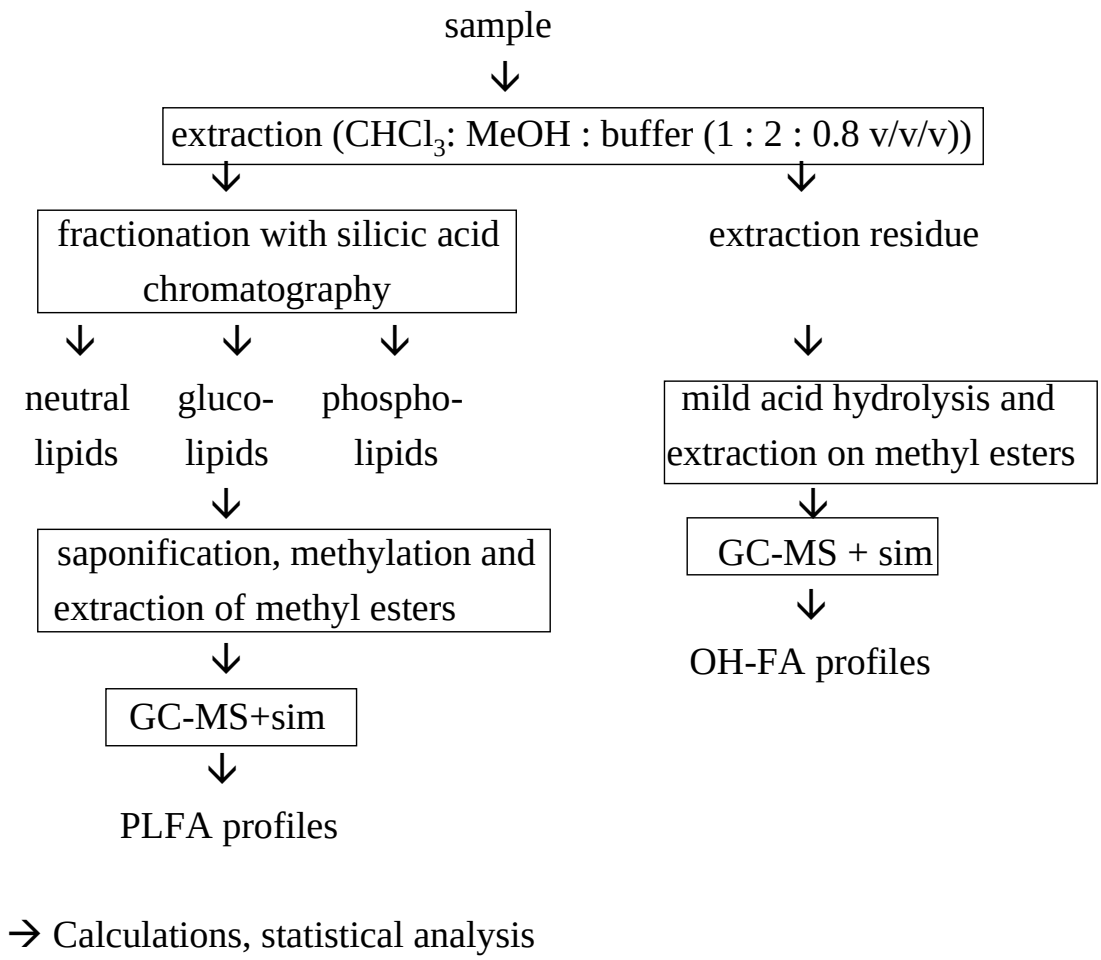

Fig. 5. The procedure of lipid biomarker analyses.

4.4.1 Glassware and chemicals (I- IV)

For lipid analyses of waters and biofilms the glassware was heated at 550°C for 6 h.

Solvents were from Rathburn Ltd (Peeblesshire, United Kingdom), non-hydroxy fatty acid standards and hydroxy substituted fatty acid standards from Sigma (St. Louis, MO, USA), except for 3-hydroxytridecanoic and 2-hydroxyoctadecanoic fatty acid methyl esters which were from Larodan AB, (Malmö, Sweden), NaOH from FF-Chemicals (Yli-Ii, Finland), HCl from Riedel- de Haën (Seelze, Germany), acetylchloride from Fluka (Buchs, Switzerland), and other reagents from Merck (Darmstadt, Germany).

4.4.2 Lipid extraction and fractionation (I-IV)

The water and extracts of biofilms were filtered through 0.2 µm (Pall Europe Ltd, Portsmouth, England) or 0.45 µm filters (Sartorius GmbH, Goettingen, Germany) with filtration equipment (Sartorius SM 16274; Sartorius GmbH, Goettingen, Germany (I-IV). Sediment, water and biofilm samples were lyophilised (Edwards 4 K Modulyo freeze-dryer, Edwards, Crawley, England). All samples were stored at -20°C until the lipid analyses.

The duplicate samples of reference without environmental material were analysed identically with the samples. The lipids were extracted and stored at -20°C under an atmosphere of N_2. All samples were extracted in 28.2 ml of chloroform : methanol : buffer (1 : 2 : 0.8 vol/vol/vol) (Bligh and Dyer, 1959; Frostegård et al., 1991; White et al., 1979). For the quantification of phospholipids, an internal standard, diheptadecanoylphosphatidylcholine, was added. Lipids were separated from the solvent phase after the addition of chloroform and buffer (final ratios of solvents 1 : 1 : 0.9 vol/vol/vol).

Lipids were fractionated in a 0.75 g silica column (100-200 mesh size, Unisil, Clarkson Chemical, Williamsport, Pennsylvania, USA) to neutral, glyco- and phospholipids with 10, 20 and 10 ml of chloroform, acetone and methanol, respectively (Frostegård et al., 1991; King et al., 1977).

4.4.3 Fatty acid analyses

Internal standards, tridecanoic and nonadecanoic acid methyl esters were added to the phospholipid fraction and extraction residues of soil and sediment samples. Fatty acids and hydroxy fatty acids in soil and sediment were saponified, methylated, and extracted as methylesters (Suutari et al., 1990) (I-IV).

The internal standard, 3-OH tridecanoic acid methyl ester was added to the lipid extraction residues of biofilms and waters. LPS hydroxy-substituted fatty acids of biofilm were treated with mild acid hydrolysis in 2 M HCl in methanol and extracted as methylesters (Jantzen et al., 1989; Torkko et al., 1998) (I, II, IV).

4.4.4 GC-MS

Fatty acids methyl esters were analysed with a Hewlett-Packard (Palo Alto, California, USA) model G1800A gas chromatograph (GC) equipped with a mass selective detector (MS) and HP7673 automatic sampler. The GC conditions were as follows: HP-5 capillary column (30 m by 0.2 mm by 0.11 µm) coated with crosslinked 5 % Ph Me Silicone; carrier gas, helium (1.0 ml min^{-1}); splitless injection; injector temperature, 250°C; detector temperature, 270°C. The oven temperature was programmed to hold at 50°C for 1 min, and then to increase by 30°C min^{-1} up to 160°C, and thereafter by 5°C min^{-1} up to 270°C. The phospholipid fatty acid methyl esters were analysed with selected ion monitoring (SIM) by following ions m/z 74 and 199. As an exception, the latter ion was m/z 268 for 16:1 acids, m/z 250 for *cy*-17:0, m/z 298 for *i*-18:0 and 18:0, m/z 294 for 18:2ω6, m/z 264 for 18:1 acids, m/z 312 for 10-Me-18:0 and 19:0, m/z 278 for *cy*-19:0 and m/z 326 for 20:0. In the SIM of 2- and 3-hydroxy fatty acid methyl esters, the ions monitored were m/z 90, 103 and M-59 (Gradowska and Larsson, 1994; Wollenweber and Rietschel, 1990), except in soil and sediment where the internal standards 13:0 and 19:0 were monitored with m/z 74 and 199 (I). The 2- and 3-OH-FAs of samples were also analysed using total ion monitoring (I). The fatty acid methyl esters were identified by comparing their mass spectra and retention times with those of standards.

4.5 Nomenclature of fatty acids

Fatty acids are designated as the total number of carbon atoms : the number of double bonds followed by the position of the double bond from the methyl end (ω) of the molecule. The *cis*/*trans*-isomerism of double bond is indicated with *c*/*t*. The prefixes *i*- and *a*- indicate *iso*-branched and *anteiso*-branched, respectively, *br*- indicates an unknown methyl branch position, 10-Me- indicates a methyl group in the 10^{th} carbon atom from the carboxyl end of the molecule, and *cy*- refers to cyclopropane fatty acids. 10-Me-18:0 is designated as tuberculostearic acid (TBSA), and the prefixes 2-OH- and 3-OH- indicate 2- and 3-hydroxy fatty acids, respectively.

4.6 Calculations and statistical analyses

To calculate calibration curves for the quantification of PLFAs, calibration standards were made with known ratios of bacterial PL-fatty acids relative to the internal standard methyl nonadecanoate (19:0) (Tunlid et al., 1989). The standards contained fatty acids at four to five concentrations ranging from 0.02 to 2 nmol $µl^{-1}$ for 16:0 with 68 pmol $µl^{-1}$ of an internal standard (I-IV).

The calibration standards for the quantification of hydroxy fatty acids contained OH-FAs at four to five concentrations within the range of 0.4 µmol μl^{-1} to 2.9 nmol μl^{-1}, with 1.0 nmol μl^{-1} (soil, sediment) or 0.4 nmol μl^{-1} (biofilm) of internal standard. The SIM responses were linear with correlation coefficients of 0.996±0.004 (I).

The fatty acid content was defined as the sum of the fatty acid methyl esters. The PLFA (I-III) and LPS OH-fatty acid (I) contents were converted to cell densities using the following factors. On average, bacteria contain 100 µmol of PLFAs and 15 µmol of LPS OH-FAs g^{-1} dry weight, and 1 g of bacteria (dry wt) is equivalent to 2.0 x 10^{12} cells dry weight (Balkwill et al., 1988).

C18/C16 was calculated as the percentage ratio of 18:0, 18:1ω7*c* and 18:1ω9*c* to 16:0, 16:1ω5*c* and 16:1ω7*c* (II).

Principal component analysis (PCA) for standardised results was performed to elucidate major variation in data either with the programmes provided by SAS Institute (1989) (II) or SPSS for Windows version 10.1. (SPSS, Inc., Chicago, IL, USA) (IV). The relationship between phosphorus supplementation and lipid biomarkers was tested with Pearson correlation analyses, using SAS (II). Linear regression analysis was performed with lipid biomarkers with different water residence times or development times of biofilms (IV). Analysis of variance (2-way ANOVA) followed by Tukey's test was used to detect changes in the biomass estimated on the basis of PLFAs or LPS 3-OH-FAs (IV). All results of lipid biomarkers were presented as mean ± standard error (I, II) or mean ± standard deviation (III, IV).

5 RESULTS AND DISCUSSION

5.1 PLFA profiles

The PLFAs were analysed with GC-MS using SIM after lipid extraction, fractionation and methyl esterification. There were 21 to 25 different PLFAs present in the biofilms (I-IV), 22 (II) and 26 (III) PLFAs in drinking and warm waters, 30 PLFAs in soil and 24 PLFAs in sediment (I). In our experiments the biofilms were collected from 41.6 cm^2 (I, II) and 62.8 cm^2 of surface areas (III, IV), water samples were 20 (III) or 30 litres (II), soil and sediment samples weighed 3.1 g and 5.2 g, respectively (I). In ealier studies, the numbers of PLFAs from environmental samples have been similar or higher than in studies I-IV. In drinking water biofilms from 1540 cm^2, 29 PLFAs (Smith et al., 2000), and in drinking water biofilters from 2 to 10 g of sand, 57 to 90 have been detected (Fonseca et al., 2001; Moll et al., 1998; Moll et al., 1999). From 0.5 to 1 g of coniferous forest soils a range of 24 to 37 PLFAs have been reported (Bååth et al., 1992; Bååth et al., 1995; Frostegård et al., 1993a) whereas 31 PLFAs were detected from 3 g of arable soil (Frostegård et al., 1993b). Using extended extraction procedures on samples of 100 g of soil, Zelles et al. (1992) reported the presence of as many as 100-132 PLFAs.

In water samples and biofilms, the most abundant PLFAs with over 80 % were straight-chain saturated, characterising general biomass (White et al., 1996), and straight-chain monounsaturated fatty acids, characteristic of gram-negative bacteria (Wilkinson, 1988) (Table 3). The terminally branched PLFAs, indicative of gram-positive bacteria, and polyenoics, indicative of microeucaryotes, represented generally less than 7 % of the total PLFAs in the samples. In distribution system B, 18:2ω6, typical for eucaryotic cells (Lösel, 1988; Rattray, 1988) accounted for 4 % in biofilms and 7% in waters. High proportions of straight-chain saturated fatty acids and straight-chain monounsaturated fatty have earlier been reported from biofilm accumulation chambers, corroded concrete surfaces and drinking water biofilters (Fonseca et al., 2001; Herb et al., 1995; Moll and Summers, 1999; Moll et al., 1999; Smith et al., 2000). The proportions of terminally branched PLFAs, indicative of gram-positive bacteria, and polyenoics, typical for microeucaryotes, have been 6.3-11 % and 2.0-6.7% in drinking water biofilters, respectively (Moll and Summers, 1999; Moll et al., 1999). In groundwater, gram-negative bacteria have dominated the profile, indicated by the presence of monoenoic PLFAs, whereas gram-positive bacteria and eucaryotes have been claimed to be rare or absent on the basis of low levels of terminally branched fatty acids and absence of polyunsaturated fatty acids, respectively (Jain et al., 1997). The PLFA profile of the soils was the most diverse of the samples studied here, as the five most

abundant fatty acids accounted for 50% of the total profile, whereas in biofilm, drinking water samples and sediment samples the proportion was 80-87% (Table 3). Table 3 summarises five most common PLFAs and their percentages in different environments. In drinking water systems, the PLFA profiles were less complex compared to other environmental samples from various geographical locations.

Table 3. The proportions of the five most abundant PLFAs in samples from studies I-IV and in some other environments (mean±sd or range).

	Five most abundant PLFAs	**Proportion of all PLFAs %**	**References**
Biofilm (Finland)			
Laboratory scale, Kuopio (n=16)	14:0, 16:0, 18:0, 18:1ω7, 18:1ω9	87.0±6.8	I, II
Full scale			
A (n=16)	14:0, 15:0, 16:0, 18:0, 20:0	85.5±6.6	IV
B (n=8)	14:0, 16:0, 18:0, 18:2ω6, 18:1ω7	80.7±2.4	III, IV
Water (Finland)			
Kuopio (n=4)	16:0, 18:0, 16:1ω7, 18:1ω7, 18:1ω9	81.0±4.3	II
B (n=2)	16:0, 18:0, 18:2ω6, 16:1ω7, 18:1ω7	81.8±4.5	III
Soil (n=2) (Finland)	*i*-15:0, *i*-16:0, 10-Me-16:0, 16:0, 16:1ω7	50.4±0.3	I
Sediment (n=2) (Finland)	*i*-15:0, *a*-15:0, 14:0, 16:0, 18:0	80.2±0.1	I
Biofilm accumulation chambers (USA)	14:0, 16:0, oxirane 16:0, oxirane 18:0, 10-Me-18:0 September (summer), November (fall)	74.3, 77.9	Smith et al., 2000
Coniferous forest soil			
Pine (Sweden)	16:0, 18:2ω6, 18:1ω7, 18:1ω9, , *cy*-19:0	55.5-58.9	Frostegård et al., 1993a
Spruce (Sweden)	*i*-15:0, 16:0, 18:2ω6, 18:1ω9, 18:1ω7/*cy*-19:0	49.3-53.5	Frostegård et al., 1993a
Pine and spuce (Finland)	i-15:0/18:1ω7, 16:0, 18:2ω6, 16:1ω7, 18:1ω9	45.5-61.1	Bååth et al., 1992; Bååth et al., 1995
Soil from a tropical ecosystem	16:0, 18:2ω6, 16:1ω7, 18:1ω7, 18:1ω9	63.0-63.4	Insam et al., 1999
Peats (Sweden)	*i*-15:0/16:1ω7/18:1ω9/*cy*-19:0, 16:0, 18:0, 18:1ω7	60.1-74.8	Borga et al., 1994
Sediments (Barbados Trench)	*a*-15:0/16:1ω5c, 16:0, 16:1ω5t, 16:1ω7, 18:1ω7	58.2-76.6	Guezennec and Fiala-Medioni, 1996
Sediments (Japan)	*i*-15:0, *a*-15:0, 16:0, 14:0/16:1d9/18:1d9/18:1d11	47.7-49.6	Rajendran et al., 1997

5.2 LPS 2- and 3-OH-FA profiles

3- and 2-OH-FAs from the residues of samples, extracted according to Bligh and Dyer (1959) to remove lipids, were analysed as methyl esters directly by GC-MS using SIM without further purification, or derivatisation of hydroxyl groups. In SIM, the ion monitored to define 3-OH-FAs was m/z 103 ($CHOHCH_2COOCH_3$), and the ions for 2-OH-FAs were m/z 90 ($CH_2OHCOOCH_3$) and M-59. The ion m/z 103 appeared to be specific for 3-OH-FAs. The ion m/z 90 was detected concomitantly with m/z M-59 only in 2-OH-FAs. The lowest detectable injected amounts of 3-OH-13:0 and 2-OH-18:0 methyl esters were 78 fmol (19 pg) and 1.6 pmol (0.5 ng), respectively, measured

with a signal to noise ratio of 4. Thus, the detection limit for 2-OH-FAs was approximately 21-times higher than that of 3-OH-FAs. The higher detection limit of 2-OH-FAs compared to 3-OH-FAs could be due to the poorer specificity of m/z 90 and m/z M-59 compared to m/z 103. In general, derivatisation and purification, or the use of more efficient mass-spectrometry, for example an MS-MS system, could possibly lower the detection limit. However, the aim was to improve the hydroxy fatty acid analysis excluding further purifications or derivatisations and without the requirement of highly sophisticated MS-MS.

In the biofilms 3 (I, II) and 16 LPS 3-OH-FAs (IV), indicative of gram-negative bacteria, were detected in laboratory experiment and full-scale drinking water distribution system B, respectively. In the biofilms from the distribution system A, the concentrations of LPS 3-OH-FAs were below the detection limit. In drinking and warm waters, 3 LPS 3-OH-FAs (II) were detected. 2-OH-FAs were never found in either biofilms or in waters. In soil and sediment we found a total of 25 2-OH-FAs and 25 to 27 3-OH-FAs, indicative of gram-negative bacteria, gram-positive bacteria, fungi and plants (I). In agricultural soils with different vegetation and agricultural managements, 36 to 65 LPS OH-FAs have been detected from a sample of 100 g of soil (Zelles et al., 1992). The absence of 2-OH-FAs in samples from drinking water systems might be due to higher detection limit for 2-OH-FAs than for 3-OH-FAs. In addition, it seems likely that microbes have lower concentrations of 2-OH-FAs than 3-OH-FAs, as can be seen from the quantitative amounts of the soil and sediment samples (Table 6). Zelles et al. (1992) reported 2-OH-FAs only from one soil sample, whereas 3-OH-FAs were present in every soil of their eight soil samples.

Straight-chain, even-numbered carbon containing 3-OH-FAs, typical for gram-negative bacteria (Wilkinson, 1988) dominated the profile in biofilms and water (Table 4). Soil and sediment samples contained more complex 3-OH-FA profiles, since the most abundant 3-OH-FAs accounted for a smaller proportion of all 3-OH-FAs than samples from drinking water systems. The hydroxy fatty acids in sediment originated from different organisms than those in soil, because 3- and 2-OH-FAs with a carbon chain length greater than 14 carbons were generally more abundant in sediment, and hydroxy fatty acids with a chain length shorter than 14 carbons generally were more abundant in soil. In sediments and indoor air environments, the 3-OH-FAs typically found in drinking water systems have accounted for most of the profile (Table 4). There is much less data available on the most abundant 3-OH-FAs and 2-OH-FAs from environmental samples in the literature than for the PLFAs.

Table 4. The proportions of the five most abundant 3- and 2-OH-FAs in samples from studies I, II, and IV, and in some other environments (mean±sd or range).

	Five most abundant 3-OH-FAs	**Proportion of all 3-OH-FAs %**	**Five most abundant 2-OH-FAs**	**Proportion of all 2-OH-FAs %**	**References**
Biofilm (Finland)					
laboratory scale, Kuopio (n=16)	10:0, 12:0, 14:0	100	ND		I, II
full scale B (n=8)	10:0, 12:0, 14:0, 16:0, 18:0	87.8±5.0	ND		IV
Water, Kuopio (n=4) (Finland)	10:0, 12:0, 14:0	100	ND		II
Soil (n=2) (Finland)	8:0, 10:0, 12:0, 14:0, *i*-15:0	73.1±0.5	12:0, 13:0, 14:0, 16:0, 24:0	51.9±0.1	I
Sediment (n=2) (Finland)	14:0, 16:0, *a*-15:0, *i*-17:0, *a*-17:0	66.4±0.7	*i*-15:0, 16:0, 24:0, 25:0, 26:0	45.8±0.5	I
Estuarine sediment (USA)	12:0, 14:0, 16:0, 18:0	100			Parker et al., 1982
Sediments (Barbados Trench)	14:0, 12:0/ *i*-15:0/*a*-15:0, 16:0, 18:0	59.3-83.2			Guezennec and Fiala-Medioni, 1996
Indoor air in two schools (USA)	12:0, 14:0, 16:0, 18:0	100			Liu et al., 2000
Indoor air in a water damaged building	10:0, 12:0, 14:0, 16:0, 18:0	100			Larsson and Larsson, 2001
Indoor dust in a stable or a dairy (USA)	10:0, 12:0, 14:0, 16:0, 18:0	100			Krahmer et al., 1998

ND, not detected

5.3 Differences in microbial communities

5.3.1 Waters (II, III)

The microbial community structure present in drinking water differed from warm water in samples from Kuopio (II, Fig. 1A). More PLFAs 14:1, 16:1ω7*c*, 18:1ω7*c*, 18:1ω9*c* and LPS 3-OH-10:0, typical for gram-negative bacteria and *i*-14:0, which is frequently found in gram-positive bacteria were common in the drinking than warm water. In contrast, microbial biomass characterising PLFAs 16:0, 18:0, gram-negative bacteria characterising *cy*-17:0, *cy*-19:0, 16:1ω5*c* and LPS 3-OH-14:0, and sulphate-reducing bacteria and actinomycetes characterised by 10-Me-16:0 and TBSA, were more common in warm than in drinking water (II, Fig. 1B, Table 1). At low temperatures microbes may decrease the ratio of C18 to C16 acids and fatty acid cyclization, and increase fatty acid unsaturation to maintain membrane fluidity (Suutari and Laakso, 1994; Suutari et al., 1990), all of which were differences observed between drinking and warm water. The hydroxy fatty acid content is known to vary with the growth temperature (Suutari and Laakso, 1994). In drinking water biofilters, PLFAs indicated that there was an increasing gradient for gram-negative bacteria and microeucaryotes when the biofilter operation temperature decreased, replacing markers for microbial biomass, gram-positive bacteria and sulphate-reducing bacteria (Moll and Summers, 1999; Moll et al., 1999). In drinking waters from distribution system B, the water residence times of 22 or 62 hours had only a minor effect on the microbial community structure (III).

5.3.2 Biofilms and waters (II, III)

Microbial community structures differed from each other in drinking and warm waters, and laboratory biofilms from Kuopio, Finland (II, Fig. 1A). The 14 to 16 carbon PLFAs, characteristic of gram-positive bacteria *(i*-14:0, *i*-15:0, *i*-16:0, *a*-15:0, 10-Me-16:0), and gram-negative bacteria (14:1, 16:1ω7*c*, LPS 3-OH-14:0) were more abundant in water samples than in biofilms (II, Fig. 1B, Table 1). Typical PLFAs found in the biofilms were 17 and 18 fatty acids which are commonly present in gram-positive bacteria (*a*-17:0, *i*-18:0), gram-negative bacteria (18:1ω9*c*), and eucaryotic cells (18:2ω6*c*), in addition to the microbial biomass indicating PLFAs (14:0, 15:0 and 18:0). Furthermore, gram-negative bacteria characterising LPS 3-OH-10:0 and 3-OH-12:0 were more abundant in biofilms than in waters. The ratio of C18 to C16 acids was the highest in the biofilms (21°C), intermediate in warm water (45°C), and lowest in drinking water (7°C). The lower ratio of C18 to C16 fatty acids in free-living bacteria than in adhered bacteria has been related with the heterogeneity present in the culture, and the rapid selection of community according to which surface the microbes have become

attached (Valeur et al., 1988). The differences between waters and biofilms might partly be explained by the different temperatures. At low temperatures, the ratio of C18 to C16 acids and fatty acid cyclization decrease, and fatty acid unsaturation increases (Suutari et al., 1990).

In drinking water distribution system B there were differences in the composition of PLFAs in the water and biofilm samples (III). In drinking waters, the proportion of gram-negative bacteria characterising straight-chain unsaturated and cyclopropane fatty acids was on avarege 2.0 and 5.3 times higher than in those biofilms with water residence times of 22 h and 62 h, respectively. In contrast, the proportion of microbial biomass characterising straight-chain saturated fatty acids was 3.5 times higher in a biofilm with water residence time of 22 h hours compared to drinking waters, and 2.4 times higher in biofilm with water residence time of 62 h also compared to drinking waters. The amount of eucaryotic cell marker, polyunsaturated fatty acids was slightly higher in drinking water than in biofilms. Methyl-branched monounsaturated fatty acids were detected only in drinking waters.

5.3.3 Effects of phosphorus-P supplementation on biofilms (II)

The microbial communities of biofilms grown without phosphate supplementation, or those developed for 4 weeks with 1 µg l^{-1} phosphate-P addition were separated from those growing for 4 or 11 weeks with 2 or 5 µg l^{-1} phosphate-P supplementation (II; Fig. 1A). The proportion of 16:1ω7*c* and 18:1ω7*c* increased linearly with the increase in the phosphate-P concentration. Phosphorus also increased the proportion of 3-OH-14:0 and decreased the proportion of 3-OH-12:0. Hence, the proportion of gram-negative bacteria increased and their community changed with the increase in phosphate-P in biofilms grown for 11 weeks (II; Fig. 2; Table 1). Generally, microbial communities in biofilms were similar already after four weeks growth, and this was independent of the amount of phosphate in water.

5.3.4 Full-scale biofilms (III, IV)

The microbial community structures in the biofilms collected from the full-scale drinking water distribution systems A and B differed (IV, Fig. 1A). The methyl-branched PLFAs *a*-17:0, *br*-15:0b, 10-Me-16:0, TBSA, characteristic of gram-positive bacteria, straight-chain saturated acids 16:0, 18:0, 20:0, typical for microbial biomass, unsaturated 18:2, common for eucaryotic cells and *cy*-19:0, found in gram-negative bacteria were more abundant in the biofilms from distribution system A than biofilms in the distribution system B. The biofilms in distribution system B were characterised with PLFAs methyl-branched *i*-15:0, *a*-15:0 and *br*-15:0a, common for gram-positive bacteria, monounsaturated 16:1ω5, 16:1ω7, 16:1ω9, 18:1ω7 and 18:1ω9, typical for gram-negative bacteria, polyunsaturated

18:2ω6 characteristic of eucaryotic cells, and straight-chain saturated 14:0 and 15:0, indicative of microbial biomass. The proportion of gram-negative bacteria was higher in the biofilms of distribution system B than in those of distribution system A. The amount of 18:1ω7 represented on average 11.6±6.7 % of the total in distribution system B compared to only 1.4±2.1% in system A. The differences in microbial community structures in biofilms collected from the two distribution systems could be associated with several factors, such as differences in raw water sources, water purification processes, hydraulic conditions including flow velocity and pattern, and pipe materials.

According to the PLFA profiles, the microbial community structure changed in the course of the biofilm development time in distribution system A. The microbial community was more complex in biofilms with a development time of 6 weeks than in biofilms grown for 23 or 40 weeks (IV, Fig. 1A). The biofilms collected after 6 weeks from the beginning of the experiment were characterised with PLFAs present in gram-positive bacteria (*i*-15:0, *i*-16:0, *i*-17:0, *a*-15:0, *br*-17:0), gram-negative sulphate-reducing bacteria (10-Me-16:0), actinomycetes (TBSA), and microbial biomass (14:0, 15:0, 16:0), whereas in the biofilms which had developed for 23 or 40 weeks the percentage of microbial biomass characteristic 18:0 and 20:0 were among the commonest (IV, Fig. 1B). The water residence time also affected the PLFA profile, and it correlated with several fatty acids after 6 (TBSA, 18:1ω7, and 16:1ω7) and 23 (16:0, 18:0) weeks of growth (IV, Fig. 2). However, the PLFAs in biofilms after 40 weeks development time did not correlate with the water residence time, which might reflect the more steady-state nature of microbial community in system compared to the younger biofilms.

In distribution system B, the biofilm development time affected the gram-negative bacteria community structure. LPS 3-OH-10:0 and 3-OH-16:0 correlated positively, and 3-OH-12:0, 3-OH-15:0, and 3-OH-17:0 negatively with the biofilm development time in biofilms with a water residence time of 22 hours (IV, Fig. 3).

5.4 Biomass

5.4.1 Biofilms (I-IV)

The viable microbial biomass, estimated from the PLFA content, was at the same level in biofilms of the full-scale distribution systems A and B (Table 5; III, IV), but it was on average 4.2 (A) and 4.6 (B) times lower than in the drinking water biofilms in the laboratory experiment (Table 5; I, II). R2A plate counts also showed that biofilms from full-scale distribution systems A and B contained on average

4.6 times and 10.8 times less CFU cm^{-2} (A: (4.15±6.79) x 10^5 CFU cm^{-2}, n=8, B: (1.75±0.64) x10^5 CFU cm^{-2}, n=4, O. Zacheus, personal communication) than biofilms in the laboratory experiment ((1.89±3.09) x 10^6 CFU cm^{-2}, n=8, M. Lehtola personal communication). The mean counts of heterotrophic bacteria in drinking water biofilms have been from 10^3 to 10^6 cfu cm^{-2}, and total cell counts from 10^5 to 10^6 cells cm^{-2} (van der Kooij et al., 1995; Pedersen, 1990; Rogers et al., 1994; Zacheus et al., 2000) Our PLFA contents were ten to a hundred times higher than in biofilms reported by Smith et al. (2000). In contrast, biofilms from the full-scale distribution system B contained on average 3.4 times more LPS 3-OH-FAs than the biofilms which developed in the laboratory experiment (Table 6), whereas in distribution system A the levels of LPS 3-OH-FAs were below the detection limit. These differences might be related to the different number of LPS 3-OH-FAs in the samples. The biofilm formation has been similar in many conditions independent of pipe materials PVC, PE or stainless steel and glass or teflon (van der Kooij et al., 1995; Niquette et al., 2000; Pedersen, 1990; Zacheus et al., 2000). Temperature could be one reason for the different PLFA levels. In the full-scale distribution systems A and B, the biofilms were grown with water temperature of 11.2-22.5°C (n=3) and 3.5-11°C (n=7), respectively, for water leaving the waterworks (IV). In the laboratory experiment, the biofilms were developed at a higher water temperature of 21°C (II).

Phosphate-P availability did not affect microbial, or even gram-negative bacteria biomass in the laboratory biofilms from Kuopio, as judged by the contents of PLFAs and LPS 3-OH-FAs, (II). However, Lehtola et al. (2002a) showed that phosphate-P addition increased the total cell counts of bacteria, heterotrophic plate counts, and the content of ATP in biofilms. The increase in heterotrophic plate counts, and content of ATP can be explained with the increase in microbial activity. In addition, phosphate supplementation has been shown to encourage microbial growth in drinking water (Miettinen et al., 1996b; Miettinen et al., 1997b; Sathasivan and Ohgaki, 1999; Sathasivan et al., 1997).

Table 5. Amount of PLFAs and cell estimates[1] in studies I-IV (mean±sd) and some other environments (mean±sd or range).

PLFA content	g	mol	Cells	References
Biofilm cm^{-2} (Finland)				
Laboratory scale, Kuopio (n=16)	$(3.32 \pm 1.74) \times 10^{-7}$	$(1.18 \pm 0.63) \times 10^{-9}$	$(2.37 \pm 1.27) \times 10^{7}$	I, II
Full scale				
A (n=16)	$(7.95 \pm 7.58) \times 10^{-8}$	$(2.85 \pm 2.73) \times 10^{-10}$	$(5.70 \pm 5.46) \times 10^{6}$	IV
B (n=8)	$(7.21 \pm 1.65) \times 10^{-8}$	$(2.6 \pm 0.60) \times 10^{-10}$	$(5.22 \pm 1.19) \times 10^{6}$	III, IV
Water l^{-1} (Finland)				
Kuopio				
Cold (n=2)	$(5.40 \pm 3.03) \times 10^{-7}$	$(1.96 \pm 1.10) \times 10^{-9}$	$(3.92 \pm 2.20) \times 10^{7}$	II
Warm (n=2)	$(1.05 \pm 0.17) \times 10^{-6}$	$(3.75 \pm 0{,}61) \times 10^{-9}$	$(7.50 \pm 1.23) \times 10^{7}$	II
B (n=2)	$(1.47 \pm 0.43) \times 10^{-5}$	$(5.23 \pm 1.56) \times 10^{-8}$	$(1.05 \pm 0.31) \times 10^{9}$	III
Soil g^{-1} (n=2) (Finland)	$(1.01 \pm 0.23) \times 10^{-4}$	$(3.67 \pm 0.83) \times 10^{-7}$	$(7.35 \pm 1.66) \times 10^{9}$	I
Sediment g^{-1} (n=2) (Finland)	$(4.09 \pm 2.23) \times 10^{-6}$	$(1.50 \pm 0.82) \times 10^{-8}$	$(3.00 \pm 1.64) \times 10^{8}$	I
Biofilm accumulation chambers cm^{-2} (USA)		$(1.1\text{- } 2.2) \times 10^{-11}$		Smith et al., 2000
Groundwaters l^{-1} (USA)		2.0×10^{-11}- 1.4×10^{-8}		Chang et al., 2001
Coniferous forest soils g^{-1} org. matter				
Pine (Sweden)		$(1.32\text{- } 1.71) \times 10^{-6}$		Frostegård et al., 1993a
Spruce (Sweden)		$(1.05\text{- } 1.61) \times 10^{-6}$		Frostegård et al., 1993a
Pine and spruce (Finland)		$(1.85\text{- } 2.85) \times 10^{-6}$		Bååth et al., 1995
Pine and spruce (Finland)		$(2.4\text{- } 2.9) \times 10^{-6}$		Pennanen et al., 1998a; Pennanen et al., 1998b
Successional forest soils (Finland)		$(1.00\text{- } 1.43) \times 10^{-6}$		Merilä et al., 2002
Forest soils g^{-1} (five European countries)		1.68×10^{-8}- 1.42×10^{-7}		Zelles et al., 1995a
Peats g^{-1} dry wt (Sweden)		5.0×10^{-12}- 1.9×10^{-8}		Borga et al., 1994
Agricultural soils with different vegetations and agricultural managements g^{-1}				
		1.69×10^{-8}- 1.08×10^{-7}		Zelles et al., 1992
(Germany)		4.19×10^{-8}- 1.63×10^{-7}		Zelles et al., 1994
(Germany)		1.68×10^{-8}- 2.04×10^{-7}		Zelles et al., 1995a
org C (Germany)		$(2.56\text{- } 6.14) \times 10^{-6}$		Zelles et al., 1995b
(USA)	7.4×10^{-6}- 4.56×10^{-5}			Steenwerth et al., 2003
Soils from a desert g^{-1} (Israel)		$(1.47\text{- } 3.69) \times 10^{-10}$		Steinberger et al., 1999
Soil for rice production g^{-1} (USA)		$(1.06\text{- } 2.30) \times 10^{-8}$		Bossio and Scow, 1998
Chinese red soils g^{-1} (China)		2.37×10^{-9}- 4.22×10^{-8}		Yao et al., 2000
Soil from a tropical ecosystem g^{-1}		$(2.97\text{- } 3.25) \times 10^{-9}$		Insam et al., 1999
Silt loam soils g^{-1} dry wt (USA)		$(2.88\text{- } 7.14) \times 10^{-8}$		Ibekwe and Kennedy, 1998

Table 5 continues

Subsurface sediment contaminated with hydrocarbons g^{-1} dry wt			
	(USA)	4.80 x 10^{-11}- 3.84 x 10^{-10}	Fang and Barcelona, 1998
	(Australia)	3.28 x 10^{-11}- 2.98 x 10^{-10}	Franzman et al., 1996
Sediments g^{-1} dry wt (Barbados Trench)	(3.21-8.95) x 10^{-6}		Guezennec and Fiala-Medioni, 1996
Sediments g^{-1} (Japan)	2.60 x 10^{-7}- 3.79 x 10^{-6}		Rajendran et al., 1992
	5.60 x 10^{-7}- 2.97 x 10^{-6}		Rajendran et al., 1994
	7.0 x 10^{-7}- 1.1 x 10^{-6}		Rajendran et al., 1997

[1]Conversion factor: 100 µmol of PLFAs g^{-1} dry weight, and 1 g of bacteria (dry wt) is equivalent to 2.0 x 10^{12} cells dry weight (Balkwill et al., 1988).

Table 6. Amounts of 3-OH-FAs and 2-OH-FAs and cell numbers[1] in studies I, II, IV (mean±sd) and in some other environments (mean±sd or range). For biofilm and water samples the cell numbers are derived from 3-OH-FAs.

	3-OH-FAs		**2-OH-FAs**			**References**
	g	**mol**	**g**	**mol**	**Cells**	
Biofilm cm^{-2} (Finland)						
Laboratory scale, Kuopio (n=16)	$(5.04 \pm 1.22) \times 10^{-10}$	$(2.13 \pm 0.51) \times 10^{-12}$	ND[2]		$(2.84 \pm 0.68) \times 10^{5}$	I, II
Full scale						
A (n=16)	ND					IV
B (n=8)	$(1.70 \pm 0.75) \times 10^{-9}$	$(6.45 \pm 2.85) \times 10^{-12}$	ND		$(8.60 \pm 3.80) \times 10^{5}$	IV
Water, Kuopio l^{-1} (Finland)						
Cold (n=2)	$(3.49 \pm 1.67) \times 10^{-9}$	$(1.45 \pm 0.68) \times 10^{-11}$	ND		$(1.93 \pm 0.91) \times 10^{6}$	II
Warm (n=2)	$(9.15 \pm 1.34) \times 10^{-9}$	$(3.74 \pm 0.51) \times 10^{-11}$	ND		$(4.99 \pm 0.68) \times 10^{6}$	II
Soil g^{-1} (n=2) (Finland)	$(1.68 \pm 0.03) \times 10^{-5}$	$(7.21 \pm 0.12) \times 10^{-8}$	$(2.70 \pm 0.07) \times 10^{-6}$	$(1.01 \pm 0.01) \times 10^{-8}$	$(1.10 \pm 0.02) \times 10^{10}$	I
Sediment g^{-1} (n=2) (Finland)	$(3.14 \pm 0.26) \times 10^{-4}$	$(1.14 \pm 0.10) \times 10^{-6}$	$(2.9 \pm 0.27) \times 10^{-5}$	$(9.08 \pm 0.87) \times 10^{-8}$	$(1.64 \pm 0.14) \times 10^{11}$	I
Agricultural soils with different vegetations and agricultural managements						
g^{-1}		5.24×10^{-10}- 2.30×10^{-9}				Zelles et al., 1992
All OH-FAs g^{-1} (Germany)		$(1.1\text{- } 3.9) \times 10^{-9}$				Zelles et al., 1994
All OH-FAs g^{-1} (Germany)		7.0×10^{-10}- 4.4×10^{-9}				Zelles et al., 1995a
Forest soils g^{-1} (five European countries)		3.80×10^{-9}- 1.68×10^{-8}				Zelles et al., 1995a
Soils from a desert all OH-FAs g^{-1} (Israel)		$2.4\ 0 \times 10^{-11}$- 1.22×10^{-10}				Steinberger et al., 1999
Indoor air in two schools m^{-3} (USA)	$(1.17\text{- } 8.96) \times 10^{-9}$					Liu et al., 2000
Indoor dust in a stable m^{-3} (USA)	$(4.60 \pm 2.20) \times 10^{-9}$					Krahmer et al., 1998
Indoor dust in a dairy m^{-3} (USA)	$(7.50 \pm 3.40) \times 10^{-9}$					Krahmer et al., 1998

[1] Conversion factor: 15 µmol of LPS OH-FAs g^{-1} dry weight, and 1 g of bacteria (dry wt) is equivalent to 2.0×10^{12} cells dry weight (Balkwill et al., 1988)

[2] ND, not detected

The microbial biomass increased in biofilms with increasing water residence time in full-scale distribution systems A and B, as judged by the PLFA content (III, IV). In drinking water distribution system A, biofilms with water residence time of 141 hours contained on average 2.5 and 3.5 times more PLFAs than the biofilms with water residence time of 11 hours and 39 hours, respectively. Also R2A plate count results showed that biofilms from full-scale distribution system A with water residence time of 141 hours ((1.37±0.90) x 10^6 CFU cm^{-2}, n=2) contained on average 15.1 and 12.8 times more viable counts of heterotrophic bacteria than those with water residence time of 10 h ((9.07±4.24) x10^4 CFU cm^{-2}, n=3) and 39 h ((1.06±0.77) x 10^5 CFU cm^{-2}, n=3), respectively (O. Zacheus, personal communication). In the biofilms of the full-scale distribution system B, the PLFA content was on average 1.5 times higher in biofilms with a water residence time of 62 hours compared to those collcted with a water residence time of 22 hours. Similarly, biofilms with a water residence time of 62 hours (2.1 x10^5 CFU cm^{-2}, n=1) contained on average 1.3 times more viable counts of heterotrophic bacteria than those with a water residence time of 22 hours (1.64±0.73) x10^5 CFU cm^{-2}, n=3) in distribution system B (III). The bacterial biomass has been higher in the dead ends of a distribution system compared to main pipes, because of longer residence times and lower chlorine residuals (Niquette et al., 2001). However, Block et al. (1993) have reported a decrease in microbial biomass after a residence time of 40 hours due to nutrient limitation. In the water distribution system B, the biomass of gram-negative bacteria seemed to increase in biofilms with the development times, as judged by the increase in level of LPS 3-OH-FAs. The biofilms developed for 41 weeks contained on average 2.1 times more LPS 3-OH-FAs than those which had only grown for 11 weeks (IV). However, the increase of LPS 3-OH-FAs, might also be related to an accumulation of extracellular polymeric substances (EPS), as the biofilm development time did not affect the total amount of PLFAs. The greatest accumulation of extracellular polymeric substances is known to occur in the late stationary growth phase, when cells are subjected to maximal stress (Uhlinger and White, 1983; Williams and Winpenny, 1977).

5.4.2 Water samples (II, III)

There were on average 27.3 and 14.0 times more PLFAs in drinking water from distribution system B than in the drinking and warm water from Kuopio, respectively (Table 5). R2A plate counts showed a similar trend. There was on average 80 times more viable counts of heterotrophic bacteria in drinking water from distribution system B (8.00±0.85) x10^6 CFU l^{-1} (O. Zacheus, personal communication) than the corresponding number in drinking water from Kuopio (1.00x 10^5 CFU l^{-1}) (II). Biomass in

groundwater samples from a uranium mill tailings site have varied from 2.0 x 10^{-11} to 1.4 x 10^{-8} mol l^{-1} PLFAs (Chang, 2001), and thus were of the same order of magnitude as those found in drinking and warm waters from Kuopio and distribution system B (Table 5).

The content of PLFAs was on average 1.9 times greater in warm water than in drinking water (Table 5; II). The LPS 3-OH fatty acid content in warm water also was on average 2.6 times greater than the amount in drinking water (Table 6; II). The bacterial biomass, presented as ng C /ml, has been found to be greater in warm water than in cold water samples from several buildings (Zacheus and Martikainen, 1995). In distribution system B, the PLFA content in drinking water was at the same level with a water residence time of 22 or 62 hours (III).

5.4.3 Soil and sediment samples (I)

The quantitative amount of PLFAs in the soil was almost the same as found earlier for agricultural soils, but smaller than that reported for coniferous forest soils (Table 5). The OH-FA content in studied soil was higher than that reported for soils ealier (Table 6). In the sediment, the PLFA content was in the same range as found earlier from Japan or Barbados, but much higher than in subsurface sediments contaminated with hydrocarbons (Table 5). The amount of lipid biomarkers is, however, strongly dependent on the environmental conditions (Table 5 and 6).

5.4.4 Use of conversion factors (I-III)

Cell numbers can be calculated from the amount of lipid biomarkers using conversion factors. However, the conversion factors for sediments (Balkwill et al, 1988) were inaccurate at least for OH-FAs in drinking water systems, soil and sediment (I-III, Tables 5 and 6). The ratio of PLFAs to LPS OH-FAs might be different in drinking water biofilms compared to subsurface sediments, where the conversion factors have been originally used. Specific conversion factors should be created for different environments. In aquifers, PLFA conversion factors have failed due to the small sizes of cells resulting from the extreme low nutrient availability (Haldeman et al., 1995). Other factors affecting conversion factors could be taxonomic differences, physical state of microbes and physical conditions of environment (for a review, see Green and Scow, 2000).

5.5 Practical implications

Lipid biomarkers are sensitive methods to analyse microbial communities, biomass and physiological status of microbes without need for culturing, and a great amount of information is achieved with one analysis. PLFAs give information on viable microbes that are not necessarily culturable. Total changes in microbial communities from totally different systems can be compared. Samples can be stored in a freezer several months or even years before analysis. Reagents are rather cheap, although purchase costs for GC-MS can be high. However, good working practices are needed to avoid lipid contamination when small amount of samples, *e.g* biofilms, are analysed. In this study, 20 to 30 liters of water and biofilms from surface area of 42-63 cm^2 were sufficient for analysis. If we want to include also minor fatty acids in our lipid profile, then larger samples or more sophisticated detection techniques (*e.g.* MS-MS system) will be needed. Intepretation of results needs good knowledge of literature.

6 CONCLUSIONS

The lipid biomarkers proved to be applicable, sensitive and specific molecules for the analysis of microbial communities, biomass and physiological state of microbes in drinking water systems.

1. The analytics was developed to determine 2- and 3-hydroxy fatty acids directly from the extraction residue of lipids without any further purification or derivatisation. The sensitivity of the method allowed detection of 3 or 16 LPS 3-OH-FAs from drinking water biofilms and drinking waters. 2-OH-FAs were not detected in biofilms or waters. Both 2- and 3-OH-FAs were detected from soil and sediment samples.

2. A total of 21 to 26 PLFAs were detected in water or biofilm samples, with most of the detected PLFAs being typical for microbial biomass or gram-negative bacteria (16:0, 18:0, 16:1ω7, 18:1ω7). The most abundant LPS 3-OH-FAs were typical for gram-negative bacteria (3-OH-10:0, -12:0, -14:0, -16:0, -18:0). Analyses of PLFAs and OH-FAs complemented each other.

3. The lipid biomarker profiles differed mostly between water and biofilm samples. In the laboratory experiments, an increase in the phosphate-P availability, increased the proportion of PLFAs 16:1ω7*c* and 18:1ω7*c* and LPS 3-OH-14:0 and decreased the proportion of 3-OH-12:0 after 11 weeks of growth, indicating that an increase had occurred in the gram-negative bacteria and possible changes in their community structure. However, the total amount of lipid biomarkers was unaffected. The microbial communities in two full-scale distribution systems differed, possibly reflecting the differences in raw water, water purification processes and distribution systems. In distribution system A, the microbial community structure in biofilms which had developed in 6 weeks was more complex than those growing for 23 or 40 weeks. The viable microbial biomass, estimated on the basis of PLFAs, increased with increasing water residence time in full-scale distribution systems. In distribution system B, the quantities of LPS 3-OH- FAs increased in parallel with the development time of the biofilms.

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