Quantitative Sampling of Indoor Air Biomass by Signature Lipid Biomarker Analysis: Feasibility Studies in a Model System

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Standard methods for characterizing the microbial content of indoor air rely on detection of viable microbes that are collected in water or impacted onto growth substrata. Viable counts consistently underestimate microbial numbers in environmental samples by 90–99.9%. Assays of biochemical components characteristic of all cells provide an assessment method independent of the ability to culture the organisms. This article provides evidence that lipid analysis provides quantitative recovery of known volumes of culturable bacteria. Monocultures of *Escherichia coli, Bacillus subtilis,* and *Legionella bozemanii* and two mixtures of these organisms were deposited onto glass fiber filters using an air test stand constructed as a modification of the ASTM 1215 standard. Filter deposited biomass was determined by three methods: (1) viable counts of bacteria sampled using an impinger, (2) phospholipid ester-linked fatty acid (PLFA) analysis, and (3) hydroxy fatty acid (OH FA) analysis. PLFA and OH FA were quantified by gas chromatography-mass spectrometry. Bacterial counts determined as colony forming units/filter following impinger sampling ranged between 25.6% (*B. subtilis*) to 18.5% (*L. bozemanii*) of the counts determined by PLFA analysis. Sampling efficiency and mechanical and dehydration stresses to the bacteria during aerosolization could have caused the decreased culturable viable counts in comparison to PLFA analysis of viable cells. Signature lipid analysis provides insight into the community composition. Analysis of the glass filters after aerosolization showed that capture was not selective, and monocultures and mixtures gave the expected signature lipid patterns enabling differentiation between species.

Keywords: bacteria, indoor air, lipid biomarkers

There is an ever-increasing concern regarding airborne microorganisms within indoor air environments. Closed indoor environments with a high density of people are susceptible to the spread of airborne infections. Exposure to airborne microorganisms may give rise to infectious diseases,[1–3] allergenic responses,[4,5] and respiratory problems.[5,6] Biocontaminants typically found in indoor air environments include bacteria, fungi, algae, protozoa, and dust mites.[7] Mycotoxins, endotoxins, pollens, and residues of organisms found in indoor air are also known to cause adverse health effects.[5–10] A quantitative detection/identification technique independent of culturability that assays both culturable and nonculturable microbes, antigenic fragments of microbes, and immuno-potentiators is critical in defining risks from indoor air biocontamination.

Traditionally, methods employed for the monitoring of microorganism numbers in indoor air environments involve classical culture-based techniques and/or direct microscopic counting. It has been repeatedly documented that viable counts of microorganisms from environmental samples only account for between 0.1–10% of the total community detectable by direct counting.[11–15] The classic viable microbiologic approach does not provide accurate estimates of microbial fragments or other indoor air components that can act as antigens and induce or potentiate allergic

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responses. Despite techniques designed to damage the microbes as little as possible,[16] microbial stress has been shown to result from air sampling, aerosolization, and microbial collection.[17–20] Commonly used sampling devices (e.g., Andersen samplers, all glass impingers, Mattson-Garvin slit-to-agar samplers) differ in their collection efficiency and microorganism recovery. The highest collection efficiency often results in microbial injury and lower viable bacterial recovery.[20] Filtration devices can collect particles at almost 100% efficiency, but captured microorganisms may become dehydrated and damaged resulting in nonculturability.[21,22]

The assays described herein do not rely on cell culturability. In these assays microbial biomass is determined from universally distributed lipid biomarkers that are characteristic of all cells.[23] Specific groups of microorganisms often contain characteristic lipid patterns and lipid extraction; purification and consequent analysis via gas chromatography-mass spectrometry (GC-MS) has enabled determination of the microbial biomass, nutritional status, and community composition from different environments including sediment, soil, and water.[13–15,24] Extractable phospholipids are found in all cell membranes and walls of microorganisms, are actively metabolized during growth, have a relatively rapid turnover, and have been used as biomarkers in many different environments.[14] The 3-hydroxy fatty acids (3-OH FA) are found in lipopolysaccharide (LPS) of gram-negative bacteria,[25] and have been used as specific markers for these bacteria and for endotoxin in air and dust samples.[26,27]

This study was conducted to determine the applicability of lipid biomarker analyses to the quantitative characterization of air biocontaminants. Microorganisms were released into a contained Biocontaminant Indoor Air Quality Test Stand (BIAQTS) and collected on glass fiber filters. The phospholipid ester-linked fatty acid (PLFA) and hydroxy fatty acid (OH FA) patterns were recovered from the filters to determine total microbial biomass independent of cell culturability from the known biomass of aerosolized microbes and compared with the culturable microbes collected in an all-glass impinger (AGI).

MATERIALS AND METHODS

BIAQTS

The BIAQTS was an adaptation of the ASTM 1215 test stand[28] (Figure 1) and was built for the authors by Dr. Wayne Davis (University of Tennessee). The ASTM 1215, the BIAQTS and the TSI Model 3076 Constant Output atomizer used to aerosolize the bacteria have been described in detail elsewhere.[28–30] Briefly, the BIAQTS provides the ability to expose a filter to a constant airflow containing a consistent concentration of microorganisms. The BIAQTS also has ports installed above and below the filter holder enabling the airflow to be sampled isokinetically and organism numbers to be quantified. Filtered and dried air passed through the atomizer containing the bacteria in buffer (0.1 M sodium phosphate buffer (0.025 M NaH_2PO_4, 0.075 M Na_2HPO_4; pH 7.4). This produced an atomized aerosol that contained droplets. The aerosol then passed into the test stand where it was mixed and diluted with the prefiltered air to produce a dried aerosol, thereby ensuring a monodispersed aerosol of bacteria. This aerosol passed through the test stand at 424.5 L/min (15 ft^3/min).

Bacterial Strains and Culture Conditions

Escherichia coli (ATCC 8739) and *Bacillus subtilis* (obtained from A. Arrage at the Center for Environmental Biotechnology) were grown in Luria Broth Base (Miller's LB Broth Base; Becton Dickinson, Cockeysville, Md.) overnight at 37°C and harvested in stationary phase. Growth medium was removed by centrifugation at 8000 g for 20 min, and the cells were washed three times in cold sodium phosphate buffer (0.1 M; pH 7.4). *Legionella bozemanii* (strain MI-15; obtained from W. Mayberry at East Tennessee State University), was grown on buffered charcoal yeast extract agar (BCYE; Becton Dickinson) at 37°C and harvested in stationary phase. Cells were suspended and washed in cold sodium phosphate buffer (0.1 M; pH 7.4). Cell suspensions were diluted to optical density of 1.0 at 610 nm (Diode Array Spectrophotometer 8450A; Hewlett Packard). Viable counts (colony forming units (CFU)/mL) were determined using spread plate counts on either Luria Agar (Miller's LB agar; *E. coli* and *B. subtilis,* 24 hr growth) or buffered charcoal yeast extract agar (*L. bozemanii,* 72 hr growth).

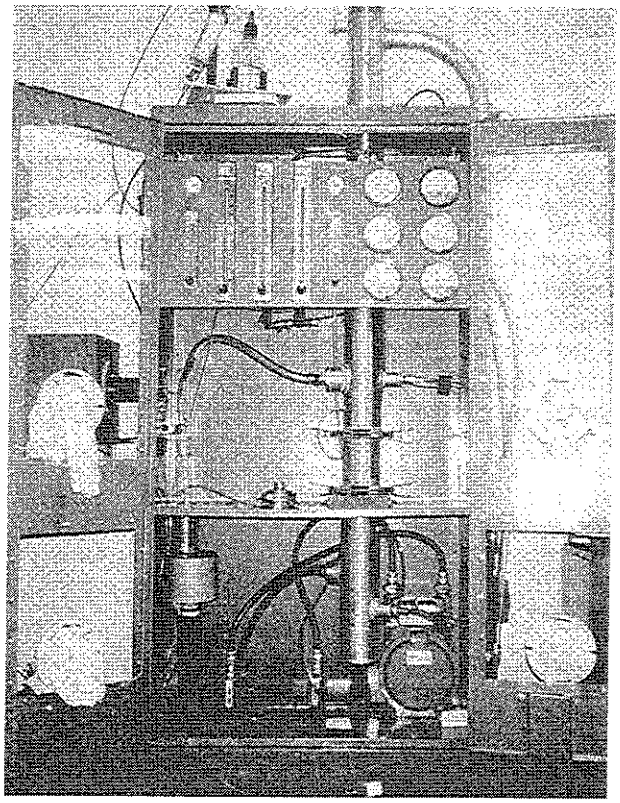

FIGURE 1. Biocontaminant indoor air quality test stand (BIAQTS)

BIAQTS Calibration

Isokinetic sampling at a constant flow rate is important for accurate and quantitative data collection from the BIAQTS airstream. The sampling probes for the single stage Andersen samplers (Graseby-Andersen, Inc. Atlanta, Ga.) and the AGI (Wheaton; Millville, N.J.) were sized to obtain isokinetic samples from the BIAQTS. To ascertain that sampling was constant and quantifiable, the BIAQTS was calibrated using the upstream Andersen sampler, which had a flow rate of 28.3 L/min (1 ft^3/min). *Escherichia coli* in 0.1 M sodium phosphate buffer (pH 7.4; 10^6 CFU/mL) was aerosolized and introduced into the BIAQTS at a flow rate of 424.5 L/min (15 ft^3/min). Samples were collected using the Andersen sampler over time periods of 10 sec, 30 sec, and 1, 2, 5, and 10 min. The Luria agar plates from the Andersen

sampler were incubated at 37°C for 24 hr prior to counting colony numbers using the positive hole method.

Lipid biomarker analysis required quantification of a substantially greater number of bacteria than those that could be reliably quantified using an Andersen sampler due to the finite number of holes in the Andersen impactor plate. For this purpose an AGI sampler was used. However, the AGI required calibration to determine sampling efficiency in comparison to the Andersen sampler. The AGI was attached to the upstream probe and an Andersen sampler attached downstream of the AGI. Bacteria were collected into 0.1 M sodium phosphate buffer (pH 7.4, 80 mL; 4°C) contained in the AGI. In addition, another Andersen sampler was attached to the downstream probe (Figure 1). *Escherichia coli* in 0.1 M sodium phosphate buffer (pH 7.4; approximately 10^7 CFU/mL) was aerosolized and introduced into the BIAQTS at a flow rate of 15 ft^3/min. Bacteria were sampled into the Andersen sampler for 10 sec (in triplicate) and into the AGI for 1 hr (in triplicate). Phosphate buffer was decanted into a sterile bottle and the AGI washed out with sterile phosphate buffer (10 mL × 2). The phosphate buffer and the two 10-mL washes were pooled, and viable counts were made on Luria agar. Plates were incubated overnight (*E. coli*) at 37°C prior to colony counting.

Bacterial Sampling

Glass fiber filters (15 cm diameter glass microfiber filters; GF/D; pore size 2.7 µm; Whatman International Ltd., Maidstone, England) were sterilized and carbon contaminants incinerated by heating overnight in a muffle furnace at 450°C. Using the BIAQTS, *E. coli* (O.D. 1.0; 8.6×10^8 viable bacteria/mL), *B. subtilis* (O.D. 1.0; 2.0×10^8 viable bacteria/mL), and *L. bozemanii* (O.D. 1.0; 9.0×10^8 viable bacteria/mL) (each in 0.1 M phosphate buffer, pH 7.4) were deposited over 30 min onto separate filters in duplicate. In addition, two mixtures of bacteria containing *E. coli:B. subtilis:L. bozemanii* at ratios of either (1:1:1 v/v/v) or (1:1:2 v/v/v) were deposited over 30 min onto separate glass microfiber filters again in duplicate. Filters were stored at –20°C prior to lipid biomarker analysis as described below.

To enable a comparison between lipid biomarker data and cell numbers, bacteria were sampled concurrently into the upstream AGI containing 0.1 M sodium phosphate buffer (80 mL, pH 7.4) at a flow rate of 28.3 L/min (1 ft^3/min). When collecting the buffer after sampling, fresh sterile buffer was used to wash the sampler (10 mL × 2). The samples were diluted by one order of magnitude and 100 µL of each was plated out on either Luria agar (*E. coli, B. subtilis*) or BCYE agar (*L. bozemanii*). Plates were incubated at 37°C (*E. coli, B. subtilis*) or for 72 hr (*L. bozemanii*) prior to colony counting. Any viable bacteria passing through the filters were sampled using the Andersen sampler attached downstream of the glass fiber filters. Media plates were again incubated overnight (*E. coli, B. subtilis*) at 37°C, or for 72 hr (*L. bozemanii*) prior to colony counting.

Lipid Biomarker Analysis

All solvents were of GC grade and were obtained from Baxter Scientific Products (McGaw Park, Ill.). All glassware was washed in a 10% (v/v) Micro cleaner solution (Baxter Diagnostics, Deerfield, Ill.), rinsed five times in tap water and then five times in deionized water. The glassware was then heated overnight in a muffle furnace at 450°C.

Polar Lipid Extraction and Analysis

Sample filters, pure sample bacteria (1 mL of each pure organism), controls (glass fiber filter onto which no bacteria had been deposited and a buffer blank) were extracted for lipids using the modified Bligh and Dyer extraction.[23] In comparison with pure culture, 80±15% extraction efficiency was determined for pure bacteria deposited on glass fiber filters (data not shown). Briefly, samples were extracted in separatory funnels containing a single phase system (chloroform:methanol:phosphate buffer (50 mM, pH 7.4); 1:2:0.8 v/v/v) for a minimum of 4 hr before adding chloroform and deionized water (final solvent ratios, chloroform:methanol: phosphate buffer/water; 1:1:0.9 v/v/v) to form two phases. The phases were allowed to separate for 24 hr, and the lipid phase was dried by rotary evaporation. The lipids were fractionated into neutral-, glyco- and polar lipids on a silicic acid column as described.[31] The phospholipid containing polar lipid fraction was subjected to a mild alkaline methanolysis, transesterifying the fatty acids into methyl esters.[31]

Fatty acid methyl esters were separated and quantified by GC-MS. Samples were dissolved in hexane containing nonadecanoic acid methyl ester (C 19:0; 50 pmoles/µL). Samples were injected into a Hewlett-Packard HP5890 series II gas chromatograph interfaced with a HP5972 series mass selective detector (Hewlett Packard, Wilmington, Del.). The gas chromatograph was equipped with a nonpolar column (HP1; 50 m, 0.25 mm internal diameter, 0.25 df; Hewlett Packard). Splitless injections were made using a Hewlett-Packard model 7673 autosampler. The carrier gas (helium) was used at an inlet pressure of 2.1 kg/cm^2 (30 lb/in^2). The column was programmed from an initial temperature of 100°C to 150°C at 10°C per min, held at this temperature for 1 min, and then raised at 3°C per min to 282°C where it was held for 5 min. The injector and source housing temperatures were maintained at 270°C and 290°C, respectively. Mass spectra were collected at an electron energy of 70 eV. Fatty acids were identified both by relative retention times compared with authentic standards (Matreya Inc., Pleasant Gap, Pa.) and by the mass spectra. Fatty acids are designated as described by Ringelberg et al.[32]

Hydroxy Fatty Acid Extraction and Analysis

Sample filters and controls (glass fiber filter onto which no bacteria had been deposited and a buffer blank) were extracted for hydroxy fatty acids using an acid hydrolysis method [33] with some modifications. Samples were hydrolyzed overnight at 100°C in 10 mL 6 M HCl in PTFE capped glass tubes. Hydrolysates were partitioned using chloroform (5 mL) and water (1 mL). The organic phase was collected and dried under a stream of nitrogen. Internal standard was added (3-OH 13:0; 100 ng) and samples were esterified in 1 mL of 1 M HCl in methanol for 1 hr at 80°C, after which the hydroxy acid methyl esters were twice extracted in hexane (2 mL).[34] The hexane layers were pooled, dried down under nitrogen, redissolved in hexane:dichloromethane (1:1 v/v), and applied onto a disposable silica gel column (1 mL, Bond-Elut, Analytichem, Harbor City, Calif.) to separate the methyl esters of hydroxylated acids from those of nonhydroxylated acids.[34] Prior to use the column was washed with 1 mL of diethyl ether and 1 mL hexane:dichloromethane (1:1 v/v). After applying the samples, 2 mL of hexane:dichloromethane was added. Hydroxy fatty acids were eluted with 2 mL of diethyl ether. The solvent was evaporated and the sample redissolved in hexane (250 µL) and trimethylsilyl (TMS). Derivatives were made by adding Bis(trimethylsilyl)tri fluoroacetamide (BSTFA; Sigma Chemical Company, St. Louis, Mo.; 50 µL), pyridine (5 µL) and then heating at 80°C for 15 min. After pyridine and BSTFA evaporation under nitrogen, samples were redissolved in hexane and analyzed by GC-MS. Details of the GC-MS system and column type are the same as for the PLFA analysis. The column was programmed from

an initial temperature of 120°C, which was held for 3 min, then rising to 280°C at 20°C/min. Both the injector and the interface temperature were kept at 290°C. Mass spectra were collected at an electron energy of 70 eV. Hydroxy fatty acids were identified from relative retention times and mass spectra.

RESULTS

BIAQTS

Viable counts of bacteria collected from the BIAQTS using the Andersen air sampler were linearly correlated over time (r^2=0.99, Figure 2), indicating that the BIAQTS was running at a constant rate.

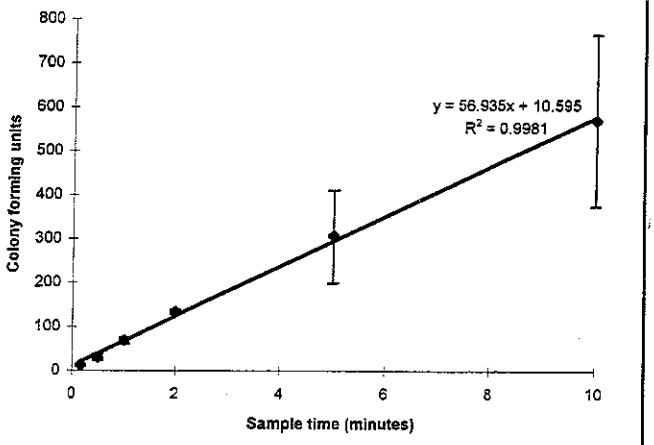

FIGURE 2. Correlation between sampling time and *Escherichia coli* (CFU) showing constant airflow within the BIAQTS

Viable bacterial counts sampled from the BIAQTS using the AGI were 1.08 ± 0.13 × 10^5 CFU. Viable bacterial counts from a separate upstream Andersen sampler were 3.05 ± 0.14 × 10^5 CFU. The percent efficiency of the AGI was 35.4%, taken as a percentage of the upstream Andersen count.

Lipid Biomarker Content and Bacterial Sampling

Biomass content for the mono- and mixed-culture biomass loaded filters was measured as PLFA (nmoles/filter), LPS-hydroxy fatty acids (3-OH FAs; gram-negative bacteria) and, for comparison, as CFU/filter using the AGI (Table I). PLFA content was converted to equivalent cell number using PLFA per cell conversion values obtained from the PLFA analysis of known numbers of the appropriate bacteria. The PLFA content of the control filter was determined to be 0.12 nmoles (2.4 × 10^3 cells per filter based on 2.0 × 10^{12} cells per gram dry weight of cells and 10^8 picomoles of phospholipid/g dry weight cells.)[35]

No culturable bacteria were recovered below the filter. The glass fiber filters used (GF/D; pore size 2.7 µm) have a high loading capacity and are recommended for filtering particles of approximately 1 µm in size (inclusive of all bacteria used here). Any bacteria that were forced through the filter would have been subject to mechanical damage, and it is likely that they would no longer be viable.

PLFA Profile Analysis

The fatty acids profiles detected in these samples are shown in Table II. A high proportion of monoenoic PLFAs generally indicative for the presence of gram-negative bacteria[36] were extracted from the glass fiber filter containing the monoculture of *E. coli* (Figure 3b), in particular 16:1w7c, 18:1w7c and cy17:0.

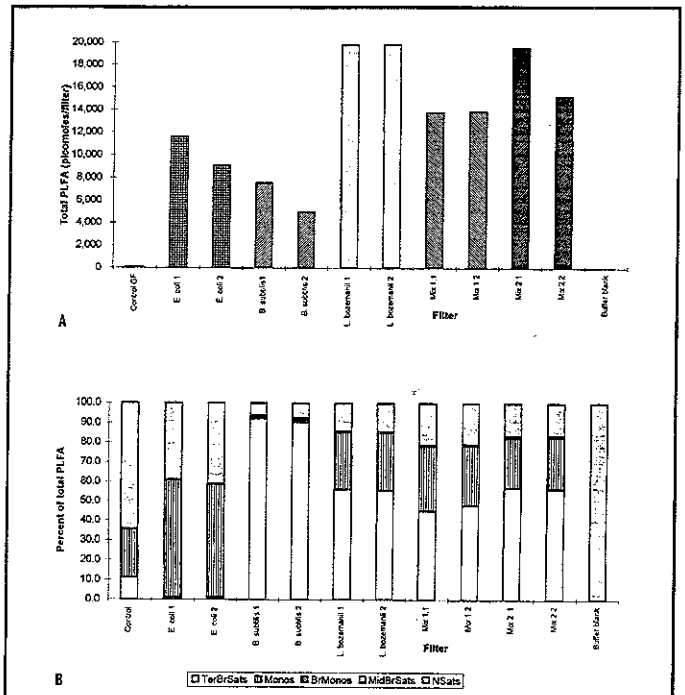

FIGURE 3. (a) Phospholipid fatty acid biomass content (*Escherichia coli, Bacillus subtilis,* and *Legionella bozemanii,* Mix 1 and Mix 2); (b) Relative phospholipid fatty acid profiles of biomass extracted from duplicate sample glass fiber filters containing either *E. coli, B. subtilis, L. bozemanii,* Mix 1 or Mix 2. Abbreviations: TerBra Sats=Terminally branched saturate fatty acids; Monos=Monoenoic fatty acids; BrMonos=Branched monoenoic fatty acids; MidBrSats=Mid-chain branched saturate fatty acids; Nsats=Normal saturate fatty acids

A high proportion of terminally branched saturate PLFA, which has been shown to be typical of gram-positive bacteria,[37] were recovered from the glass fiber filter containing the monoculture

TABLE I. Quantitative Assessment of Indoor Biocontamination by Signature Lipid Biomarker Analysis

Bacteria	PLFA nmoles/filter	Equivalent Cell Number (ECN; 10^8)[A]	CFU/filter (all-glass impinger) (AGI; 10^7)	Ratio ECN (filter)/CFU (AGI)/filter	LPS-OH FA (nmoles/filter)
E. coli	10.3 ± 1.2	3.2 ± 0.37	6.4 ± 0.15	5	0.049[B]
B. subtilis	6.3 ± 1.3	0.9 ± 0.18	2.3 ± 0.6	3.9	<0.01
L. bozemanii	19.8 ± 0.04	8.0 ± 0.02	14.9 ± 3.9	5.4	0.053[C]
Mix 1 (1:1:1 v/v/v)	13.8 ± 0.05	3.9 ± 0.03	11.9 ± 1.6	3.3	0.029
Mix 2 (1:1:2 v/v/v)	17.4 ± 3.0	5.5 ± 0.94	11.3 ± 1.9	4.9	0.033

[A]Calculated from the PLFA nmoles/filter values; cell equivalent calculated with pure biomass of known concentrations; *E. coli* = 3.08, *B. subtilis* = 1.39, and *L. bozemanii* = 4.08 x 10^{12} cells/g dry weight and 10^8 pmoles phospholipid/g dry weight cells[36]

[B]Lipopolysaccharide hydroxy fatty acids measured as 3-OH 14:0 (*E. coli*)

[C]LPS-OH FA 3-OH 14:0, i15:0, 15:0, i16:0, 17:0, 18:0, 19:0, 20:0 (*L. bozemanii*)

TABLE II. Mean Relative Proportions of PLFA Present in *E. coli, B. subtilis, L. bozemanii,* Mix 1, and Mix 2

Sample Name	*E. coli*	*B. subtilis*	*L. bozemanii*	Mix 1	Mix 2
Terminally branched saturated fatty acids					
i14:0	0.1	0.8	1.9	1.0	1.2
i15:0	0.0	51.6	0.4	17.1	19.4
a15:0	0.2	20.0	27.0	15.7	18.9
i16:0	0.0	2.9	12.9	4.9	6.0
i17:0	0.0	11.5	1.2	3.8	4.3
a17:0/17:1w8c	0.7	5.3	12.1	5.5	6.5
i19:0	0.0	0.1	0.1	0.1	0.1
Monoenoic fatty acids					
15:1	0.1	0.0	0.9	0.4	0.4
16:1ω49c	0.0	0.1	0.0	0.1	0.2
16:1ω7c/9c	15.0	0.2	14.9	9.7	9.2
16:1ω7t	0.2	0.0	0.0	0.1	0.1
16:1ω5c	0.2	0.0	1.2	0.5	0.6
cy17:0	13.3	0.0	12.2	8.8	8.2
18:1ω9c	1.9	0.4	0.0	0.8	0.5
18:1ω7c	23.2	0.2	0.2	8.7	6.4
18:1ω7t	0.4	0.0	0.0	0.2	0.1
19:1ω6c	0.2	0.0	0.0	0.1	0.1
cy19:0	3.2	0.0	0.1	1.2	0.8
20:1ω7c	0.1	0.0	0.0	0.0	0.0
Branched chain monoenoic fatty acids					
i16:0	0.0	0.1	0.0	0.1	0.1
i17:1ω7c	0.0	0.5	0.1	0.4	0.5
Midbranched chain saturate fatty acids					
br15:0	0.0	0.0	0.0	0.1	0.1
br16:0	0.1	0.0	0.2	0.0	0.1
10me16:0	0.0	0.1	0.1	0.2	0.2
Normal saturate fatty acids					
14:0	1.8	0.8	0.2	1.0	0.8
15:0	4.7	0.2	1.0	2.1	1.6
16:0	28.9	4.7	8.2	14.0	10.8
17:0	3.6	0.1	2.3	1.9	1.6
18:0	2.2	0.6	2.0	1.6	1.2
20:0	0.0	0.0	0.6	0.2	0.2
21:0	0.0	0.0	0.1	0.0	0.0

B. subtilis. Filter deposited *L. bozemanii* PLFA contained a high proportion of monoenoic fatty acids. In general, all *Legionella* spp. are distinguishable from other gram-negative bacteria by the presence of an unusually high proportion of branched chain fatty acids.[36] Here, the profiles were distinguishable from *E. coli* by the high proportion of terminally branched saturate PLFA present (especially a15:0 and i16:0).

All three bacteria contained normal saturate PLFA although in different proportions dependent on bacterial strain (Figure 3b). The PLFA profiles for all three monocultures deposited on glass fiber filters using the BIAQTS (Figure 3b) were very similar to those of the pure monoculture biomass (Figure 4), which indicated that there was no substantial contamination of the glass fiber filters, and that the filters did not interfere with PLFA extraction.

Principal Components Analysis

Principal components analysis projected the multivariate data onto a reduced number of dimensions (principal components). The PLFA profiles of the filter samples required multivariate statistical analysis to provide a representation of the data so that relationships

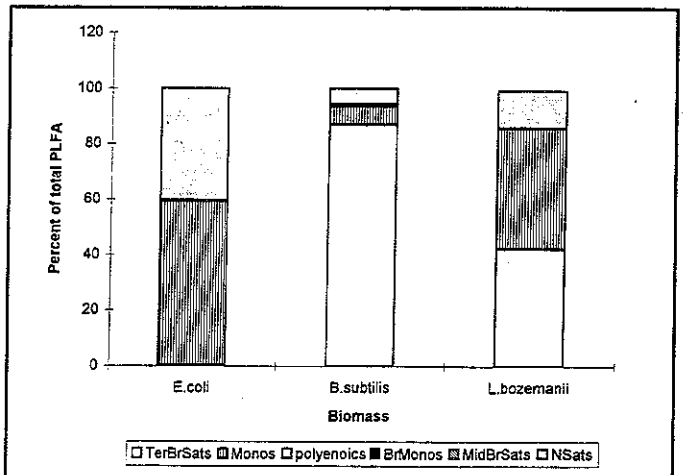

FIGURE 4. Phospholipid fatty acid profiles of pure *Escherichia coli, Bacillus subtilis* and *Legionella bozemanii*. Abbreviations: TerBra Sats=Terminally branched saturate fatty acids; Monos=Monoenoic fatty acids; BrMonos=Branched monoenoic fatty acids; MidBrSats=Mid-chain branched saturate fatty acids; Nsats=Normal saturate fatty acids

between the samples could be easily observed.[38] This enabled the major relationships between samples to be visualized by examining the relative similarity of their principal component scores, while the causes of any variation are determined by examining the correlation of the original variables to the principal components.[39]

Hierarchical cluster analysis of the PLFA profiles demonstrated grouping of the duplicate samples of both the monoculture bacteria extracted from the filters and of the mixtures (Figure 5a). Of the monocultures, *L. bozemanii* clustered closest to the two mixes as would be expected from the excess of *L. bozemanii* biomass present in the mixtures. *Bacillus subtilis* clustered furthest away from *E. coli, L. bozemanii*, and the two mixes reflecting the substantial difference between the gram-positive PLFA profile in comparison

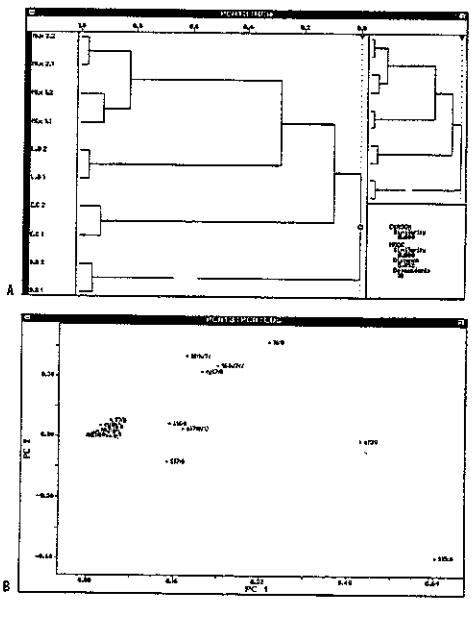

FIGURE 5. (a) Hierarchical cluster analysis of PLFA profiles of biomass extracted from duplicate sample glass fiber filters containing either *Escherichia coli* (E.C 1, E.C 2), *Bacillus subtilis* (B. S 1, B. S 2), *Legionella bozemanii* (L. B 1, L. B 2), Mix 1 (Mix 1.1, Mix 1.2) or Mix 2 (Mix 2.1, Mix 2.2); (b) Principal components analysis of the specific fatty acids extracted from glass fiber filters containing either *E. coli, B. subtilis, L. bozemanii,* Mix 1 or Mix 2

to those of the two gram-negative bacteria present and the two mixes (Figure 5a).

Principal components analysis of the PLFA showed which specific PLFA most significantly accounted for the patterns (Figure 5b). Terminally branched saturated PLFA showed a marked effect. Gram-negative *E. coli* has only trace amounts of terminally branched saturated PLFA (specifically i14:0, a15:0 and a17:0/17:1w8c) (Table II). The i15:0, which is rare to absent in either *E. coli* or *L. bozemanii*, accounts for the distinctiveness of the *B. subtilis* compared to the gram-negative bacteria and the mixes.

3-OH FA Profiles

Figure 6 shows the relative distribution of 3-OH FA. No 3-OH FA were detected in *B. subtilis*. The monoculture of *E. coli* showed exclusively 3-OH 14:0, which also represented 33% of *L. bozemanii* monoculture 3-OH FAs. The 3-OH FA composition of *L. bozemanii* includes substantial proportions of 3-OH i15:0, 15:0, i16:0, 16:0, 18:0, and 20:0. The mixtures contained the full range of 3-OH FAs detected, with Mix 2 having higher proportions of *L. bozemanii* representative 3-OH FAs as would be expected from the higher proportion of that bacteria present in Mix 2.

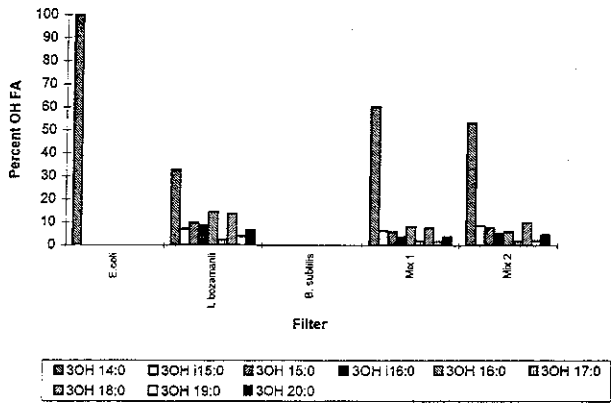

FIGURE 6. Relative amount of hydroxy fatty acid moiety per filter

DISCUSSION

The BIAQTS was established as having a constant airflow rate. Regression analysis of the viable counts of bacteria aerosolized into the BIAQTS over time periods ranging between 10 sec to 10 min showed a constant flow rate (r^2=0.99).

To compare the efficiency of lipid biomarker analysis to culturable detection methods for aerosolized viable bacteria, an all-glass impinger (AGI) was used as the classical sampling system. Because the aerosol was collected in liquid there was no limitation on the duration of sampling or on the concentration of the bacteria sampled. The efficiency of the AGI in comparison with Andersen sampler was 35.4 % where Andersen sampler efficiency was calculated using the positive hole method (airflow rate 28.3 L/min [1 ft^3/min]). This compares with a previously published figure for an AGI (AGI-30) efficiency in comparison with an Andersen sampler of 46.7% (airflow rate 28.3 L/min (1 ft^3/min).[22] All viable counts obtained using the AGI were taken as 35.4% of the total value.

Total viable biomass of aerosolized viable monocultures and mixtures of monocultures was measured as PLFA and 3-OH FA from glass fiber filters and compared with viable counts sampled using the AGI (Table I). The discrepancy in bacterial numbers between viable counts and equivalent cell numbers from PLFA was clearly demonstrated to be between 18.5 and 25.6% of the viable bacteria actually there. This could be due to the low efficiency of the AGI in comparison with collection on filters, which is highly efficient.[21,22] It is also possible the discrepancy was in part due to mechanical damage to the bacteria cause during aerosolization and sampling with a loss of culturability. When viable counts of bacteria (*E. coli*) collected in the AGI were compared with acridine orange direct counts of the same sample, the viable count was approximately 50% of the total acridine orange direct count (data not shown). Also, previous studies have shown that bacterial cell damage can occur on mechanical stress, e.g., after impingement or impaction into collection media.[20,40]

Following aerosolization and sampling, a higher percentage of the gram-positive *B. subtilis* (25.6) were culturable than were the gram-negative *E. coli* (20) and *L. bozemanii* (18.5). The thick peptidoglycan cell wall[41] of the gram-positive *B. subtilis* may provide greater protection against the environmental stress caused by aerosolization and sampling than the thinner gram-negative cell wall of the *E. coli* and *L. bozemanii*. This difference has been detected previously when sampling aerosols.[20] To ensure a monodispersed aerosol of bacteria in the BIAQTS it was necessary to dilute the wet bacterial aerosol with preconditioned, purified, and dry air producing a dried aerosol. It is possible that the drying process could have damaged the gram-negative cells to a greater extent than the gram-positive cells leading to a higher percentage of culturable *B. subtilis* in comparison with the equivalent cell number than was the case with the *E. coli* and *L. bozemanii*. Previous studies have shown that the outer lipid membrane of gram-negative cells is damaged by dehydration, rehydration, and changes in temperature.[16]

The PLFA profiles provided quantitative detection of all three bacterial monocultures and the two mixed cultures in a single analysis. The viable culturing required the use of two different media (buffered charcoal yeast extract agar specific to *Legionella* spp. and Miller's LB agar for *E. coli* and *B. subtilis*). This illustrates a further major advantage of signature lipid biomarker analysis as it provides biomass analysis irrespective of the ability to culture on "selective" media. Each of the bacteria showed a unique pattern of PLFA so the monocultures and expected ratios in the mixed cultures were readily detected. In actual indoor air samples, there will be a far greater diversity of PLFA detected. From such complex data sets, lipid biomarker analysis can provide for quantitative definition of groups of microorganisms containing characteristic PLFA patterns.[42] Phospholipid analysis should also provide a means to determine the nutritional/physiological status of the microorganisms,[43,44] an important feature of the technique considering the loss of culturability of microorganisms that are dehydrated or injured but still viable.[16]

Currently the detection limit of the GC-MS analysis is approximately 10^5 cells. This being the case, lipid biomarker analysis of indoor air will require the rapid sampling of large air volumes. When sampling such large air volumes, it is highly likely that the air in confined spaces will be effectively "stripped" of biomass, lowering the concentration of airborne particulates. To counter this effect it will be important to sample from ventilated sites containing large air volumes and to never sample over 100% of the air space. Such sites include office space, hospital wards, industrial sites (factories), and agricultural sites. Preliminary PLFA analyses of such spaces have enabled detection of biomass at levels of approximately two to three orders of magnitude greater than the culturable microorganisms (data not shown). However, until improvements are made to the detection limits for PLFA, this

analysis will not be useful for certain applications, e.g., personal air samplers.

Analysis of indoor air frequently includes quantitation of endotoxin, which comprises fragments of gram-negative bacteria and can act as an immuno-potentiator.[10,27,45] Two assays of endotoxin, the *Limulus* amebocyte lysate test (LAL) and GC-MS are used. The LAL is the most common. A major advantage of GC-MS analysis of hydroxy fatty acids over the traditional *Limulus* amebocyte lysate (LAL) test, is that variation in patterns in OH FA can be extremely useful when determining the origin of endotoxin. Specific groups of gram-negative bacteria contain different 3-OH FA. For example, *enterobacteriaceae* (including *E. coli*) contain predominantly 3-OH 14:0, while the *Pseudomonadaceae* contain predominantly 3-OH 10:0, 12:0, and 16:0.[36] *Legionella* spp. contain many different 3-OH FA, ranging from 3-OH 12:0 to 3-OH 23:0. This study shows that the 3-OH FA analysis provided detection/identification of the two gram-negative bacteria and showed their absence from gram-positive bacteria (Figure 6).

CONCLUSIONS

The signature lipid analysis technique for aerosolized monocultures or mixtures of monocultures of viable bacteria recovered from glass fiber filters was three to five times more efficient, dependent on species, in detecting known quantities of culturable bacteria than culturing on growth media. Aerosolization and sampling using conventional procedures that were dependent on viable counts were clearly not efficient in recovery and detection. The signature lipid detection methods were quantitative, independent of cell culturability, and allowed identification of the bacteria that have distinctive PLFA and OH FA patterns in a single analysis. The ready detection of OH FA indicates the analysis system will also detect and in many cases allow identification of endotoxin. The present study was limited to analysis of culturable bacteria to enable comparison between the different analysis techniques. However, further research will concentrate on using lipid analysis techniques to detect and quantify the presence of nonculturable airborne particles such as endotoxins, pollens, and dust mites as well as the culturable microorganisms, thereby enabling quantification of airborne biocontaminants from a single sample.

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