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Gas chromatographic–mass spectrometric detection of 2- and 3-hydroxy fatty acids as methyl esters from soil, sediment and biofilm

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Abstract

Hydroxy fatty acids (OH-FAs) can be used in the characterization of microbial communities, especially Gram-negative bacteria. We prepared methyl esters of 2- and 3-OH-FAs from the lipid extraction residue of soil, sediment, and biofilm samples without further purification or derivatization of hydroxyl groups. OH-FA methyl esters were analyzed using a gas chromatograph equipped with a mass selective detector (GC–MS). The ions followed in MS were m/z 103 for 3-OH-FAs and m/z 90 and M-59 for 2-OH-FAs. The rapid determination of 3- and 2-OH-FAs concomitantly with phospholipid fatty acids provided more detailed information on the microbial communities present in soil, sediment, and drinking water biofilm.

Keywords: Microbial community structure; Hydroxy fatty acid methyl esters; PLFA

1. Introduction

Hydroxy fatty acids typically are constituents of lipopolysaccharides (LPS), which are endotoxins located in the outer membrane of Gram-negative bacteria. One part of the LPS molecule is lipid A, which contains glucosamine disaccharide moiety with phosphoryl groups bound with an ester or amide linkage to 2- and 3-hydroxy fatty acids (2- and 3-OH-FAs). On average, 1 mol of lipid A contains 4 mol of fatty acids [1–5]. The amount of LPS in a bacterial cell is relatively constant, and LPS OH-FAs can be used for determinations of biomass [6]. 2- and 3-OH-FAs have also been detected in fungi and Gram-positive bacteria [7–9]. The mycobacterial 3-OH-FAs have 14–28 carbon atoms [10].

LPS OH-FAs have been used to describe the communities of Gram-negative bacteria present in environmental samples such as in sediments and soils (e.g. Refs. [11,12]). The fatty acids bound to LPS have conventionally been methylated by mild acid hydrolysis [13] from the extraction residue after

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extraction according to Bligh and Dyer [14] to remove lipids. 2-OH- and 3-OH-FAs are then purified, e.g. using thin-layer chromatography, and the hydroxyl groups are further derivatized, for example, to trimethylsilyl or pentafluorobenzoyl methyl esters followed by gas chromatography [1,11,15,16]. These purification and derivatization steps are often time and resource consuming and the chemicals used for derivatization may be health hazards for the scientists performing the procedures.

The aim of this study was to simplify the LPS 2- and 3-OH-FAs analyses after their methylation from the lipid extracted residue [14] by eliminating the purification of hydroxy fatty acid methyl esters, and derivatization of OH groups. The usefulness of the method was determined in three completely different types of environment, including soil, sediment and drinking water biofilm. Information on microbial community structures obtained from gas chromatography–mass spectrometry analyses of 2-OH and 3-OH fatty acid methyl esters using selected ion monitoring was compared with that from phospholipid fatty acids (PLFAs).

2. Experimental

2.1. Chemicals

Reagents were from Merck (Darmstadt, Germany) except for solvents (Rathburn, Peeblesshire, UK), 3-hydroxytridecanoic and 2-hydroxyoctadecanoic acid methyl esters (Larodan, Malmö, Sweden), other fatty acid standards (Sigma, St Louis, MO, USA), NaOH (FF-Chemicals, Yli-Ii, Finland), HCl (Riedel-de Haën, Seelze, Germany) and acetylchloride (Fluka, Buchs, Switzerland).

2.2. Sample collection and preparation

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 dry mass of the soil was determined by oven drying at 105 °C for 24 h. The sample was stored at −20 °C until the lipid analyses.

The sediment sample was taken in April 15, 1997 from Lake Ahmasjärvi in Finland (64°39′N, 26°27′E). Two parallel 4-m long cores were collected in plastic pipes using a Livingstone-type piston sampler [17]. The depth of the water column at the sampling point was 2.2 m. The sealed cores were stored at 4 °C until opening on December 3, 1997. The sediment layer of 310–320 cm was frozen and lyophilized (Edwards 4 K Modulyo freeze-dryer, Edwards, Crawley, UK), and stored at −20 °C until lipid analysis.

For the collection and analyses of the biofilm, the glassware was heated for 6 h at 550 °C (Hobbyceram, Milan, Italy). The formation of biofilm was studied with drinking water taken from Kuopio waterworks (61°51′N, 27°45′E) in Finland. The biofilm was collected 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) for 4 weeks, and prepared as presented in Keinänen et al. [18]. Heterotrophic bacteria in the biofilm were determined on R2A agar after 7 days of incubation at 22 °C [19].

2.3. Lipid extraction

The duplicate samples of reference without environmental material were analyzed in a manner identical to the samples. The lipids were extracted and stored under an atmosphere of N_2. Duplicate soil samples of 3.05±0.04 g wet wt. (2.29±0.03 g dry wt.), sediment samples of 5.24±0.21 g dry wt., and biofilm retentates on filters were extracted in 28.2 ml of chloroform/methanol/buffer (1:2:0.8 v/v/v) by shaking for at least 2 h (biofilm) or overnight (soil and sediment) at 21±2 °C [14,18,20,21]. The buffer for soil and sediment samples was 0.15 *M* citrate buffer at pH 4, and for biofilm samples it was 50 m*M* phosphate buffer at pH 7.4. For the quantification of phospholipids, an internal standard, diheptadecanoylphosphatidylcholine was added, and the samples were shaken for 5 min. The solvents were separated by centrifuging (2000 *g*), chloroform and buffer were added to obtain final ratios of chloroform/methanol/buffer 1:1:0.9 v/v/v, and samples were centrifuged for 10 min (2000 *g*). The solvent layer was separated and evaporated to dryness in a

centrifugal evaporator (Jouan RC10.10, Jouan, Saint-Herblain, France).

2.4. Lipid fractionation

A glass column (height 100 mm, I.D. 6 mm) was packed with 0.75 g of silicic acid (100–200 mesh size, Unisil, Clarkson Chemical, Williamsport, PA, USA) activated at 120 °C for 2 h and washed with chloroform. The lipid extract was applied in chloroform (3–6×100 μl) on the top of the packed column, and neutral lipids were eluted with 10 ml of chloroform, glycolipids with 20 ml of acetone, and phospholipids with 10 ml of methanol [18,20,22]. The phospholipid fraction was evaporated to dryness in the centrifugal evaporator.

2.5. Fatty acid analyses

Internal standards, tridecanoic and nonadecanoic acid methyl esters (phospholipid fractions, the extraction residues of soil and sediment samples) or 3-hydroxytridecanoic and 2-hydroxyoctadecanoic acid methyl esters (the extraction residue of biofilm), detected in low amounts in samples, were added as internal standards. Fatty acids were saponified, methylated, and extracted as methylesters [18,23]. LPS hydroxy-substituted fatty acids of biofilm were treated with mild acid hydrolysis in 2 *M* HCl in methanol [13,24]. Fatty acids methyl esters were analyzed with a Hewlett-Packard (Palo Alto, CA, 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×0.2 mm×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 mass spectra were recorded at an electron energy of 70 eV and a trap current of 300 μA. The temperature of the ion source was 180 °C, and the temperature of the molecular separator was 135 °C. The phospholipid fatty acids were analyzed with selected ion monitoring (SIM) mode 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. To calculate calibration curves for the quantification of fatty acids, calibration standards were made with known ratios of bacterial fatty acids relative to the internal standard methyl nonadecanoate (19:0) [18,25]. 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. In the SIM of 2- and 3-hydroxy fatty acid methyl esters, the ions monitored were *m*/*z* 90, 103 and M-59 [5,26], and *m*/*z* 74 and 199 for internal standards 13:0 and 19:0. The calibration standards contained fatty acids 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. The 2- and 3-OH-FA samples were also analyzed using total ion monitoring. The methyl ester peaks of fatty acids were identified by comparing their mass spectra and retention times with those of standards. The fatty acid content was defined as the sum of the fatty acid methyl esters. The PLFA and LPS OH-fatty acid 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 2.0×10^{12} cells dry weight [27]. All results are presented as mean±standard error.

2.6. Fatty acid nomenclature

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 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 10th 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.

3. Results and discussion

3.1. GC–MS analyses of hydroxy fatty acid methyl esters

In this work, we methylated 3- and 2-OH-FAs from the residues of soil, sediment and biofilm samples extracted according to Bligh and Dyer [14] to remove lipids, and analyzed hydroxy fatty acid methyl esters directly by GC–MS using SIM mode without further purification, or derivatization 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 [5,26]. The SIM chromatograms of 3- and 2-OH-FAs from the sediment sample are presented in Fig. 1. The ion m/z 103 appeared to be specific for 3-OH-FAs (Fig. 1A). The soil and sediment samples were also analyzed using total ion monitoring, and the 3-OH fatty acid peaks presented in Fig. 1A could even be identified from the mass spectra. However, the identity of small peaks seen in Fig. 1A could not be confirmed from the mass spectra, although at least part of them were likely to be monoenoic derivatives of 3-OH fatty acids, known to occur in environmental fatty acids [28]. The ion m/z 90 was less specific for 2-OH-FAs, and additional peaks were detected in the GC–MS chromatogram (Fig. 1B). However, the ion m/z 90 was detected concomitantly with m/z M-59 only in 2-OH-FAs. Thus, the intensity ratio of ion m/z 90 to M-59, and the information on relative retention times (t_R) (Tables 1 and 2) can be used to confirm the identities of each 2-OH-FA. As can be seen from the relative retention times of 3- and 2-OH-FAs with

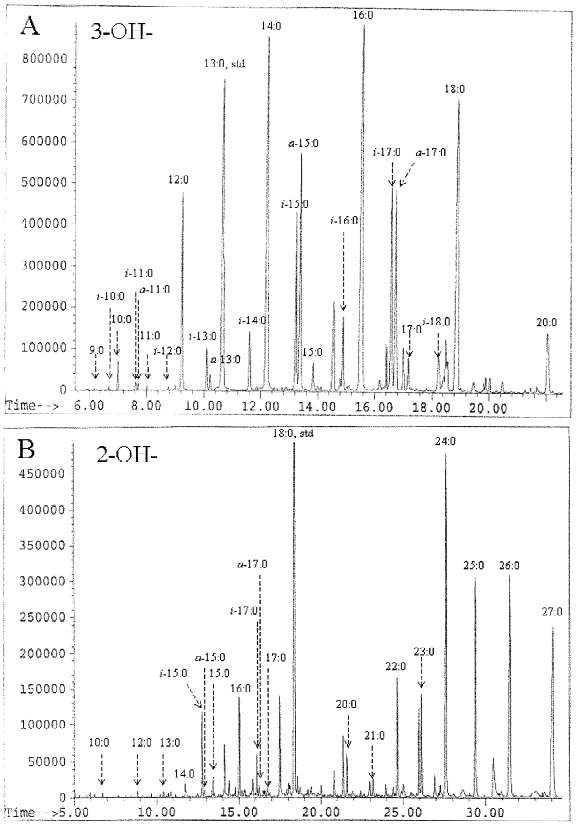

Fig. 1. The GC–MS chromatograms for selected ion monitoring of 3-hydroxy (m/z 103) (A) and 2-hydroxy (m/z 90) (B) fatty acid methyl esters extracted from sediment.

Table 1
Percentages of 3-hydroxy fatty acids (mean±SE, $n=2$) in soil, sediment and biofilm, and relative retention times (RT_{rel})

3-OH-	RT_{rel}	Soil (%)	Sediment (%)	Biofilm (%)
i-8:0	0.587	0.41±0.02	0.03±0.00	nd
8:0	0.611	8.69±0.30	0.14±0.02	nd
i-9:0	0.654	0.27±0.04	0.08±0.01	nd
9:0	0.682	2.24±0.07	0.06±0.00	nd
i-10:0	0.734	0.17±0.01	0.13±0.01	nd
10:0	0.767	26.20±0.25	0.77±0.04	8.25±1.86
i-11:0	0.832	2.02±0.07	0.27±0.03	nd
a-11:0	0.840	nd	0.14±0.01	nd
11:0	0.873	0.83±0.03	0.14±0.02	nd
i-12:0	0.951	tr	0.09±0.00	nd
12:0	1.000	13.33±0.28	6.24±0.43	28.00±0.81
i-13:0	1.091	3.12±0.19	1.59±0.05	nd
a-13:0	1.103	0.95±0.01	0.58±0.02	nd
13:0	1.147	0.70±0.02	0.70±0.03	nd
i-14:0	1.250	1.81±0.00	3.01±0.08	nd
14:0	1.311	17.88±0.49	18.61±0.48	63.75±1.05
i-15:0	1.421	7.02±0.12	6.41±0.02	nd
a-15:0	1.436	3.06±0.03	12.33±0.26	nd
15:0	1.487	0.56±0.06	1.04±0.02	nd
i-16:0	1.600	1.24±0.07	2.54±0.11	nd
16:0	1.668	5.65±0.06	20.07±0.22	nd
i-17:0	1.783	1.16±0.03	7.38±0.11	nd
a-17:0	1.800	0.64±0.01	7.97±0.41	nd
17:0	1.851	0.19±0.00	0.63±0.01	nd
i-18:0	1.987	nd	1.19±0.06	nd
18:0	2.032	1.38±0.15	7.21±0.26	nd
20:0	2.384	0.36±0.07	0.64±0.01	nd

nd, not detected; tr, trace amount.

Table 2
Percentages of 2-hydroxy fatty acids (mean±SE, $n=2$) in soil and sediment, and relative retention times (RT_{rel})

2-OH-	RT_{rel}	Soil (%)	Sediment (%)
7:0	0.553	4.37±0.01	0.67±0.04
8:0	0.617	2.58±0.10	1.51±0.14
a-9:0	0.663	2.52±0.12	nd
9:0	0.688	3.85±0.08	1.97±0.14
i-10:0	0.755	2.84±0.08	nd
10:0	0.772	3.78±0.16	3.60±0.30
11:0	0.875	1.07±0.03	1.85±0.11
12:0	1.000	20.70±0.20	2.66±0.03
13:0	1.153	7.63±0.39	2.83±0.02
14:0	1.314	6.33±0.22	4.81±0.04
i-15:0	1.426	6.01±0.10	9.36±0.09
a-15:0	1.441	1.72±0.06	1.69±0.17
15:0	1.494	2.40±0.07	4.84±0.13
16:0	1.681	9.09±0.55	13.19±0.26
i-17:0	1.801	1.21±0.13	3.18±0.08
a-17:0	1.817	1.35±0.25	0.91±0.06
17:0	1.873	2.14±0.32	2.13±0.11
18:0	2.061	1.24±0.07	2.49±0.19
19:0	2.248	nd	0.80±0.01
20:0	2.431	1.85±0.35	1.84±0.01
21:0	2.609	0.74±0.05	1.90±0.00
22:0	2.780	3.38±0.69	4.86±0.02
23:0	2.946	2.67±0.10	3.31±0.23
24:0	3.107	8.11±1.05	9.34±0.14
25:0	3.279	1.31±0.12	7.14±0.24
26:0	3.482	1.12±0.04	6.78±0.10
27:0	3.729	nd	6.35±0.12

nd, not detected.

the same molecular mass, 3-OH-FAs eluted slightly earlier than 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 detection limit for 3-OH-13:0 methyl ester was 2.9-times lower than the 226 fmol reported for the heptafluorobutyric anhydride derivative of 3-OH-14:0 analyzed with electron-capture detection [11]. The lowest detectable amounts of the injected trimethylsilyl and pentafluorobenzoyl methyl ester derivatives of 3-OH-14:0 using SIM have been 9 fmol (3 pg) and 2 fmol (1 pg), respectively [1], which are 9- and 39-times lower than our detection limit of 3-OH-13:0. Our detection limit for 2-OH-18:0 was 800-times higher than the 2 fmol reported by Odham et al. [29], in which study 2-OH-18:0 was derivatized with pentafluorobenzyl bromide and analyzed with GC–MS equipped with negative ion detection in the selected ion mode. The use of 2- and 3-OH-FAs analysis in microbial environmental monitoring was determined by analyzing soil, sediment and biofilm, and comparing the results with microbial community structures obtained with PLFAs.

3.2. Soil

The numbers of 3- and 2-OH-FAs detected in soil were 25 (Tables 1 and 2). The most abundant 3-OH-FAs, 3-OH-8:0, 3-OH-10:0, 3-OH-12:0, 3-OH-14:0, 3-OH-*i*-15:0 and 3-OH-16:0, represented 79% of all 3-OH-FAs, while 2-OH-12:0, 2-OH-13:0, 2-OH-14:0, 2-OH-*i*-15:0, 2-OH-16:0 and 2-OH-24:0 accounted for 58% of all 2-OH-FAs. Such high percentages of several straight-chain, and *iso*- and *anteiso*-branched LPS OH-FAs with 10–18 carbons, typical for Gram-negative bacteria [4], may be related to the species diversity. Indeed, the PLFA profiles supported this assumption, due to the presence of 28.7% of monoenoic and cyclopropane fatty acids, at least partly indicative of Gram-negative bacteria [4]. The markers for Gram-negative sulfate-reducing bacteria, 10-Me-16:0 and *br*-17:1 [30] were also detected in amounts of 8.6%.

The 3- and 2-OH-FAs not typical for Gram-negative bacteria, such as 3-OH-20:0, 2-OH-20:0, 2-OH-22:0, 2-OH-23:0, 2-OH-24:0, 2-OH-25:0 and 2-OH-26:0 (Tables 1 and 2), might originate from several different species, such as yeasts and other fungi, and Gram-positive actinomycetes [7–10]. In the PLFAs, 30 in number, terminally branched saturated fatty acids indicating Gram-positive bacteria [31] and straight-chain saturated fatty acids indicating general biomass, were found in almost equal amounts to monoenoics (26.8–29.0%) (Table 3). TBSA and 18:2ω6, characteristic of actinomycetes and fungi [8,9,30] were also detected in amounts of 2.0 and 1.9%, respectively. The previously reported PLFA profiles of soils have been rather similar to our results, containing 20 to 48 phospholipid fatty acids, with straight-chain, unsaturated, and methyl-branched chain fatty acids in the range 15–25, 30–50 and 24–40%, respectively [12]. The quantitative amounts

Table 3
Phospholipid fatty acid (PLFA) profiles (mean±SE, $n=2$) in soil, sediment and biofilm

PLFA	Soil (%)	Sediment (%)	Biofilm (%)
Saturates			
14:0	2.72±0.49	10.79±0.04	9.38±0.55
15:0	1.26±0.07	3.39±0.32	4.50±0.75
16:0	18.93±0.88	38.97±0.14	29.41±2.37
18:0	3.11±0.54	19.48±0.31	24.88±0.81
20:0	0.83±0.09	nd	nd
Total saturates	26.84±0.77	72.63±0.44	68.17±0.26
Monoenoics			
16:1ω9	2.27±0.41	tr	nd
16:1ω7	8.07±0.24	0.47±0.01	0.47±0.11
16:1c	nd	0.04±0.01	nd
16:1ω5	2.38±0.00	0.16±0.01	0.78±0.07
18:1ω9	4.66±0.39	0.93±0.01	8.06±0.68
18:1ω7	4.78±0.47	1.18±0.05	14.39±0.20
18:1c	0.36±0.10	0.38±0.01	nd
18:1ω5	0.72±0.11	nd	nd
cy-17:0	2.47±0.44	nd	1.13±0.20
cy-19:0	3.02±0.14	nd	tr
Total monoenoics	28.73±0.73	3.16±0.03	24.83±0.64
Polyenoics			
18:2ω6	1.89±0.30	1.39±0.02	1.53±0.31
Terminally branched saturates			
i-14:0	0.75±0.10	2.14±0.04	0.18±0.04
i-15:0	10.60±0.79	6.35±0.08	0.51±0.09
i-16:0	7.26±0.01	2.96±0.04	0.47±0.05
i-17:0	3.07±0.22	3.65±0.06	0.47±0.05
i-18:0	0.50±0.24	0.03±0.02	0.42±0.03
a-15:0	5.18±0.43	4.61±0.09	0.63±0.09
a-17:0	1.66±0.06	2.08±0.08	1.09±0.12
Total Ter branched saturates	29.02±1.29	21.82±0.39	3.76±0.08
Middle branched saturates			
br-15:0a	1.31±0.10	0.11±0.01	1.01±0.77
br-15:0b	0.41±0.06	0.37±0.00	0.24±0.05
10-Me-16:0	5.50±0.33	0.38±0.01	0.28±0.02
br-17:0	0.69±0.07	nd	nd
TBSA	2.03±0.07	0.10±0.01	nd
Total Mid branched saturates	9.94±0.17	0.96±0.00	1.52±0.79
Branched monoenoics			
br-15:1a	0.13±0.02	nd	nd
br-15:1b	0.38±0.04	nd	nd
br-17:1	3.06±0.06	0.04±0.00	nd
Total branched monoenoics	3.58±0.08	0.04±0.00	nd

nd, not detected; tr, trace amount.

Table 4
Quantitative amounts of PLFAs, 3-OH-FAs and 2-OH-FAs in soil, sediment and biofilm and calculated cell numbers [27]

FA type	Mean±SE ($n=2$)		
	Soil (g^{-1})	Sediment (g^{-1})	Biofilm (cm^{-2})
PLFAs			
g	$(1.01\pm0.16)\times10^{-4}$	$(4.09\pm1.58)\times10^{-6}$	$(2.21\pm0.32)\times10^{-7}$
mol	$(3.67\pm0.59)\times10^{-7}$	$(1.50\pm0.58)\times10^{-8}$	$(7.90\pm1.10)\times10^{-10}$
cells	$(7.40\pm1.20)\times10^{9}$	$(3.00\pm1.16)\times10^{8}$	$(1.58\pm0.22)\times10^{7}$
3-OH-FAs			
g	$(1.68\pm0.02)\times10^{-5}$	$(3.14\pm0.19)\times10^{-4}$	$(4.08\pm0.43)\times10^{-10}$
mol	$(7.21\pm0.09)\times10^{-8}$	$(1.14\pm0.07)\times10^{-6}$	$(1.66\pm0.17)\times10^{-12}$
2-OH-FAs			
g	$(2.70\pm0.05)\times10^{-6}$	$(2.92\pm0.19)\times10^{-5}$	nd
mol	$(1.01\pm0.01)\times10^{-8}$	$(9.08\pm0.62)\times10^{-8}$	nd
cells[a]	$(1.10\pm0.10)\times10^{10}$	$(1.64\pm0.10)\times10^{11}$	$(2.21\pm0.23)\times10^{5}$

nd, not detected.
[a] Calculated using the sum of the quantitative amounts of 3- and 2-OH-FAs, expect in biofilm using the amount of 3-OH-FAs.

of PLFAs and OH-FAs in soil (Table 4) were slightly higher than the 15–202 nmol g^{-1} of PLFAs and 1.5–27 nmol g^{-1} of OH-FAs reported by Zelles [12], probably due to the different soil types. The cell densities of 7.4×10^{9} and 1.1×10^{10} calculated from PLFAs and OH-FAs contents of the soil, respectively, were quite similar, when the conversion factors by Balkwill et al. [27] were used.

3.3. Sediment

There were 27 of the 3-OH-FAs and 25 of the 2-OH-FAs detected in sediment (Tables 1 and 2). The characteristic 3-OH-FAs were 3-OH-12:0, 3-OH-14:0, 3-OH-*i*-15:0, 3-OH-*a*-15:0, 3-OH-16:0, 3-OH-*i*-17:0, 3-OH-*a*-17:0 and 3-OH-18:0, accounting for 86% of total 3-OH-FAs. The following 2-OH-FAs 2-OH-*i*-15:0, 2-OH-16:0, 2-OH-24:0, 2-OH-25:0, 2-OH-26:0 and 2-OH-27:0 accounted for 52% of the total 2-OH-FAs. As in the soil, straight-chain and *iso*- and *anteiso*-branched LPS OH-fatty acids with 10–18 carbons at least partly originated from Gram-negative bacteria [4]. However, a part of the hydroxy fatty acids might indicate other sources, such as Gram-positive actinomycetes [7,10], sedimentation of dead plant material [12,28], and possibly also yeast and other fungi [8,9]. The 3- and 2-OH-FAs with a carbon chain length greater than 14 carbons had a tendency to be more abundant in sediment, and hydroxy fatty acids with a chain length shorter than 14 carbons generally were more abundant in soil (Tables 1 and 2). Altogether, the hydroxy fatty acids in the sediment originated from different organisms than those in soil.

The PLFAs of sediment had a high content of saturated PLFAs (72.6%), indicating the general biomass. The second common PLFA group was terminally branched saturated acids (21.8%), characteristic of Gram-positive bacteria and also detected in Gram-negative bacteria [4,31]. Of the 24 PLFAs detected in sediment, 14:0, 16:0, 18:0, *i*-15:0 and *a*-15:0 acids dominated. The percentages of monoenoics, 10-Me-16:0 and *br*-17:1, TBSA and 18:2ω6 indicative of Gram-negative bacteria, Gram-negative sulfate-reducing bacteria, actinomycetes and fungi were low at 3.2, 0.4, 0.1 and 1.4%, respectively (Table 3). The overlap in these fatty acids between different microbial groups is also possible, such as monoenoics may also originate from microbial groups other than Gram-negative bacteria [7–9,31]. Bacteria may also synthesize polyunsaturated fatty acids at low temperatures [32,33]. However, oxygen-dependent desaturation typical for fungi and mycobacteria [34] was not likely to be found in the lake sediment at a depth of 3.1–3.2 m.

The PLFAs content of 15.0±5.8 nmol g^{-1} in lake sediment at the depth of 3.1–3.2 m (Table 4) was rather similar to the value of 28±9 nmol g^{-1} found

in estuarine sediment, whereas the OH-FAs amount of 1.2 μmol g^{-1} in the lake sediment was much higher than the 5.0 nmol g^{-1} reported in estuarine sediment [11]. Consequently, the cell estimate was 547-times higher when calculated on the basis of OH-FAs content compared to cell estimation on the basis of PLFAs content. The turnover rate of phospholipids is known to be rapid [21]. In the completely closed and separate lake sediment with radiocarbon ages of approximately 8000 years [35] at the depth of 3.1-3.2 m the amounts of hydroxy fatty acids excreted outside the cell, and originating from dead organisms and even from the deposited plant material could be higher than in the estuarine sediment values reported by Parker et al. [11]. According to Balkwill et al. [27], the ratio of PLFAs to LPS-OH-FAs for subsurface sediments was proposed to be 100:15≈6.7, whereas in this study the ratio was 0.013±0.006.

3.4. Biofilm

Three 3-OH-FAs, 3-OH-10:0, 3-OH-12:0 and 3-OH-14:0, which are typical of Gram-negative bacteria [4], were detected in biofilm (Table 1), whereas the 2-OH-FAs were below the detection limit. The most common 3-OH-FA was 3-OH-14:0 and 3-OH-10:0 was the least common. The PLFA profile indicated that a community of Gram-negative bacteria was probably predominant, because the proportion of monoenoics was high (24.8%) (Table 3 [4]). According to many studies, the most abundant microbial group in drinking water systems is Gram-negative bacteria [36–43]. The amounts of 18:2ω6 and terminally branched fatty acids indicative of eukaryotes and Gram-positive bacteria were as low as 1.5 and 3.8%, respectively. There were 21 PLFAs present in the biofilm with a high content (68.2%) of saturated PLFAs indicating general biomass. The fatty acids 14:0, 16:0, 18:0, 18:1ω7 and 18:1ω9 accounted for 86% of PLFAs.

The PLFAs content in biofilm was 800 pmol cm^{-2} (Table 4), and the estimated cell numbers $(1.58\pm0.22)\times10^{7}$ cells cm^{-2}. The result 2.46×10^{4} cfu cm^{-2} obtained by culture on R2A agar was close to that estimated from the PLFAs content, when one considers that in Finland less than 0.5% of ground water microbes are culturable [44]. The cell density calculated from the OH-FAs content was $2.21\pm0.23\times10^{5}$, which is 71-times smaller than that obtained from PLFAs. The ratio of PLFAs to LPS OH-FAs might be different in a drinking water biofilm compared to subsurface sediments, for which these conversion factors were originally created [27]. On the other hand, the analysis of 3-OH-FAs was close to the detection limit and 2-OH-FAs were not detected. The measured quantitative amount of OH-FAs in biofilm was possibly smaller than the real amount.

3.5. Concluding remarks

This study showed that when the selected ion monitoring mode was used in mass spectrometry, LPS 2- and 3-hydroxy fatty acid methyl esters could be analyzed directly from the lipid-extracted residue of environmental samples, such as soil, sediment and drinking water biofilm, without further purification or derivatization of hydroxyl groups. However, a great part of impurities had already been removed in the lipid extraction [14] before the fatty acid methylation, and in the extraction of fatty acid methyl esters. 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), corresponding to cell amounts of 1×10^{4} and 2×10^{5}, respectively [27]. In environmental samples, however, the detection limits for hydroxy fatty acids were likely to be higher than for standard hydroxy fatty acids, due to the greater background levels of impurities. In the biofilm, the estimation of 2.2×10^{8} cells on the basis of PLFAs was not sufficiently high enough for the detection of 2-OH-FAs. The combined rapid analysis of 2- and 3-OH-FAs with results from PLFAs provided more detailed information on the microbial communities present in different types of environmental samples.

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