Am. Indus. Hyg. Assn

QUANTITATIVE LIPID BIOMARKER ANALYSIS OF AIRBORNE MICROORGANISMS IN INDOOR ENVIRONMENTS

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INTRODUCTION

There is increasing concern over airborne microorganisms in indoor air environments. Exposure to such biocontaminants can give rise to large numbers of different health effects including infectious diseases [1-3], allergenic responses [4, 5] and respiratory problems [5, 6]. Standard microbiological methods based on microorganism culture have severe limitations for the analysis of indoor air samples and a quantitative detection/ identification technique independent of culturability that assays both culturable and non culturable biomass is critical in defining risks from indoor air biocontamination.

Counts of culturable bacteria from environmental samples consistently underestimate the actual viable community by 90-99% [7-10]. Additionally, although bioaerosol samplers are designed to damage the microbes as little as possible [11], microbial stress which inhibits cell culture has been shown to result from air sampling, aerosolization and microbial collection [12-15]. Higher collection efficiency (e.g. using filtration) results in greater cell damage while less cell damage often results in lower collection efficiency [15-17]. The lipid biomarker assays described herein do not rely on cell culture. Lipids are components that are universally distributed throughout cells providing a means to assess biomass independent of culturability [18]. Extractable phospholipid fatty acids (PLFA) are found in all cell membranes, are actively metabolized during growth and have a relatively rapid turnover rate [10]. Specific groups of microorganisms often contain characteristic lipid patterns which can be analyzed using gas chromatography-mass spectrometry (GC-MS) enabling the determination of the microbial biomass, nutritional status and community composition [8-10].

To determine if there is a connection between viable biomass/community composition (measured using PLFA), and "sick building syndrome", we have begun a cumulative process of compiling a database comprising the phospholipid fatty acid profiles and biomass contents of indoor air. Filtration sampling of large volumes of air allowed us to obtain and analyze airborne particulate matter from a number of test sites that included office and industrial space. For comparison with the conventional methodology, we also sampled the culturable bacterial and fungal biomass at each test location.

MATERIALS AND METHODS

Sampling sites

Sampling sites were chosen dependent on whether they were occupied during the working day, heating, ventilation and air conditioning (HVAC) system maintenance, and on air quality complaint levels. The air samples were taken from companies designated 'W, X, Y and Z'. Company W was a 20 year old building and had recently been remodeled. The buildings where Companies X and Y were located were 20 and 16 years old, respectively. Of these two buildings, that housing Company Y had the worst maintained HVAC system. A substantial percentage of employees at both companies (>25%) had complained of work based respiratory problems. Company Z was a large operations manufacturing building at a local industrial site. Complaints had been received regarding the indoor air along the aluminum manufacturing lines but not for the indoor air along the steel manufacturing lines. Possible sites of contamination, e.g the machine line sumps (Company Z) were also sampled and the PLFA extracted and analyzed.

High Volume Air Sampling

High volume air sampling was done using a portable air sampler designed in conjunction with Graseby Andersen Inc. (Atlanta, GA). The sampler was 65 inches in diameter, and was able to pull air at over one cubic meter per minute over a glass fiber filter (GF/D; 2.7 µm) onto which the particulate matter was loaded. The unit was modular and enabled samples to be taken from the breathing zone. Using the sampler, particulate matter was collected from the air at companies W, X, Y and Z. Concurrent outdoor air samples were taken for all sites. Companies W and X were sampled during both summer and winter months. Samples taken comprised approximately 1000-1500 m^3 of air collected over 24 hours. Prior to lipid analysis all sample filters were stored at -20 °C.

Low Volume Sampling

Total culturable heterotrophs were sampled using single stage Andersen viable particle samplers (Graseby Andersen Inc). Collection plates for the Andersen sampler were prepared by pouring either 30 mL of R2A agar (R2A; Becton Dickinson and Co., Cockeysville, MD) or 30 mL malt extract agar (MEA; Sigma Chemical Co., St. Louis, MO) aseptically into sterile plastic petri dishes (diameter, 100 mm; height, 15 mm). The R2A agar contained cycloheximide (50 µg/mL) to inhibit fungal growth. Five replicate air samples were collected for 2 and/or 5 min at 28.3 liters/min at each of the indoor and outdoor sample locations (Companies W, X and Y), with an extra R2A agar plate sample collected for 30 sec at Company Z. Media plates were incubated at 30°C for up to 14 days. Colony counts were made on days 2, 5, 8, 10 and 14.

Reservoir Sampling

Aluminum line sump samples (also 100 mL) were collected from Company Z. Sump was then filtered through sterile glass fiber filters (type GF/D, 2.7 µm pore size ; GF/B, 1.0 µm pore size ; GF/F, 0.7µm pore size and Anodisc, 0.2µm pore size) to obtain the particulates for lipid analysis. Prior to lipid extraction all sample filters were stored at -20 °C.

Lipid Biomarker Analysis

All solvents were of GC grade and were obtained from Baxter Scientific Products (McGaw Park, IL). All glassware was washed in a 10% (v/v) "micro" cleaner solution (Baxter Diagnostics, Deerfield, IL), 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.

All sample filters and negative controls (a field blank filter, a 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 [18, 19]. Briefly, samples were extracted in separatory funnels containing a single phase system (chloroform:methanol:phosphate buffer (50mM, pH 7.4); 1:2:0.8 v/v/v) for a minimum of 4 h 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 h, 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 [20]. The phospholipid containing polar lipid fraction was subjected to a mild alkaline methanolysis, transesterifying the fatty acids into methyl esters [20]. Fatty acid methyl esters were separated and quantified by gas chromatography-mass spectrometry (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, DE). 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.* [21].

RESULTS AND DISCUSSION

The PLFA content, prokaryote (bacterial) equivalent cell number (calculated from the PLFA content) [22],

culturable bacterial and fungal counts as well as complaint and maintenance levels, temperature, carbon dioxide levels (where available) and the range of relative humidity (where available), are all presented in Table 1. The maximum number of prokaryote cells per cubic meter was detected at Company Z along the aluminum line (Z08/96:2; 3.3×10^6), with the minimum number of prokaryote cells detected at Company X (X04/94:1; 1.8×10^3). Of the office space samples, the site with the poorest maintenance (Company Y) contained the highest level of PLFA but the lowest number of culturable organisms whereas the site with a well maintained HVAC system (Company W) contained less PLFA but higher numbers of culturable organisms. Inclusive of Company Z, the microbial biomass levels (measured as the prokaryote PLFA equivalent cell number [22]), and culturable bacteria/fungi in indoor air were compared to complaint levels. A significantly larger amounts of prokaryote cells were detected in the high complaint sites compared to the low complaint sites ($p<0.05$, student t test). Conversely, levels of culturable microorganisms (both fungi and bacteria)from sites with high and low levels of complaints regarding the air quality were not significantly different ($p>0.05$). Not all causes of poor indoor air quality are microbial or maintenance related, however, and this may well have been the case at Company X which had a medium well maintained HVAC system but a high level of complaints concerning the indoor air quality and contained low levels of prokaryote cells/m^3.

Bacterial cell counts measured as PLFA content were consistently between 1and 3 orders of magnitude higher than the counts obtained for culturable bacteria measured using conventional sampling and culture techniques (Table 1). Previous studies in different environments showed that culturable counts of microorganisms from environmental samples only account for between 0.1-10 % of the total community detectable by direct counting [7-10]. In the case of air sampling and analysis, this situation is complicated by the fact that commonly used devices as Andersen samplers, all glass impingers and Mattson-Garvin slit-to-agar samplers are designed to damage the microbes as little as possible so as to increase culturability thereby loosing efficiency compared to the more rigorous filtration devices. Consequently, when comparing PLFA based prokaryote cell counts to culturable bacterial cell numbers, we were comparing a highly efficient sampling and analysis technique to one that was far less efficient, thereby increasing any discrepancy between viable cells measured using PLFA and culturable bacteria.

The air from the office space with poorly maintained HVAC systems (Company Y) contained less culturable bacteria/ m^3 but more PLFA/ m^3 than did that at the companies with well maintained systems (W and X) (Table 1). Of the other parameters taken at each sample site, higher relative humidity correlated best with the greater numbers of culturable bacteria, i.e., Company W which had the highest relative humidity, also contained the air with the greatest number of culturable bacteria/m^3 while those companies with lower relative humidity (X and Y) contained air with lower numbers of culturable bacteria (Table 1). Previous studies have shown that the outer lipid membrane of Gram-negative cells is damaged by dehydration and rehydration [11] which can leave cells still viable but no longer culturable. Consequently, low relative humidity may well have resulted in greater cell desiccation or dehydration, leading to reduced culturable bacteria counts at Companies X and Y. Confirmation of the increased stress placed on the Gram negative cells by dehydration can be obtained using lipid biomarker analysis. Gram-negative bacteria make *trans* fatty acids to modify their cell membranes as protection against changes in the environment such as toxicity or starvation [23, 24]. For example, bacteria make *trans* (t) fatty acids in the presence of toxic pollutants like phenol [25]. Ratios (16:1ω7t/16:ω7c (*cis*) and 18:1ω7t/18:1ω7c) greater than 0.1 have been shown to indicate the effects of starvation on bacterial isolates. In indoor air samples, amounts of PLFA extracted are often very low with the necessary markers not always detectable. As a result, in these samples assessment of bacterial stress levels was not always possible. The sample set taken at Company Y provided the best measurement of bacterial metabolic stress (Table 2). For comparison, included in Table 2 is the concurrent outdoor air sample data. This data showed that the indoor air Gram negative microbial biomass at Company Y was experiencing far higher levels of environmental stress from toxicity, starvation or dehydration than were the Gram negative communities in the equivalent outdoor samples.

	Y07/96:1	Y07/96:2	Y07/96:3	Y07/96:1 out	Y07/96:2 out	Y07/96:3 out
Sum ω7t/ω7c	**0.55**	**0.52**	**0.55**	0.12	0.12	0.09

TABLE II: Metabolic stress (sum ω7t/ω7c) for the indoor and outdoor air Gram negative communities at Company Y.

To enable relationships between samples to be easily observed and the source of indoor biocontamination at Company Z to be determined, the PLFA profiles of the samples taken required multi variate statistical analysis. Specifically, the bacterial PLFA profiles from Company Z were analyzed by applying principal components analysis (PCA). Principal components analysis projects the multi variate data (PLFA profile) onto a reduced number of dimensions (principal components). The results of this PCA are shown in Figure 1. In this example, the PCA showed the source of contamination in the aluminum line air to be the aluminum line sumps (containing the liquid used to clean the machine parts). Principal components analysis also showed that the primary PLFA contributing the most variance between the samples were *iso*17:0 and *anteiso*17:0, 16:1ω5c, *iso*15:0 and *anteiso*15:0. The comparatively high relative proportion of all of these PLFA is principally associated with the presence Gram negative sulfate reducing bacteria such as *Desulfovibrio*. This finding corresponds with the sulfur odor reported by the work force. The steel line air PLFA profiles, however, clustered with those of the outdoor air showing that along that along the steel machine lines outdoor air was the main contributor to the airborne microbial community.

CONCLUSIONS

With the exception of the data obtained from Company X, these initial studies and analyses indicated a measurable correlation between complaints concerning the indoor air quality and increased biomass (measured as PLFA) contents of air particulate matter. There was a difference of between 1 and 3 orders of magnitude between the equivalent cell numbers (obtained from the PLFA yield) and the number of colony forming units (culturable bacteria) at the sites we analyzed. Generally, the best maintained systems contained larger numbers of culturable bacteria and smaller amounts of PLFA, while the badly maintained systems contained smaller numbers of culturable bacteria and higher amounts of PLFA. A possible reason for this phenomenon was the impact of environmental stress, e.g. starvation desiccation and/or dehydration, on the bacteria in the indoor air environment resulting in the presence of viable but non-culturable cells. Particularly clear evidence of such environmental stress was detected in the samples from Company Y. With PLFA providing a measure of actual viable biomass (both culturable and non-culturable) these assays should give more valid data concerning bacterial contamination of indoor air than does the conventional quantification using culturable microorganism counts.

ACKNOWLEDGMENTS

This work was supported by a SBIR grant from the National Aeronautical Space Agency and by a grant from the Center for Indoor Air Research. The authors are grateful to Michael Wimpee Jane Doll, and Gregory A. Davis for help throughout data analysis.

REFERENCES

1. **Riley, R.L.; Mills, C.C.; O'Grady, F.O., Sultan, L.U.; Wittestadt, F.; Shivpuri, D.N.:** Infectiousness of air from a tuberculosis ward. Ultraviolet irradiation of infected air: comparative infectiousness from different patients *Am.Rev. Respir. Dis.*, *84*: 511-525 (1962)

2. **Fraser, D.W.:** Legionellosis: evidence of airborne transmission. *Ann. Natl. Acad. Sci. USA.*, *353*: 61-66 (1980)

COMPANY	W12/95:1	W8/96:2	W8/96:3	X4/96:1	04/96:2	X4/96:3	X4/96:4	X4/96:5	X8/96:6	X8/96:7	X8/96:8	Y7/96:1	Y7/96:2
Complaints	Low	Low	Low	High	High	High	High	High	High	High	High	High	High
Maintenance	High	High	High	Medium	Medium	Medium	Medium	Medium	Mediu m	Mediu m	Medium	Low	Low
Range Rel. Hum.	-	40-69	37-61	-	-	-	-	-	35-50	35-50	35-50	31-45	34-44
Temp. range (°C)	16-24	23-33	24-31	21-25	25-31	23-27	22-28	22-29	22-30	22-30	22-30	22-29	23-30
PLFA (pM/m^3)	2.3	8	7	1.8	2.1	3.5	3.5	2.0	4.0	3.5	4.2	31.4	25.5
Prokaryote PLFA/m^3	0.34	0.32	0.5	0.09	0.23	0.22	0.32	0.15	0.36	0.38	0.48	3.96	3.44
Prokaryote cells/m^3	$6.8x10^3$	$6.4x10^3$	$1.0x10^4$	$1.8x10^3$	$4.6x10^3$	$4.4x10^3$	$6.4x10^3$	$3.0x10^3$	$7.2x10^3$	$7.6x10^3$	$9.6x10^3$	$7.9x10^4$	$6.9x10^4$
Bacteria (cfu/m^3)	-	417	3533	115	193	364	526	257	52	17	81	64	150
Fungi (cfu/m^3)	-	1703	178	12	4	6	4	300	47	20	37	88	61

COMPANY	Y7/96:3	Z8/96:1	Z8/96:2	Z8/96:3	Z8/96:4	Z8/96:5	Z8/96:6	Z8/96:7	Z8/96:8	Z8/96:9	Z8/96:10	08/96:11	Z8/96:12
Compaints	Low	High	High	Low	Low	High	High	Low	Low	High	High	Low	Low
Maintenance	Low	High	High	High	High	High	High	High	High	High	High	High	High
Range Rel. Hum.	32-38	-	-	-	-	45-71	45-71	45-71	45-71	39-76	39-76	39-76	39-76
Temp. range	24-28	18-32	18-32	18-32	18-32	23-30	23-30	23-30	23-30	25-37	25-37	25-37	25-37
PLFA (pM/m^3)	16.8	263	617	70	53	200	422	10	16	183	452	44	28
Prokaryote PLFA/m^3	2.29	81.76	165.44	3.83	1.42	63.7	102.53	0.65	0.61	65.13	124.43	3.48	1.41
Prokaryote cells/m^3	$4.6x10^4$	$1.6x10^6$	$3.3x10^6$	$7.6x10^4$	$2.8x10^3$	$1.3x10^6$	$2.1x10^6$	$1.3x10^4$	$1.2x10^4$	$1.3x10^6$	$2.5x10^6$	$7.0x10^4$	$2.8x10^4$
Bacteria (cfu/m^3)	100	35000	9900	580	560	24000	1100	190	130	20000	1600	69	76
Fungi (cfu/m^3)	72	60	81	34	85	170	300	75	65	140	95	61	69

TABLE 1: Summary of the biomass data obtained for Companies W, X, Y and Z.

3. **Hickman, C.; MacDonald, K.L.; Osterholm, M.T.; Schecter, G.F.; Royce, S.; Vugia, D.J.; Procter, M.E.; Davis, J.P.; Bur, S.; Dywer, D.:** Exposure of passengers and flight crew to *Mycobacterium tuberculosis* on commercial aircraft, 1992-1995. *Morbid. Mortal. Weekly Rep.*, *44*: 137-140 (1995)

4. **Bernstein, R.S.; Sorenson, W.G.; Garabrant, D.; Reaux, C.; Treitman, R.D.:** Exposures to respirable, airborne *Penicillium* from a contaminated ventilation system: clinical, environmental and epidemiological aspects *Am. Ind. Hyg. Assoc. J.*, *44*: 161-169 (1983)

5. **Croft, W.A.; Jarvis, B.B.; Yatawara, C.S.:** Airborne outbreak of trichothecane toxicosis *Atmos. Environ.* *20*: 549-552 (1986)

6. **Jacobs, R.R.** Airborne endotoxins: an association with occupational lung disease. *Appl. Ind. Hyg.*,*4*: 50-56 (1989)

7. **Skinner, F.A.; Jones, P.C.T.; Mollison, J.E. :** A comparison of a direct and a plate counting technique for the quantitative estimation of soil microorganisms. *J. Gen. Microbiol.* *6*: 261-271, (1952)

8. **Bakken, L.R.:** Separation and purification of bacteria from soil. *Appl. Environ. microbiol.*, *49*: 1188-1195 (1985)

9. **White D.C.:** Analysis of microorganisms in terms of quantity and activity in natural environments. In *Microbes in their natural environments.* J.H. Slater, R. Whittenbury and J.W.T. Wimpenny, eds. Vol. 34. Society for General Microbiology, London: Cambridge University Press., 1983. pp. 37-66.

10. **Tunlid, A. and White, D.C.:** Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of the microbial communities in soil. In *Soil Biochemistry.* J-M. Bollag and G. Stotzky, eds., Vol. 7. New York: Marcel Dekker, Inc., 1992. pp. 229-262.

11. **Cox, C.S.:** Quantitative and qualitative analysis of airborne spora *Grana*, *30*: 407-408 (1991)

12. **Webb, S.J.:** Factors affecting the viability of airborne. I. Bacteria aerosolized from distilled water *Can. J. Microbiol.* *5*: 649-669 (1959)

13. **Cox, S.C.:** The survival of *Escherichia coli* sprayed into air and into nitrogen from distilled water and from solutions of protecting agents as a function of relative humidity J. *Gen. Micro.*, 1966, *43*, 383-399

14. **Marthi, B.; Fieland, V.P.; Walter, M.; Seidler, R.J.:** Survival of bacteria during aerosolization *Appl. Environ. Microbiol.* *56*: 3463-3467 (1990)

15. **Stewart, S.L.; Grinshpun, S.A., Willeke, K.; Terzieva, S.; Ulevicius, V.; Donnelly, J.:** Effect of impact stress on microbial recovery on an agar surface. *Appl. Environ. Microbiol.* *61*: 232-1239 (1995).

16. **DeCosemo, G.A.L., Stewart, I.W., Griffiths, W.D., and Deans, J.S.:** The assessment of airborne microorganisms. *J. Aerosol sci.* *23* (Supplement 1): S683-S686 (1992).

17. **Jouzaitis, A.; Willeke, K.; Grinshpin, S.A.; Donnelly, J.:** Impaction onto a glass slide or agar verses impingement into a liquid for the collection and recovery of airborne microorganisms. *Appl. Environ. Microbiol.* *60*: 861-870 (1995).

18. **White, D.C., Davis, W.M., Nickels, J.S., King, J.D., and Bobbie, R.J.:** Determination of the sedimentary microbial biomass by extractable lipid phosphate. *Oecologia* 40:51-62 (1979).

19. **Macnaughton, S.J.Jenkins, T.L., Alugupalli, S. and White, D.C.:** Quantitative sampling of Indoor Air Biomass by Signature Lipid Biomarker Analysis: Feasibility Studies in a Model System. *AIHA Journal* (IN PRESS)

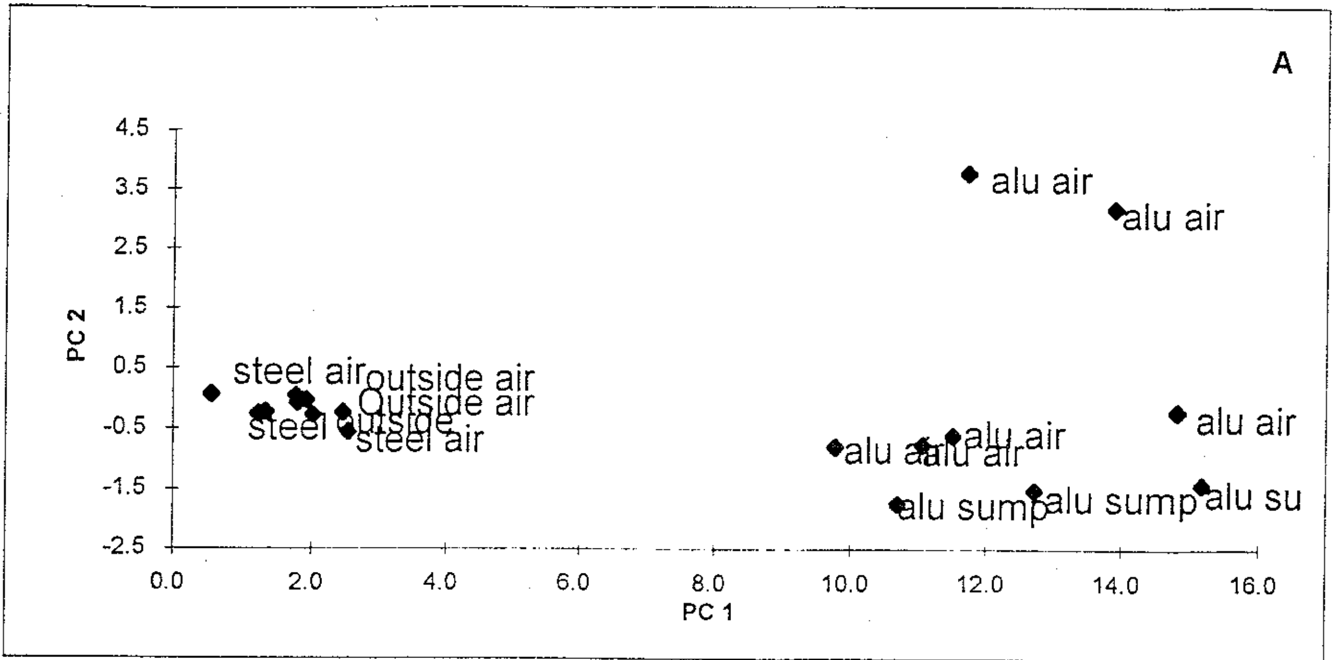
20. **Guckert, J.B., Antworth, C.P., Nichols, P.D. and White, D.C.:** Phospholipid ester linked fatty acid profiles as reproducible assays for changes in prokaryote community structure of estuarine sediments. *FEMS Microbiol. Ecol. 31*: 147-158 (1985)

21. **Ringelberg, D.B., Davis, J.D., Smith, G.A., Pfiffner, S.M., Nichols, P.D., Nickels, J.S. Henson, J.M., Wilson, J.T., Yates, M., Kampbell, D.H., Read, H.W., Stocksdale, T.T. White, D.C.:** Validation of signature polar lipid fatty acid biomarkers for alkane-utilizing bacteria in soils and subsurface aquifer materials. *FEMS Microbiol. Ecol. 62*:39-50 (1989).

22. **Balkwill, D.L., Leach, F.R., Wilson, J.T., McNabb, J.F., White, D.C.:** Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments. *Microbial Ecol. 16*:73-84 (1988).

23. **Klieft, T.L., Ringelberg, D.B., White, D.C.:** Changes in ester linked phospholipid fatty acid profiles of subsurface bacteria during starvation and dessication in a porous medium. *Appl. Environ. Microbiol.60*, 3292-3299. (1994)

24. **Guckert, J.B., Hood, M.A., White, D.C.:** Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids *Appl. Environ. Microbiol. 52*:794-801 (1986).



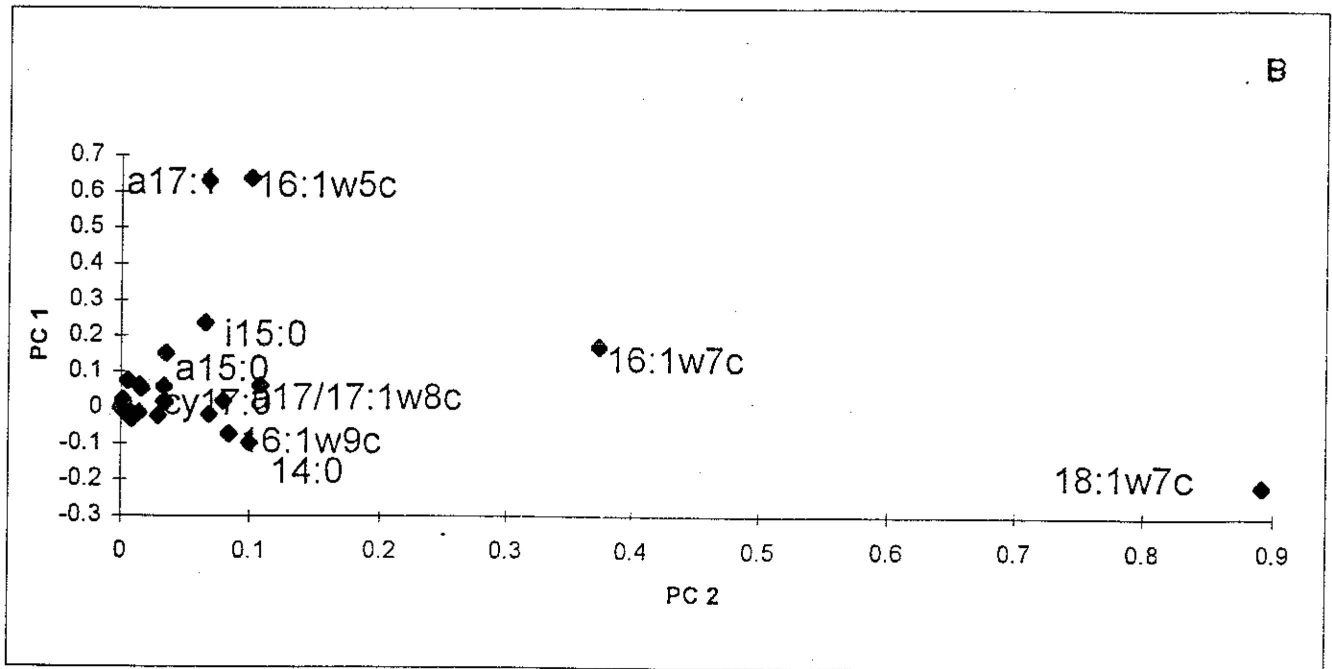


Figure 1: Principal Components Analysis:A) Scatter plot of bacterial PLFA profile B) scatter plot of the loadings derived from the PCA in A.