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Project Summary

Bioaerosols have been linked to a wide range of different allergies and respiratory illnesses. Currently, microorganism culture is the most commonly used method for exposure assessment. Such culture techniques, however, generally fail to detect between 90-99% of the actual viable biomass. Consequently, an unbiased technique for detecting airborne microorganisms is essential. In this Phase II proposal, a portable air sampling device has been developed for the collection of airborne microbial biomass from indoor (and outdoor) environments. Methods were evaluated for extracting and identifying lipids that provide information on indoor air microbial biomass, and automation of these procedures was investigated. Also, techniques to automate the extraction of DNA were explored.

After investigating many commercially available air samplers, the Portable Air Sampling Device (PASD) was designed with and then purchased from Graseby GMW with adaptations made to meet the needs of this project. The PASD was calibrated using orifice plate calibration standards to determine the air flow (changes in which were continuously recorded during sample acquisition enabling accurate determination of sample volume). Initial testing of the air sampler utilized three local businesses where complaints of respiratory problems had been voiced. Results from these studies showed that the indoor air in high-complaint areas tended to contain larger viable bacterial microbial communities that significantly differed in structure from those detected in low-complaint areas. Also, the Gram negative communities in high-complaint areas typically experienced higher levels of environmental stress from either toxicity or starvation than did those from low complaint areas.

The conventional process by which lipids were extracted was both tedious and time-consuming (requiring at least 24 hours per sample). The automation of this process, as well as other common extraction procedures, was of great interest. To achieve this goal, an Automated Solvent Extractor (ASE 200) was purchased. The ASE 200 combines high temperatures and pressures to increase extraction efficiency while reducing analyst time. Several lipid extraction methods were evaluated and improvements were made for the extraction of phospholipid fatty acids from pure biomass and environmental samples (including air); poly β-hydroxyalkanoates from soil; and polyunsaturated fatty acids, certain sterols, and lipopolysaccharides from different types of pure biomass. Also investigated were the extraction of aflatoxins from *Aspergillus* spp. and mycolic acids from species of *Mycobacterium*. In addition, the computer based process of identifying the fatty acids that allow for characterization of the microbial community was automated.

Finally, attempts were made to extract DNA from environmental samples using the ASE 200, however these were less efficient than the conventional mechanical extraction procedures.

In conclusion, the objectives set out in the Phase II proposal were pursued and, in general, achieved. The portable air sampling device may be used in any indoor air environment to determine indoor air quality. Automation of lipid (biomarker) extraction and identification from a wide variety of habitats was developed, improving extraction efficiency and substantially decreasing turn-around time.

Phase II Final Report

The principal objectives of this Phase II contract were: 1) to adapt or build a portable air sampling device with which it would be possible to sample the indoor air microbial community in any given indoor air environment, 2) to automate the extraction and identification of lipid biomarkers from biomass, 3) using the same technology to automate the extraction of nucleic acids (DNA) from filters, thereby enabling isolation of both DNA and lipids from one sample, and 4) to prepare a report of the SBIR Phase II.

Work carried out throughout the project

Objective 1: Air sampler design

Airborne microbial contaminants within indoor air environments are an ever increasing source of concern in public health and industrial hygiene. Exposure to such biocontaminants can give rise to large numbers of different health effects including infectious diseases, allergenic responses, and respiratory problems. Biocontaminants typically found in indoor air environments include bacteria, fungi, algae, protozoa, and dust mites. Traditionally, methods employed for monitoring microorganism numbers in indoor air environments have involved classical culture-based techniques. However, it has been repeatedly documented that such culture techniques only account for between 0.1-10% of the total community detectable by direct counting.

A large number of different types of air samplers have been designed for the collection of airborne biomass. Characterized according to collection methodology, the major groups are the inertial and the non-inertial samplers. Inertial samplers enable collection of airborne particles through settling (*i.e.* gravity), impaction, impinging, and centrifugation. The majority of inertial samplers are designed for the collection of undamaged microbial biomass enabling eventual cell culture. As such, with the exception of the centrifugation technique, the majority of such samplers enable low to mid volume biomass collection and are of low to mid efficiency. Conversely, non-inertial samplers employ filtration, electrostatic precipitation, and thermal precipitation, with, for large scale sampling, filtration being the most widely used method. However, filtration can lead to cellular damage and non culturability, and as such, is not often used where culturing of large scale samples is required.

For a successful and comprehensive lipid analysis, relatively large quantities of biomass are required (approx. 10^6 bacteria), and as such, a high volume sampling system was required. We investigated the filtration and centrifugation approaches to sampling. The filtration device (Portable Air Sampling Device, PASD) was designed to collect sufficient amounts of biomass from indoor air environments for the characterization of the microbial community through lipid analysis. Although several commercially available air samplers were investigated (the standard high volume sampler, the portable tripod sampler, and the small portable sampler (all Graseby GMW)), each was too loud for use in occupied space. Consequently, Graseby GMW modified the design of a commercially available sampler to reduce the noise levels. The final design was for a sampler approximately 25 inches overall diameter and 24 inches in height (Figure 1C). The sampler operated off 115 volt AC current at 60 hertz and used approximately 10 amps

running at maximum flow rate. It could operate 24 hours per day and 7 days a week. The sampler was able to pull air at more than one cubic meter per minute over a glass fiber filter (GF/D) onto which air particulate matter was loaded. Air volume sampled was monitored by determining the changes in the pressure drop across the filter; the pressure drop increased as the volume of biomass on the filter increased. In addition, the unit was modular and enabled samples to be taken at various heights (including the breathing zone) and/or from air ventilation systems, making it ideal for environments such as buildings or planes. The muffler system reduced noise levels to under 60 decibels, and with wheels attached, the sampler was fully and easily portable. Figure 1C shows the PASD in an office setting. This sampler was designed so that it would be easy to set up, required no observation during the sample collection time, and collected biomass from the air directly onto filters for simplicity of analysis. Throughout its use, the PASD was calibrated on a regular basis using orifice plate calibration standards obtained from Graseby GMW to determine the actual air flow of the sampler in m^3/minute.

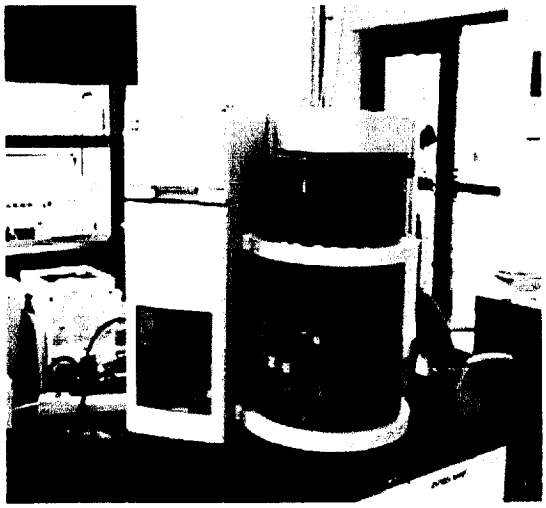
In order to have a more portable sampler, a Quick Form Air Sampler (Figure 1B) was also designed that consisted of a hard plastic filtration unit that attached directly to a common industrial vacuum cleaner. Air is pulled over the plastic attachment where a glass fiber filter is secured with sample volume determined from pressure drop across the filter. Provided an industrial vacuum cleaner was easily accessible at the sample site, this was a substantially smaller sampler than the PASD, however, it does not have any form of sound muffling and was therefore unsuitable for occupied buildings.

We also investigated the suitability of a Cyclonic Sampler manufactured by Team Technologies of Newton Upper Falls, Massachusetts. This was a form of inertial sampler. The unit was smaller than the Graseby GMW filtration sampler, but was still able to sample at a sufficiently fast rate for subsequent lipid biomarker analysis. To collect an appropriately sized sample, however, it required centrifugation of biomass into 2 liters of phosphate buffer per sample. As a result, the biomass sample obtained required filtration to enable successful lipid analysis. This being the case, the filtration device (PASD) was the most appropriate sampler design and was used throughout the remainder of this project.

Following construction and adaptation of the PASD, three sites (two office buildings and one industrial site) were selected for indoor air analysis and sampler evaluation. To enable comparison with conventional airborne biomass sampling techniques as well as with all previously published data, an Andersen sampler was used. Using a vacuum, air was pulled at a known flow rate onto growth media plates suitable for either bacteria or fungi placed within the Andersen sampler. The media plates were incubated and counts made to determine the number of culturable microorganisms in the air.

Air sampler testing:

Several different sites were chosen for sampler testing to enable observation of differences in contamination due to location. For the purposes of confidentiality, all sampling sites were designated the letters of the alphabet X, Y, and Z.



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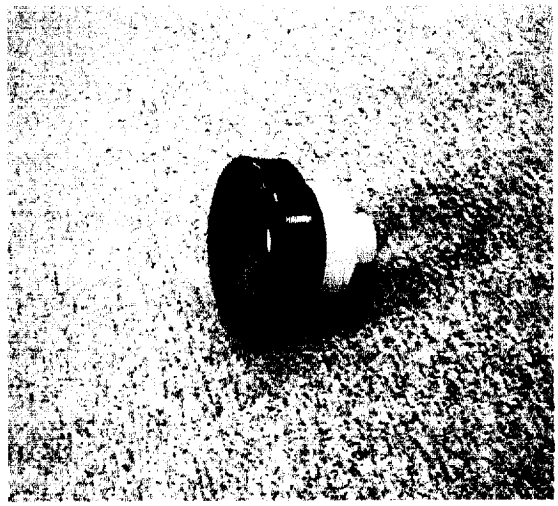

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Figure 1: A) ASE 200, B) Quick Volume Air Sampler, C) Portable Air Sampling Device in an Office Environment

The first building, Company X, was 20 years old, had carpeted floors, covered chairs and separation panels, and improperly maintained heating and air conditioning units. Over 25 % of the employees at Company X had complained of respiratory problems. Five offices were selected for air sampling and analyzed for PLFA, 3-OH fatty acids, sterols, and total culturable heterotrophs content. Company Y also had poorly maintained heating and air conditioning units as well as more than 20 % of employees complaining of respiratory illness. Three indoor and three outdoor air samples were taken. Outdoor samples are taken to ensure quality control, however, such samples also allow outdoor air contamination to be ruled out of any investigation of indoor air quality. Unlike the previous office buildings studied, Company Z, a local factory, had well maintained heating and air conditioning units, and, due to the presence of two different types of machine lines (with either aqueous based or oil based machine wash fluids), contained both "clean" (complaint free) or "dirty" (about which there had been complaints) air. Samples were collected from indoors while the machinery was running (two along machine lines with "dirty" air, and two along lines with "clean" air). Samples were taken indoors when the machinery was off (one from each of the machine lines), and outdoor samples were taken concurrent with the indoor air sampling sessions.

Filters obtained from each of Companies X, Y, and Z were analyzed for PLFA. The standard method used by the contractor for characterizing the microbial community involves extraction of total lipids from environmental samples using the modified Bligh/Dyer solvent system. This solvent system consists of a homogenous solution of methanol:chloroform:phosphate buffer (2:1:0.8 v/v/v). Samples are extracted in this solvent for 4 hrs before appropriate volumes of chloroform and nanopure water were added to give a ratio of 1:1:0.9 (v/v/v) of methanol :chloroform:water. This addition of solvent resulted in a separation of the extract into organic (chloroform) and aqueous (water and methanol) layers. To allow these two phases to completely separate, the sample is allowed to sit undisturbed for an additional 18 hrs. At this point, the lower (lipid containing) layer is removed and dried by rotary evaporation. The resulting total lipid is fractionated into neutral lipids, glycolipids, and polar lipids using silicic acid column chromatography. The phospholipid containing polar lipid fraction is subjected to mild alkaline methanolysis, transesterifying the fatty acids cleaved from the phospholipids into methyl esters. These fatty acid methyl esters are then analyzed by GC/MS to give a PLFA profile that is used to characterize the microbial community. Specific lipids serve to indicate the presence of a certain species and are biomarkers for that species. Other lipids are less specific in that their presence is indicative of larger microbe divisions {e.g. bacteria (Gram-negative, Gram-positive) or fungi}.

Additional tests on the samples taken from Companies X, Y, and Z were done to determine levels of endotoxins and sterols. Inhalation of endotoxins, measured as 3-hydroxy fatty acids (3-OH FA), have been shown to cause respiratory disorders. Sterol analysis gives information on the amount of microeukaryotes (e.g. fungi, algae, protozoa) present in a sample. As a point of comparison with published sampling and microbial biomass assessment techniques, total culturable heterotroph analysis (a standard microorganism counting procedure) was done simultaneously with all air samples taken using the Andersen sampler.

At Company X, all five indoor air samples contained relatively simple microbial communities composed of organisms typically found in the human skin and/or respiratory tract. The sterol and endotoxin (3-OH FA) analyses also revealed only those sterols and 3-OH FA commonly found in the human flora. The standard microbiology technique of total heterotroph counting of colony forming units (c.f.u.) per cubic meter of air sampled were also within normal limits for a healthy building (bacteria: approximately 10^2 c.f.u./m^3 and fungi: approximately 10^1c.f.u./m^3), however, culture based counts were 1-2 orders of magnitude lower than the amount of bacteria calculated from PLFA analysis. This deviation is due to the fact that PLFA analysis measures all viable biomass, whereas the standard counting method required the microorganisms in the sample to be culturable. Overall, each method of analysis performed on air samples from Company X indicate a building without high microbial contamination.

Compared to Company X, the level of biomass at Company Y was high. More diverse microbial communities were found in all indoor and outdoor air samples, although the indoor air contained different microbial communities as confirmed by principal components analysis[1] (Figures 2 and 3). The Gram negative communities in each of the indoor air samples were experiencing high levels of stress from either toxicity or starvation, but those in the outdoor samples were not (Figure 4). This stress was most likely due to the dry conditions indoors, caused by excessive air conditioning and heating, that cause desiccation of bacteria resulting in biomarkers indicative of environmental stress. As with Company X, total culturable heterotroph counts were approximately 2-3 orders of magnitude less than the counts obtained by PLFA analysis for all samples taken at Company Y.

In general, samples taken from the "dirty" air at Company Z contained high relative proportions of Gram negative bacteria (Figure 5a and b) that were in early stationary growth phase and undergoing low levels of metabolic stress (Figure 6), whereas those in samples from the "clean" air lines had smaller Gram negative communities (by approximately 2 orders of magnitude), slower growth rates, and were, in general, not experiencing as much environmental stress. Shutting down the machine lines resulted in a significantly decreased amount of biomass (2-3 orders of magnitude less), suggesting that aerosolization of the machine washing fluids was causing the high bacterial counts.

The community structure of the microbial biomass samples from the "clean" air were similar to each other and to those of the outdoor air samples. This was not surprising given that the machine lines were located adjacent to permanently open doors. As before, total culturable heterotroph counts were consistently approximately 2-3 orders of magnitude less than those obtained by PLFA analysis. In the initial proposal, it was reported that culturable microbe (bacteria and fungi) counts of between 200- 12000 colony forming units/ cubic meter of air had been reported in the literature. Due to the high efficiency of filtration sampling and the fact that lipid analysis does not require cell culture, the bacterial counts at Companies X, Y, and Z tended to be higher.

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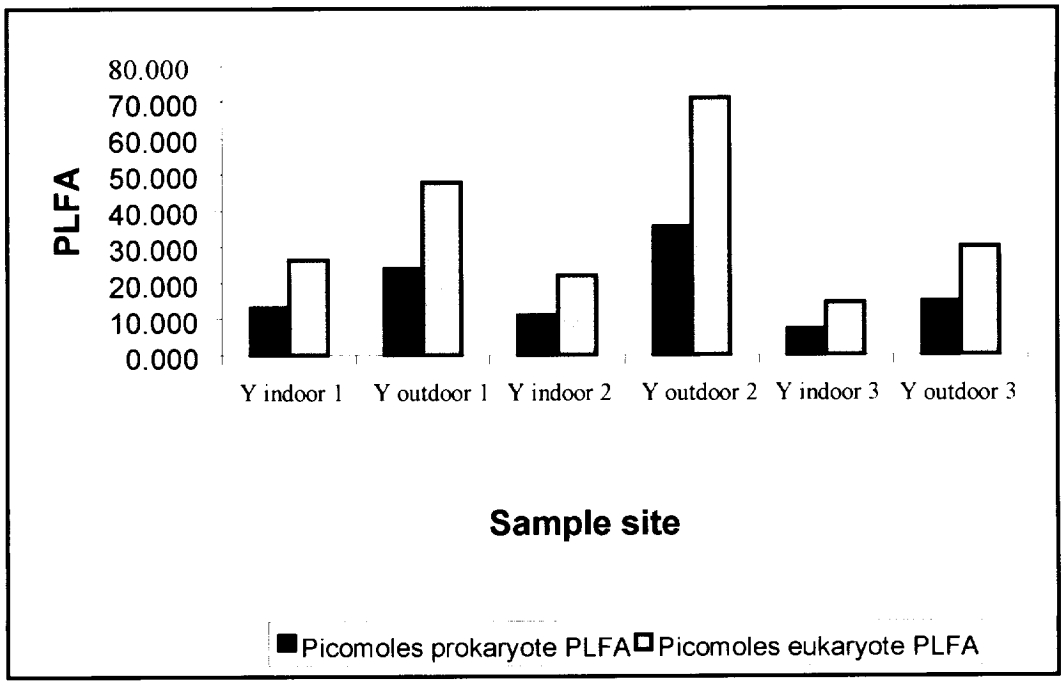
[1] Principal Components Analysis (PCA) is built on the assumption that variation implies information. In PCA, the multivariate data (PLFA profiles) is projected onto a reduced number of dimensions (principal components), thereby simplifying the data so relationships between sample sets can be easily observed. Thus, PCA analysis can show which microbial communities are similar.



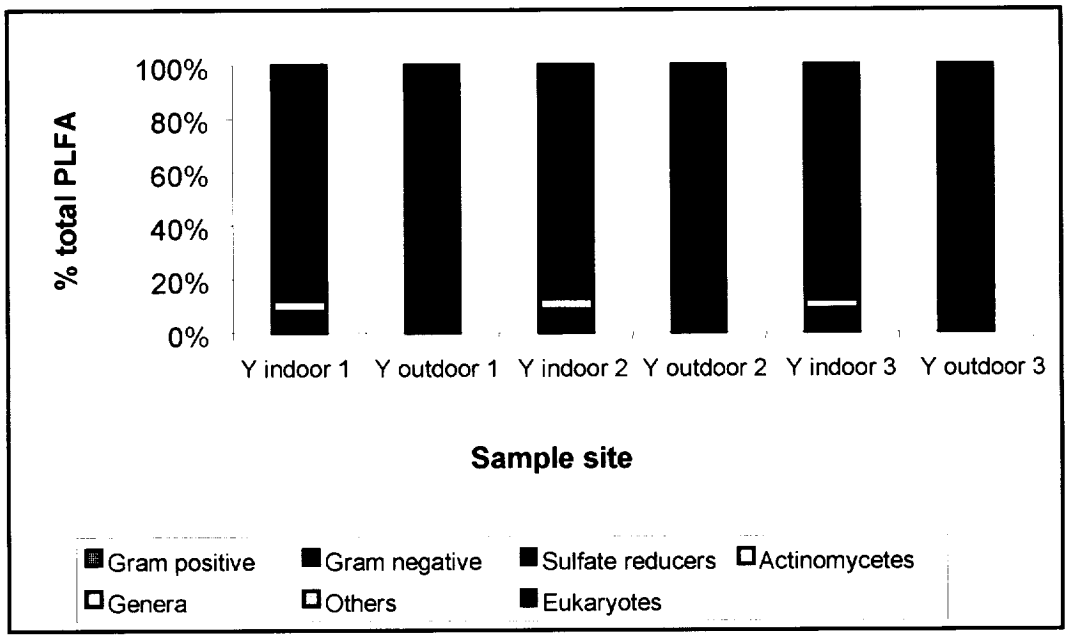


Figure 2: (a) Total biomass from company Y; (b) relative proportions of different forms of biomass at Company Y

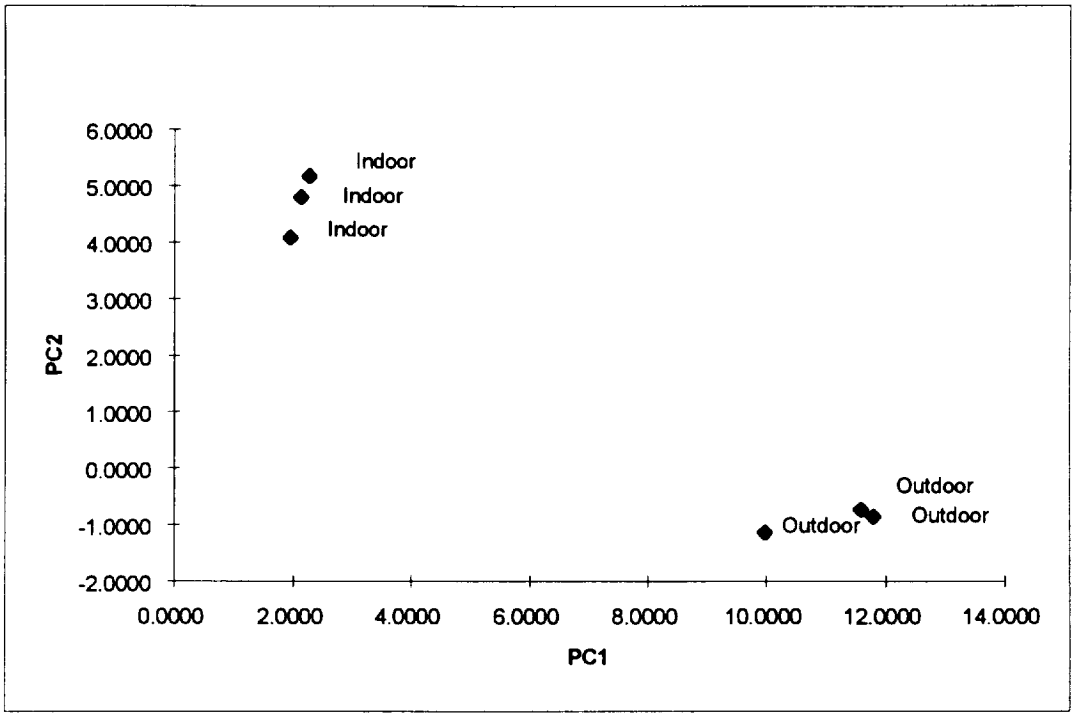


Figure 3: Principal components analysis of the PLFA extracted from the air indoors and outdoors at Company Y.

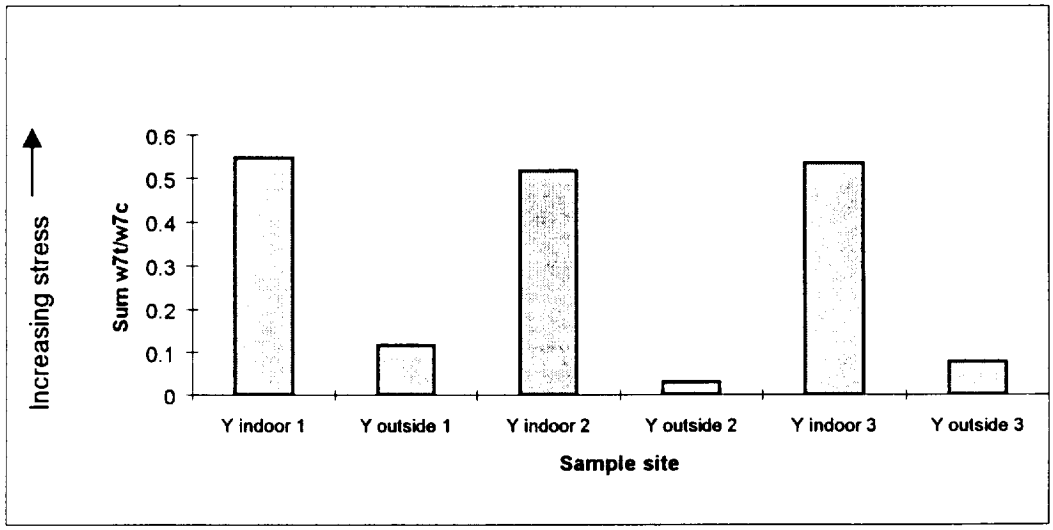
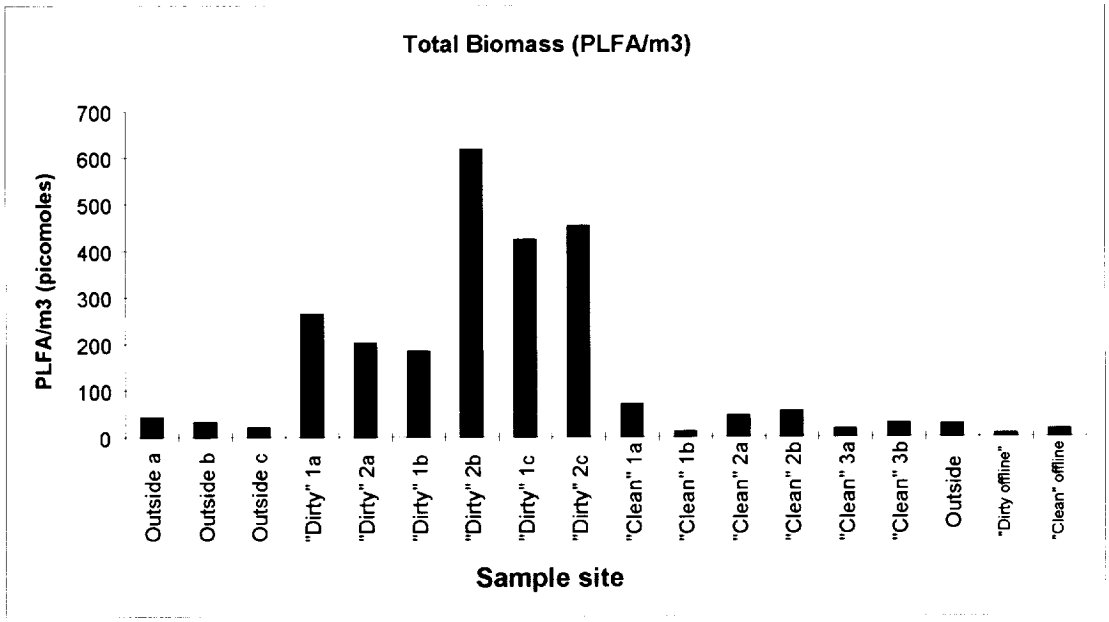


Figure 4: Increased impact of environmental stress indoors at Company Y.



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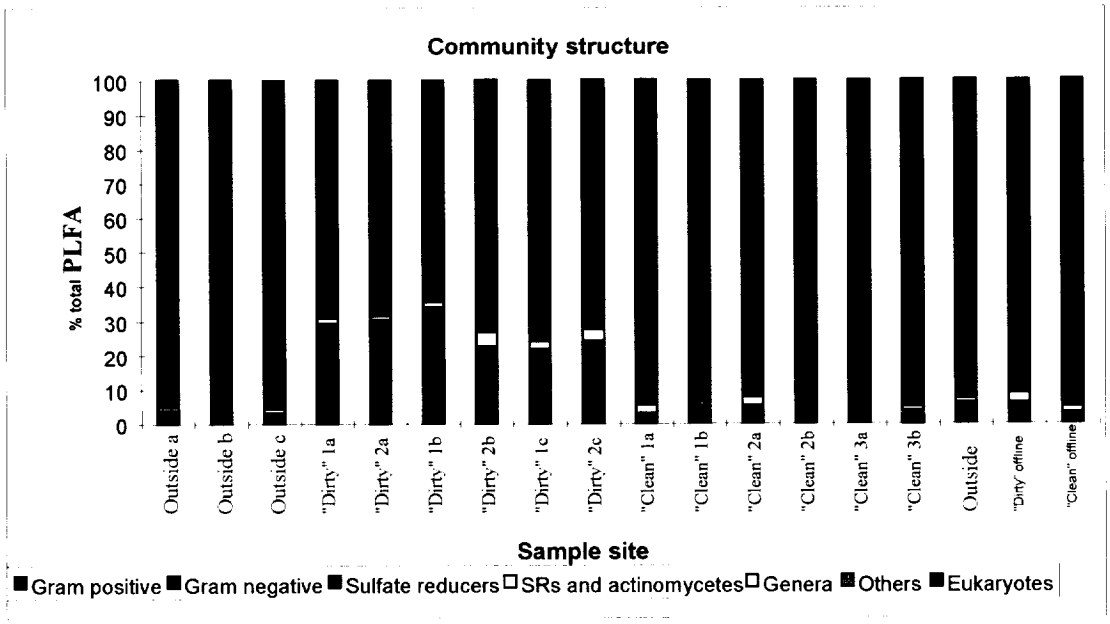

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Figure 5: (A) Total airborne biomass at Company Z along machine lines with either "clean" or "dirty" air; (B) Relative proportions of different forms of biomass at Company Z.

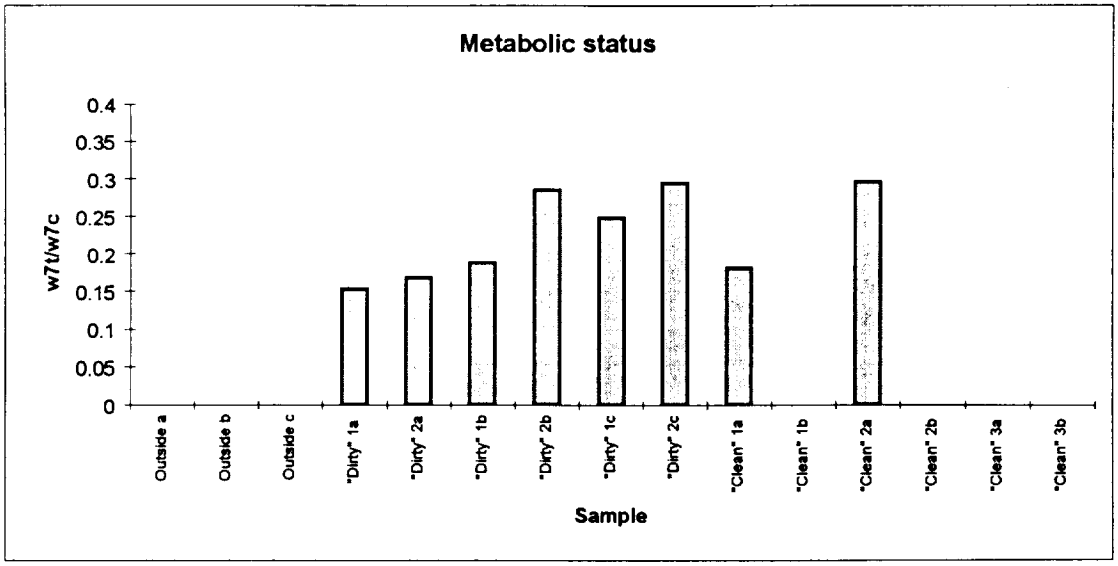

Figure 6: Metabolic stress levels of Gram negative bacteria at Company Z. Increased stress = higher ratio.

Except for Company X, higher levels of biomass were detected in areas where complaints were expressed. In all three Companies, the community structures of high-complaint areas were distinctly different from those of low-complaint areas (which more closely resembled the community structure of outdoor air samples). Typically, microorganisms from "dirty" samples were experiencing higher levels of environmental stress than were those from "clean" samples. Samples "Clean 1a" and "Clean 2a" from company Z were experiencing significantly higher levels of environmental stress possibly due to dry conditions in that area of the factory..

From this study of Companies X, Y, and Z, it is clear that the PASD was capable of effectively sampling air for subsequent lipid analysis to provide information on possible airborne contaminants. Due to the generally low biomass content per cubic meter of air, obtaining an air sample is not as simple as obtaining a typical environmental sample of soil or water. Using the PASD, however, an operator has the ability to take an air sample in any facility, even while it is occupied. As public awareness of problems associated with airborne biocontaminants increases, the simple, convenient method of indoor air analysis developed in this project will be able to meet arising needs by characterizing viable biomass in polluted air.

Objective Two:
Accelerated solvent extraction

A primary focus of the work performed during the Phase II contract has been the automated and improved extraction of PLFA and other lipids from both air filters and other environmental matrices. Generally, the wet chemistry required for lipid analysis is slow and labor intensive, often taking up to 24 hours for an initial lipid extraction. Pressurized accelerated hot solvent extraction offered the possibility of significantly improving the speed and extraction efficiency of lipid analysis. The higher temperature increases the extraction kinetics while high pressure keeps solvents below their boiling points, thereby enabling safe extractions. The accelerated hot solvent extractor (ASE

200) was developed by Dionex Corporation (P.O. Box 3603, Sunnyvale, CA 94088-9988) for the extraction of compounds such as PCBs and PAHs (Figure 1A). Whereas supercritical fluid extraction (SFE) of polar analytes is a complex, time-consuming and matrix-dependent procedure, the ASE offered the possibility of increasing the automation, speed, and efficiency of lipid extraction without such complications. The ASE 200 uses elevated temperatures and pressures to increase recovery and decrease extraction time. Solvent usage is also reduced because of the increased solute capability of heated solvents. The ASE 200 is capable of performing up to 24 extractions sequentially and is fully programmable, allowing samples to be set up and extractions to take place overnight.

In a typical extraction, samples are loaded into stainless steel extraction cells, filled with the solvent of interest, heated, and pressurized. These conditions are maintained for a predetermined amount of time (typically 5-25 min) before the solvent is purged to a collection vial. If desired, this static cycle may be repeated. At this point, the collection vial containing the total lipid is removed from the ASE 200. This basic extraction procedure is the same for whatever microbial component is under analysis. However, to optimize the extraction efficiency for a large number of different lipids, the solvent, time, pressure, temperature and static cycle number parameters were evaluated . Investigations of improved extraction for each biomarker are presented below.

Phospholipid fatty acids

Initial comparisons between the conventional Bligh and Dyer and the ASE 200 extractions were carried out using pure filter borne biomass. A wide range of different solvent extraction parameters were tested. Parameters investigated included solvent system, temperature, extraction time, and static cycle number. The pure biomass used included vegetative cells {bacterial biomass (Gram negative *Escherichia coli*, Gram positive *Staphylococcus aureus*, *Actinomycete* (*Mycobacterium fortuitum*), and yeast} and sporulated biomass {fungal spores (*Aspergillus niger*) and bacterial spores (*Bacillus subtilis*)}.

In general, two solvent systems were used. The specific extraction conditions for pure biomass are listed in Table 1.

The pure biomass studies were performed to determine the most efficient extraction procedure for the extraction of lipids from environmental samples. As such, only the most efficient extraction parameters determined from these studies were investigated with the environmental samples (Table 2). Figure 7 is a schematic diagram of the accelerated solvent extraction system used in this study. All extraction vessels were solvent rinsed in acetone prior to use to remove any lipid contaminants. The outlet end of the cell was then lined with a chloroform rinsed cellulose filter, stopping particulates from entering the system. Samples were loaded into the cells and, where necessary, excess space was filled with muffle furnace sterilized sand (Ottawa sand, 20-30 mesh, Fisher Scientific, Atlanta, GA 30091), minimizing the solvent volume required. After loading the cell into the ASE system, the cell was filled with extraction solvent by opening the pump valve, following which the cell was pressurized, achieving a high pressure seal at both ends.

Table 1: Extraction conditions investigated using the ASE 200 for pure biomass

Biomass	Temperature (°C)	Time (min)	Cycle	Solvent systems
E. coli	80 and 120	5, 10, 15	1, 2, 3	$CHCl_3$:Methanol (1:2 v/v) Methanol:$CHCl_3$: PO_4 buffer (2:1:0.8 v/v/v)
S. aureus	80 and 120	5, 10, 15	1, 2	$CHCl_3$:Methanol (1:2 v/v) Methanol:$CHCl_3$: PO_4 buffer (2:1:0.8 v/v/v)
M. fortuitum	80 and 120	5, 10, 15	1, 2	$CHCl_3$:Methanol (1:2 v/v) Methanol:$CHCl_3$: PO_4 buffer (2:1:0.8 v/v/v)
B. subtilis	80 and 120	5, 10, 15	1, 2	Methanol:CHCl3: PO4 buffer
A. niger	80 and 120	5, 10, 15	1, 2	$CHCl_3$:Methanol (1:2 v/v) Methanol:$CHCl_3$: PO_4 buffer (2:1:0.8 v/v/v)
S.cerevisiae	80 and 120	5, 10, 15	1, 2	$CHCl_3$:Methanol (1:2 v/v) Methanol:$CHCl_3$: PO_4 buffer (2:1:0.8 v/v/v)

Sample cell heating (under constant pressure) followed, and when the appropriate temperature was reached, the static extraction occurred. The extract was then transferred into the sealed sample vial after displacement with fresh solvent. Following this, further static cycles were performed where necessary. After the final static cycle extractions, the cell was purged with nitrogen for 3 minutes finishing the extraction process. The solvent was then rinsed through the system before the ASE 200 automatically loaded the next sample and collection vial. Where phosphate buffer was used in the first phase extraction solvent, an appropriate volume of chloroform and deionized water was added to give the correct final ratio (chloroform:methanol:phosphate buffer/ water; 1:1:0.9 v/v/v) to form the two phases. The chloroform layer was transferred into a new test tube and evaporated under nitrogen at 37 °C. Where no phosphate buffer was used, the methanol : chloroform (2:1 v/v) was evaporated directly under nitrogen prior to fractionation. Although using the phosphate buffer added another step to the extraction, chloroform evaporates substantially faster than 2:1 MeOH:$CHCl_3$ and, as such, the time added to the sample preparation was inconsequential.

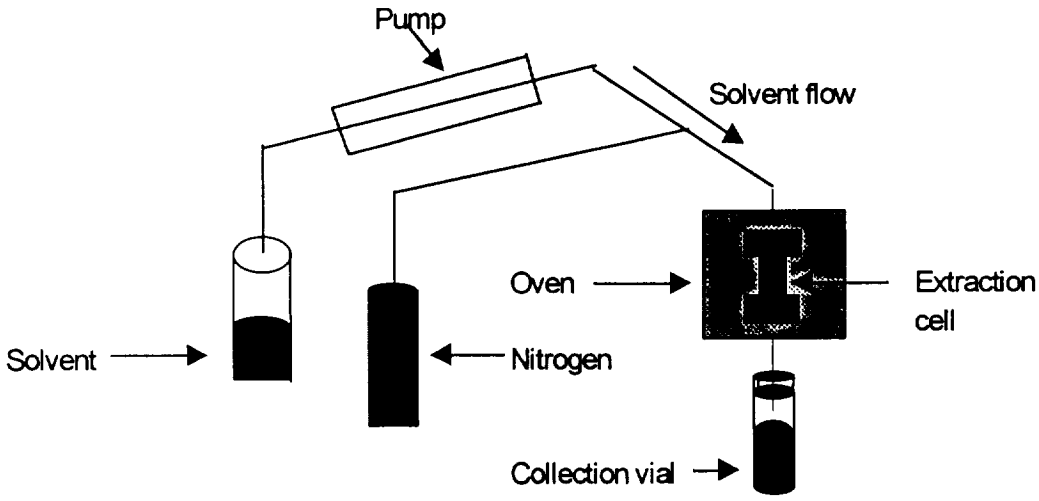

Figure 7: Schematic diagram of the ASE 200

Using the ASE 200, maximum extraction of PLFA from pure biomass was achieved with the methanol : chloroform : phosphate buffer (2:1:0.8 v/v/v) solvent system with two 15 minute static cycles at either 80 or 120 °C. Figure 8 shows the maximum recovery of PLFA using the ASE 200 as a percentage of the PLFA extracted using the modified Bligh and Dyer (defined as100%). Although there was no significant difference between extraction efficiencies obtained using the modified Bligh and Dyer or the ASE 200 for the vegetative biomass, PLFA was extracted with far higher efficiency from the spore forms analyzed (fungal and bacterial; $p<0.05$). *Bacillus* spp. spores can contain higher relative proportions of certain terminally branched saturate PLFA than do the corresponding vegetative cells (14) and the ASE 200 enabled recovery of higher relative proportions of terminally branched saturate PLFA (specifically i15:0 and i17:0) than were extracted using the modified Bligh and Dyer extraction.

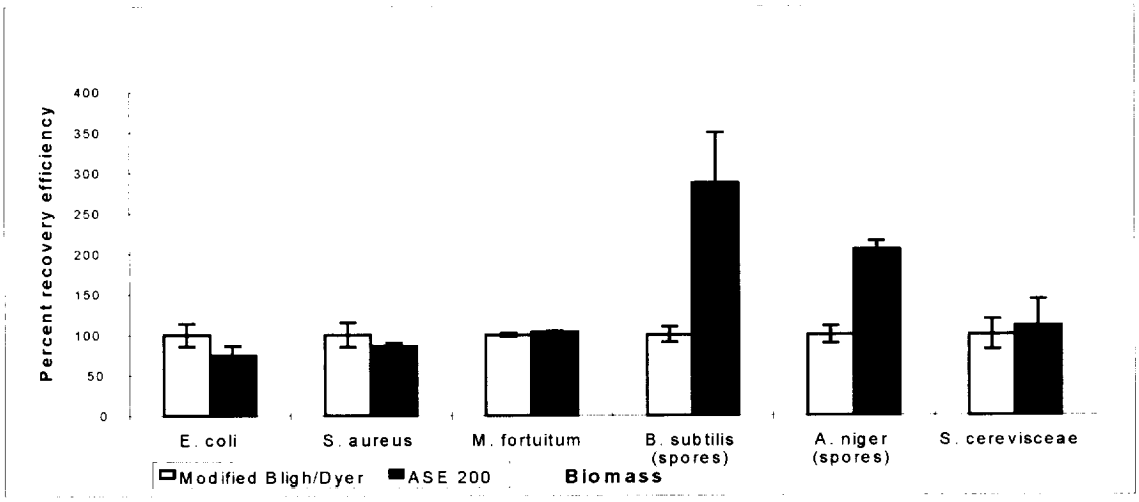

Figure 8: Extraction of phospholipid fatty acids from pure biomass samples using accelerated solvent extraction expressed as a percentage of extraction using the modified Bligh and Dyer procedure (100%)

Following comparison of different extraction parameters, it was evident that solvent system, time, and static cycle number had the greatest influence on extraction efficiency. Generally, the methanol : chloroform : phosphate buffer (2:1:0.8 v/v/v) solvent system gave more consistent and higher recoveries of PLFA than did the methanol : chloroform (2:1 v/v) solvent system. Indeed, compared to extraction efficiencies obtained using the methanol:chloroform:phosphate buffer system the extraction efficiencies obtained using 2:1 methanol:chloroform were low (data not shown). Principal components analysis (PCA) enabled comparison of the multi-variate PLFA profiles obtained following the different extraction procedures for each type of biomass. Independent of whether the modified Bligh and Dyer or the accelerated solvent extraction was used, there was no distinct trend in the extraction efficiencies achieved for specific PLFA when using the methanol : chloroform : phosphate buffer solvent system. However, where the solvent system contained only 2:1 methanol:chloroform, a decreased relative proportion of the PLFA 18:1ω9c was extracted from the *S. cerevisiae* while an increased relative proportion of the PLFA 10me18:0 was extracted from the *M. fortuitum*.

Where the temperature of the accelerated solvent extraction was too high (*i. e.* above 120 °C), using either the methanol:chloroform (2:1 v/v) or the methanol : chloroform : phosphate buffer (2:1:0.8 v/v/v) solvent system resulted in breakdown of the extraction cell and poor PLFA recovery independent of static cycle number (data not shown). Following extraction of PLFA from pure biomass, it was necessary to further test the ASE 200 using environmental samples. The common environmental matrices of air, water, and soil were chosen for extraction efficiency analysis. All of the samples underwent PLFA analysis with extraction by conventional Bligh/Dyer and ASE Bligh/Dyer with the following extraction conditions (Table 2).

Table 2: Extraction conditions investigated using the ASE in the environmental sample studies

Sample type	Temperature (°C)	Static Cycle number (15 min)	Solvent systems
Airborne biomass	80	2, 3	$CHCl_3$:Methanol (1:2 v/v) Methanol:$CHCl_3$: PO_4 buffer (2:1:0.8 v/v/v)
Water biomass	80 and 120	2, 3	$CHCl_3$:Methanol (1:2 v/v) Methanol:$CHCl_3$: PO_4 buffer (2:1:0.8 v/v/v)
Soil biomass	80 and 120	2, 3	$CHCl_3$:Methanol (1:2 v/v) Methanol:$CHCl_3$: PO_4 buffer (2:1:0.8 v/v/v)

Compared to the modified Bligh and Dyer, a greater amount of PLFA was extracted from soil using the ASE 200, however, the increase was not significant (Figure 9; $p>0.05$). Also, there was no significant difference between the efficiency of the Bligh and Dyer and the ASE 200 for PLFA extractions from water samples (Figure 9), however, using the ASE a significantly greater ($p<0.05$) amount of PLFA was extracted from the air biomass sample.

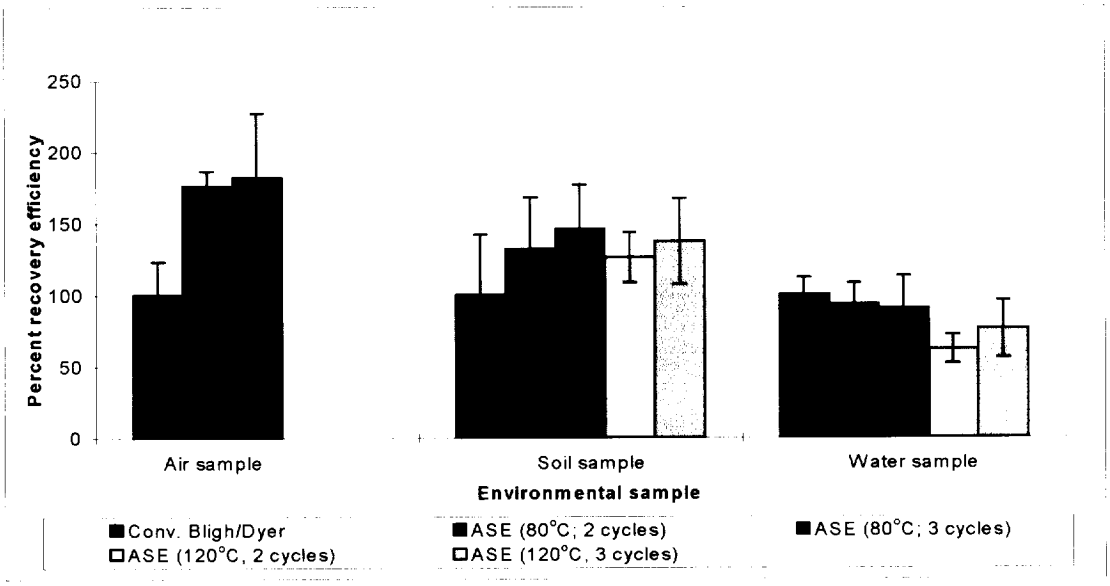

FIGURE 9: Extraction of phospholipid fatty acids from air, soil, and water samples using accelerated solvent extraction expressed as a percentage of extraction using the modified Bligh and Dyer procedure (100 %)

Although the percentage recovery of PLFA for both the air and soil samples increased with the extra 15 minute static cycle, these increases were not significant (Figure 9; $p>0.05$). The increase in the recovery of PLFA was probably due to the rinse and purge procedures within the ASE 200, both of which displaced solvent extractant from the samples into the collection vials. During the conventional modified Bligh and Dyer procedure, no such solvent displacement is performed.

Extraction recoveries at 120 °C were generally lower, although not significantly so, than those at 80 °C. It is likely that some decomposition of the PLFA was occurring at this higher temperature. Due to comparatively low recoveries of PLFA from airborne biomass samples in preliminary studies at 120 °C, additional studies were not performed at this temperature for this type of biomass.

To further investigate any impact on the types and amounts of PLFA recovered when using the ASE 200 (at either 80 or 120 °C with 2 or 3 static cycles) compared to the modified Bligh and Dyer, a PCA was performed on the PLFA profiles obtained. Although there were some out-lying profiles for both the soil and water samples (following either Bligh and Dyer or ASE extractions), there was no significant difference in the amounts of PLFA obtained dependent on extraction procedure (data not shown). Conversely, the PLFA profiles from the air samples exhibited clustering (Figure 10a). Compared to the profiles obtained using the Bligh and Dyer extraction, the PLFA profiles obtained following extraction using the ASE at 80°C contained larger amounts of typically eukaryote type PLFA (Fig 10b). The first principal component accounted for 97% of the variance and the second, 1.1 %. The first principal component was most heavily influenced by the PLFA 18:3ω3, 18:1ω9c, and 16:0, all of which are more commonly present in eukaryote biomass (Fig. 10b). The second principal component was most heavily influenced by 18:0, 18:1ω7t and 18:1ω7c. For the remainder of the PLFA, there was no difference in the relative proportion extracted using either procedure. We concluded that, compared with the Bligh and Dyer extraction, ASE extraction procedures on predominantly prokaryote samples should have no impact on the extraction efficiencies of the different PLFA. However, the accelerated solvent extraction was substantially more efficient than the Bligh and Dyer for a predominantly eukaryote sample, and this will have an impact upon community structure analysis of such samples.

In conclusion, it is clear that using a methanol : chloroform : phosphate buffer system solvent system (1:2:0.8 v/v/v) at 80°C and 1200 psi , the ASE 200 enabled rapid extraction of phospholipids from both pure biomass and environmental samples. The accelerated solvent extraction required substantially less materials, labor and time than did the conventional modified Bligh and Dyer extraction. Compared to the modified Bligh and Dyer extraction, PLFA yields obtained using the pressurized hot solvent extraction were not significantly different for the bacteria, yeast, or water samples but were significantly higher ($p<0.05$) in the case of the bacterial and fungal spores and the airborne biomass.

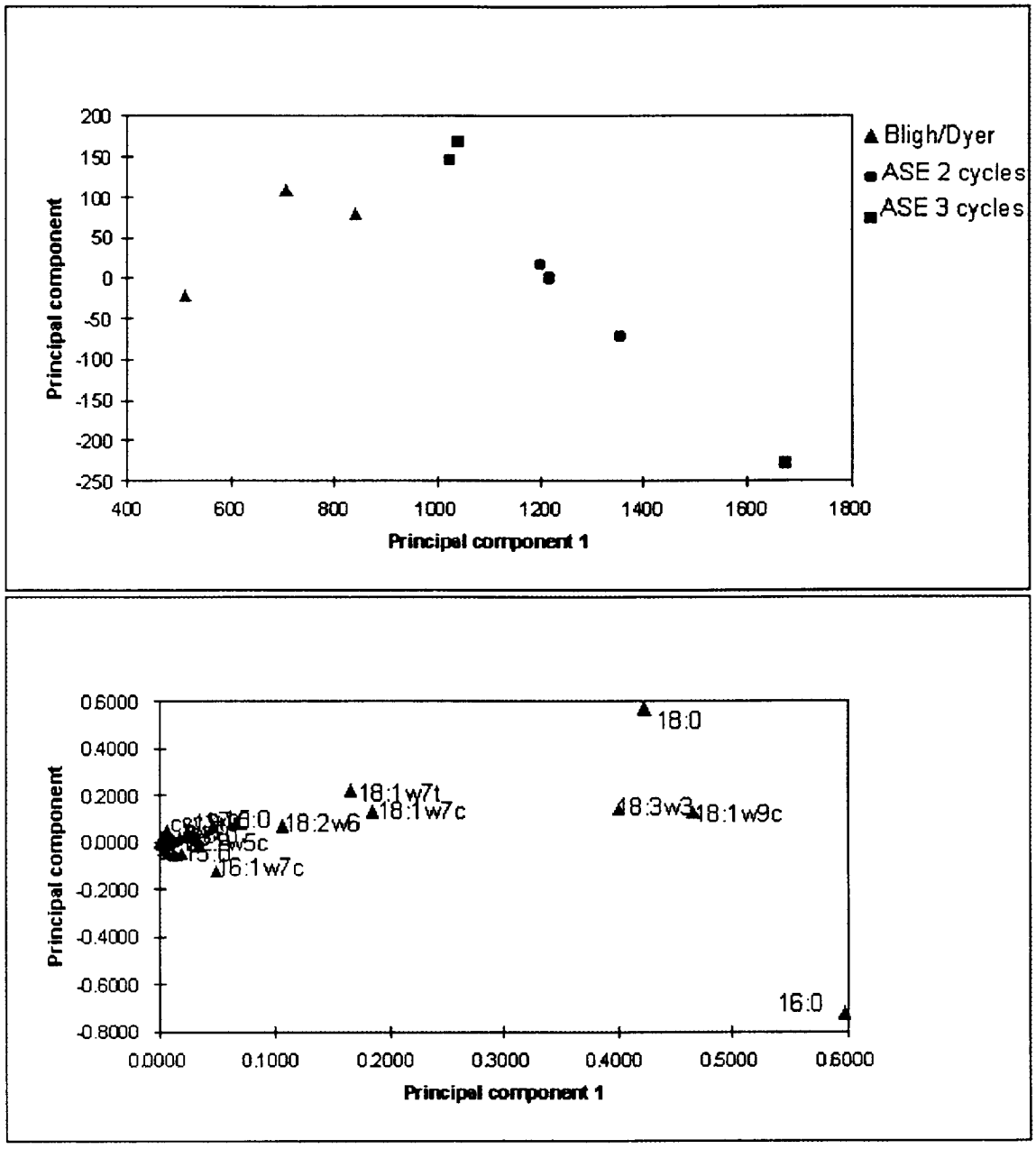

FIGURE 10: (A) A scatter plot of the scores from a principal components analysis of the PLFA profiles from Figure 3 (from airborne biomass). Principal component 1 described 98 % of the variance, and principal component 2, 1.1 %. (B) A scatter plot of the coefficient of loading derived from the principal components analysis in A.

Computer automated identification of PLFA

A major time-consuming portion of the lipid analysis is the identification of individual fatty acid biomarkers. Computer automation of this process was achieved by developing a retention time calibration table from fatty acid standards. The basic algorithm for any peak identification in a uni-dimensional analysis technique is based on

retention time matching. In order for these algorithms to operate successfully, the retention times of the calibration table (standards) must closely agree with those obtained from the sample. There are a number of instrumental and sample related parameters which can cause these values to "wander" or vary. In order to correct for this, a "window" is defined in which the peak's retention time will fall 95% of the time. This works well for systems where compounds of interest are well separated and result in comparable signal levels. When either large numbers of components or widely varying signal levels are encountered, these algorithms fail to accurately identify components of the mixture. In the case of a large number of components, this happens when multiple peaks occur within the specified retention window. This misidentification can be remedied by reducing the retention time window, but this reduction results in large numbers of components not being identified or being misidentified because of retention time "wander". When widely varying signal levels are encountered identification problems result from retention time shifts due to column overload conditions (non-linear adsorption isotherms). In the case of the PLFA analyses, both situations contribute to the difficulty in peak identification.

In order to correct for the wander in retention time and more correctly identify overloaded peaks, the retention times must be standardized. For most GC situations, a single time reference is utilized. However, when large numbers of closely eluting components must be identified (as with PLFA analyses) such standardization does not result in sufficiently reproducible retention times to allow the required narrow retention time windows to be utilized. Therefore, additional corrections must be applied. In order to accurately adjust retention times over the full elution window, a retention time standard containing the normal saturates consisting of chain lengths from 12 to 24 is analyzed. An Equivalent Chain Length (ECL) value, or retention index, is then utilized to calculate a linear conversion factor for all peaks which have retention times between those of the normal saturates. Once the conversion factors have been calculated for each region of the elution window, they can be applied to the chromatogram. Since the Chemstation software has an extremely robust retention time matching/peak identification algorithm, it was employed for the actual identifications. However, since it is much more accurate to generate retention times from ECLs than vice versa, it was decided that the most logical approach would be to convert a standard table of ECLs into their equivalent retention times and then utilize the existing identification algorithms. Standard ECL values were determined for all PLFAs of interest and entered into the Chemstation's calibration table. For each individual sample these values were converted to retention times using the calculated conversion factors. Major changes in environmental, column, and instrumental parameters were accounted for in this manner. Since most of the retention times are relatively stable over a 6-8 hr period, a retention time standard mixture is required only every 5-6 analyses. Any slight changes in retention time over this period can usually be compensated for by using the internal standard (19:0 normal saturate) as a time reference in the standard identification algorithm. Employing the outlined method, very small retention time windows (relative windows as small as +/- 0.6%) may be used and very complicated samples can be accurately identified. At this point, approximately 95% of all fatty acids in a complex environmental sample will be correctly identified. Subsequently, this computerized identification has been incorporated into our PLFA analysis.

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Poly unsaturated fatty acids (PUFA)

From the results of the separate PLFA extraction efficiencies above, there seemed to be a possibility of a discrepancy between extraction efficiencies for the bacterial type of PLFA (the majority of which have one double bond) and the eukaryote type PLFA (the majority of which have 2 or more double bonds and are therefore known as polyenoic unsaturated fatty acids). As such, we investigated the extraction efficiency of accelerated solvent extraction for these specific PLFA.

Polyenoic unsaturated fatty acids (PUFA) are eukaryotic components of PLFA. PUFA may also exhibit significant branching of the carbon chain. Using *Ulva* spp., a marine algae containing relatively high proportions of PUFA, a study was conducted to determine the best method of extraction of PUFA by the ASE 200 varying the solvent, temperature, and number of static cycles (15 minutes each). Figure 11 shows the extraction efficiencies obtained for *Ulva* PLFA.

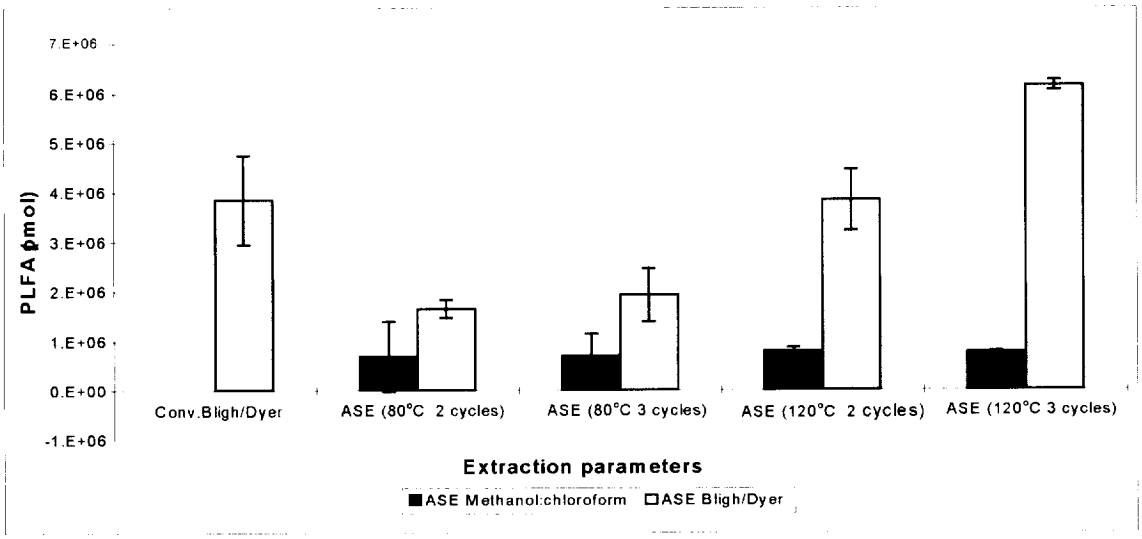

FIGURE 11: Extraction of fatty acids from *Ulva* using conventional and ASE methodologies. N=3, error bars represent standard deviation. ASE Bligh/Dyer = ASE 200 with Bligh/Dyer solvents and ASE Methanol:Chloroform = ASE 200 with 2:1 Methanol:Chloroform.

As is apparent in Figure 11, the best method of extracting PLFA from *Ulva* was the ASE Bligh/Dyer at 120°C using 3 cycles. However, to determine which method was most efficient for extracting the PUFA, it was necessary to further subdivide the PLFA findings above into the major catagories of PLFA. Figure 12 shows these major catagories of PLFA.

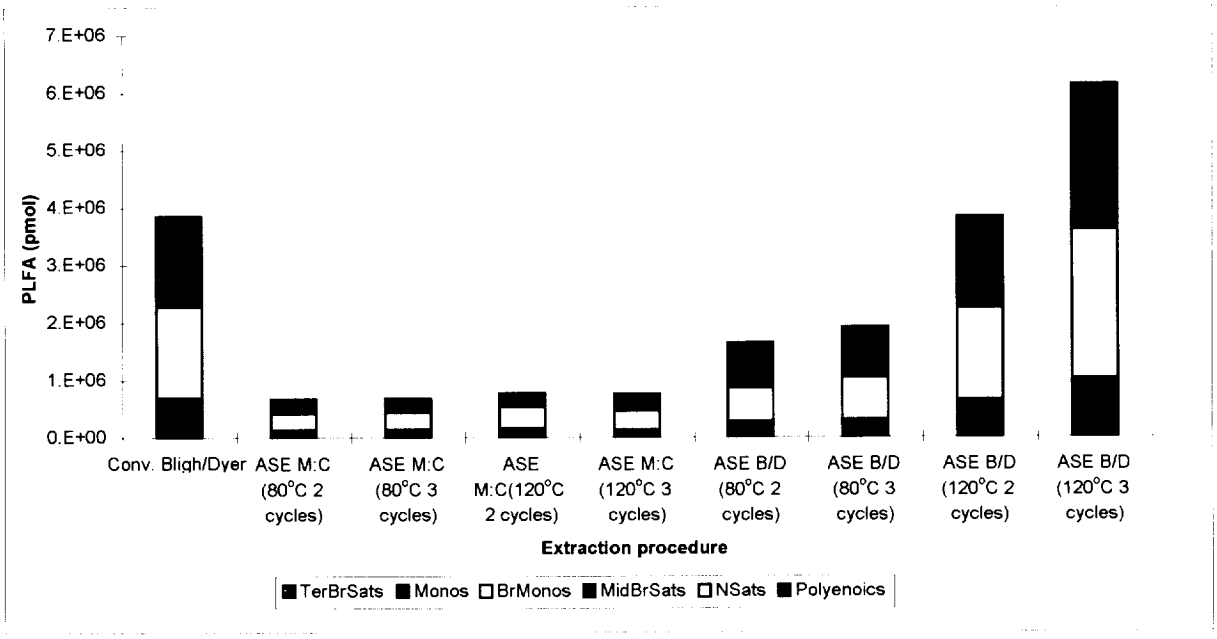

FIGURE 12: Total amounts of PLFA types recovered using different extraction procedures. N=3, maximum standard deviation = 5%.

From Figure 12, it was clear that the most efficient extraction of PUFA from *Ulva* was obtained using the ASE Bligh/Dyer at 120°C with 3-15 minute cycles. It is also clear, however, extraction efficiency is at least partly dependent on the nature of the substrate and the target molecule. The total PLFA from environmental samples were more efficiently extracted at 80°C, whereas PUFA were more efficiently extracted at 120°C.

Sterols extraction

Sterols are typically found in the glycolipid and neutral lipid fractions of the total lipid extract obtained following the Bligh/Dyer extraction and are an excellent measure of the microeukaryotes (fungi, algae, protozoa, etc.) found in the microbial community. Two important sterols have been studied in Phase II: 1) ergosterol, a common fungal sterol, and 2) isofucosterol, found in algae. Both sterols have great potential as diagnostic markers for biomass contamination, whether in indoor air or in marine environments.

Ergosterol is the primary component of fungi cell walls and, as such, has been used as an indicator of fungi present in many environmental matrices including air, soil, and grain. Fungal propagules represent one of the major forms of indoor air biocontamination. To determine the efficiency of using the ASE 200 to extract ergosterol from fungal biomass, experiments were performed comparing the established extraction procedure for ergosterol (methanolic saponification) with several ASE methods.

Because the majority of airborne fungi are present in spore rather than mycelial form, spores (*Aspergillus niger*) were analyzed during this study. The spores were cultivated, harvested, and then loaded onto glass fiber filters. Extractions of ergosterol

and Larsson, L. *J. Chromatography*, **666**: 77-84 (1995)). Initial quantification of ergosterol was done by High Performance Liquid Chromatography (HPLC). Our early results indicated that the fractionation performed to separate lipid groups resulted in a substantial loss of ergosterol (into the glycolipid fraction), and consequently, this fractionation step was not used. Figure 13 shows the ergosterol extraction efficiencies (100% recovery was defined as that of the methanolic saponification). No ergosterol was extracted using hexane as the solvent.

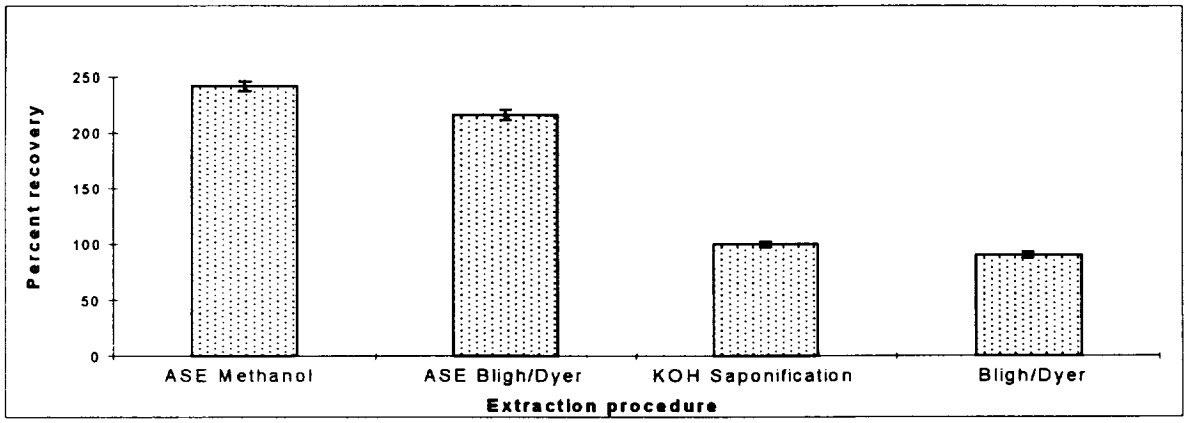

FIGURE 13: A comparison ergosterol recovery from fungal spores using established and ASE based procedures. 100%= KOH saponification, n=3, error bars = SEM.

Subsequent inclusion of the fractionation step decreased the total amount of ergosterol detected, but not the relative extraction efficiency among the four methods studied.

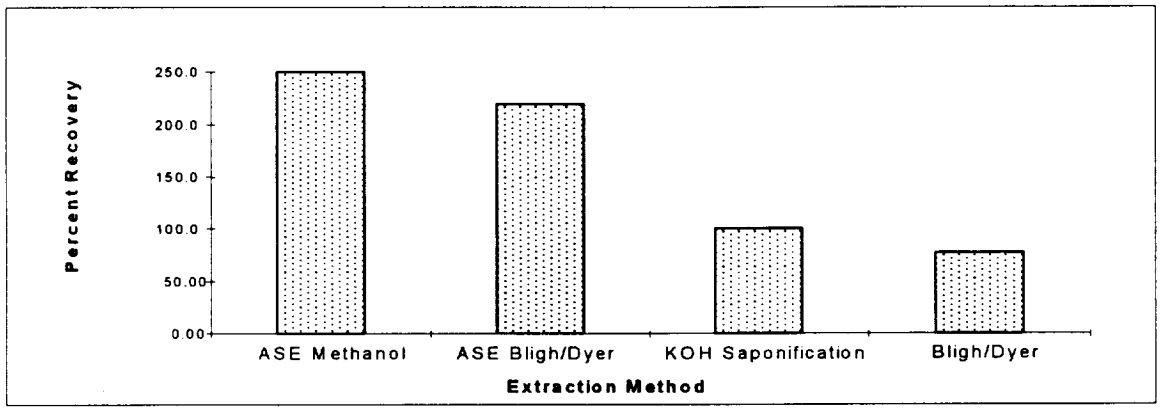

FIGURE 14: A comparison of the percent recovery of ergosterol from the glycolipid fraction, following extraction from fungal spores using both established and ASE based procedures. 100%= KOH saponification, n=3.

Initial ergosterol detection was done using HPLC with identification made by comparing retention times to that of a known ergosterol standard. Adaptation of analysis to GC/MS enabled lower limits of detection and definitive identification by mass spectrometry. Ergosterol was extracted from *A. niger* as before using ASE Methanol, ASE Bligh/Dyer, and KOH saponification. Half of these samples were fractionated while

the others were analyzed in total lipid. All ASE 200 samples were methylated, and then all samples were derivatized by the addition of trimethylsilyl group to each hydroxyl position to facilitate analysis by GC.

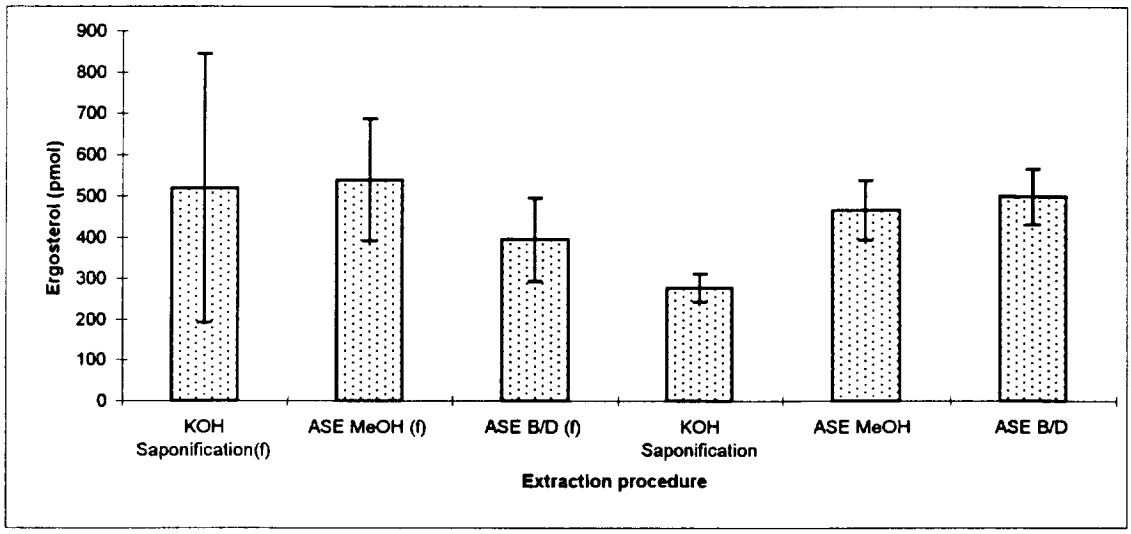

FIGURE 15: A comparison of extraction, fractionation and derivatization of ergosterol from pure biomass (*Aspergillus niger*). N=3 for all sample sets. f=fractionation. Error bars represent the standard deviations of the mean.

Figure 15 showed that ASE Methanol and ASE Bligh/Dyer were the most efficient, most consistent methods of analysis. As previously, it was also apparent that fractionation negatively impacted the extraction efficiency, significantly increasing the standard deviation for the data and should, if possible, be omitted.

From these results, ASE Methanol provided the greatest extraction efficiency, followed by ASE Bligh/Dyer, KOH saponification, and conventional Bligh Dyer. It can be concluded that when analysis is simply for sterols, ASE Methanol is the extraction method of choice, but when PLFA is also required, ASE Bligh/ Dyer followed by fractionation (saving the glycolipid and neutral lipid for ergosterol analysis and the polar lipid for PLFA) is the preferred technique.

To determine efficiency of recovery of ergosterol from airborne biomass samples, outdoor air samples were collected for 36 hours in triplicate and analyzed using KOH saponification, ASE Methanol, and ASE Bligh/Dyer techniques. All three extraction techniques were comparable in efficiency, but, because of erratic and low concentrations of fungi in the air in mid-autumn/winter, the standard deviations for ergosterol extracted from each filter was very large. Thus, no significant conclusions could be made (data not shown).

Isofucosterol is an algal sterol found in *Enteromorpha Linza*. Extraction of isofucosterol was performed using the following conditions: ASE Bligh Dyer, ASE methanol:chloroform (1:1 v/v), ASE methanol:chloroform (2:1 v/v), hand extraction methanol:chloroform (1:1v/v), and hand extraction methanol:chloroform (2:1 v/v).

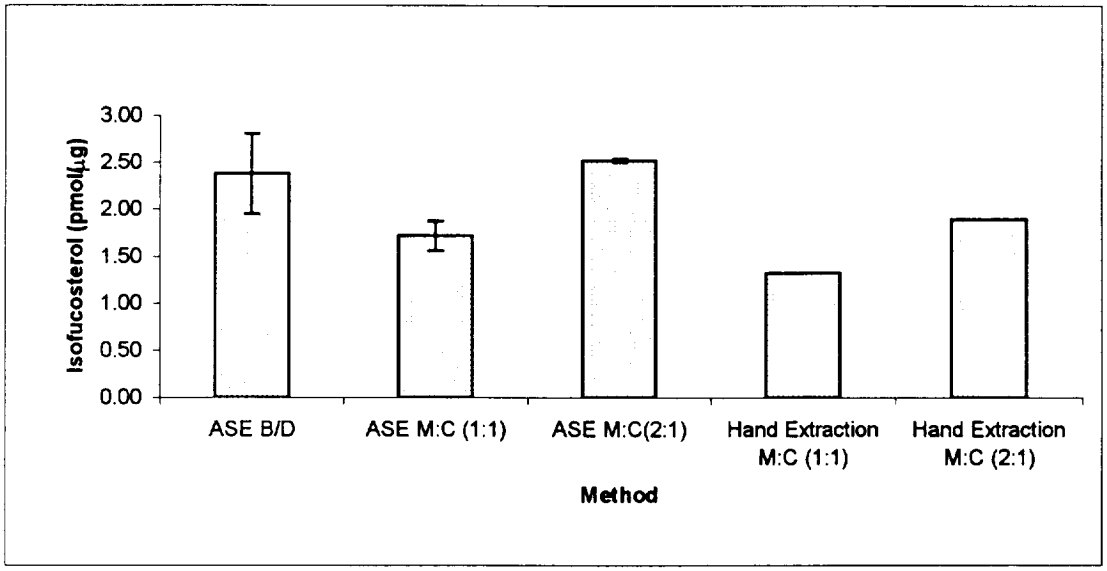

FIGURE 16: Amount of isofucosterol extracted from *Enteromorpha Linza*

As is apparent from Figure 16, the most consistent and efficient method for extracting isofucosterol from *Enteromorpha* was the ASE 200 (120°C, 3-cycles) with methanol:chloroform (2:1 v/v) as the solvent system. ASE Bligh/Dyer provided the next most efficient extraction, but the standard deviation was much greater. Figure 16 also shows that the ASE methods extract more isofucosterol per µg than the traditional, time-consuming hand extraction (requiring 30 minutes of sonication and then an 18 hour heating in a water bath). The dramatic reduction in analysis time and improvement in extraction efficiency makes the ASE 200 extremely appealing for the extraction of isofucosterol from *Enteromorpha Linza*.

Poly β- Hydroxyalkanoic Acid (glycolipid)

Another major lipid group referred to in our initial Phase II proposal was the glycolipids from which fraction the poly β- hydroxyalkanoic acids are obtained. When essential nutrients required in the formation of bacterial membrane lipids are lacking from the environment, bacteria cannot divide, formation of PLFA ceases, and instead, carbon is stored as poly β- hydroxyalkanoic acid (PHA). Therefore, analysis of PHA provides valuable information on the unbalanced growth and nutritional status of the microbial community. The current limits of detection of PHA are high relative to the amount of PHA found in a typical sample, so improvements on the extraction of PHA were required.

To compare extraction efficiency of the ASE 200 versus conventional Bligh/Dyer analysis for PHA, soil samples were obtained from near the contractor's facility. For use in the ASE 200, samples were thoroughly mixed with diatomaceous earth to remove excess water. The solvent systems used in the ASE 200 (80°C, 2 cycles) were Bligh/Dyer,

100% methanol, 100% chloroform, and 1:1 methanol:chloroform. All extractions were performed in triplicate and the resulting total lipid fractionated. The glycolipid was saved and subsequently underwent ethanolysis. Samples were then analyzed by GC/MS. Results of this analysis are shown in Figure 17.

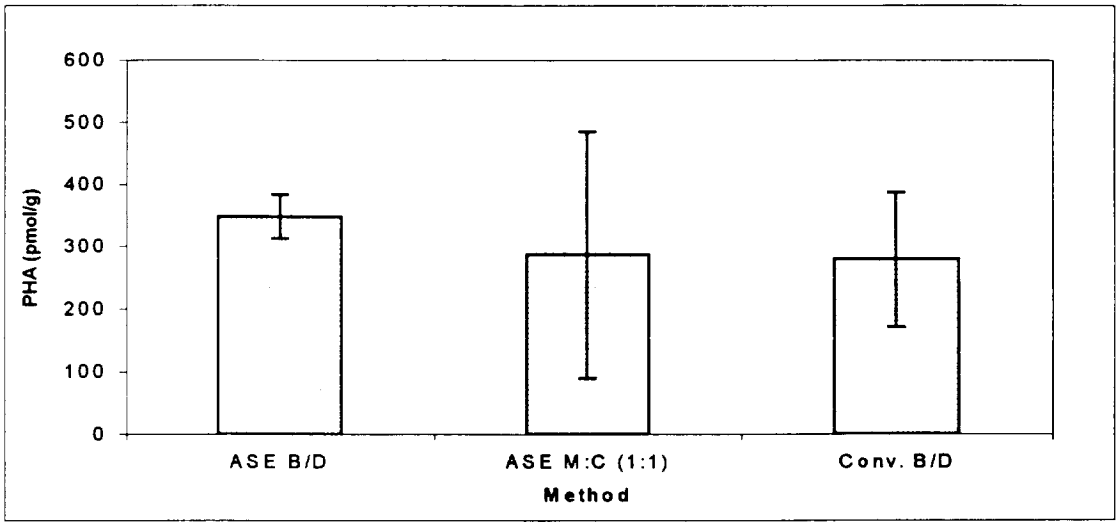

FIGURE 17: Extraction of PHA from soil by various methods. Error bars represent the standard deviation from the mean.

The ASE-methanol and the ASE-chloroform based methods failed to extract any PHA from the samples, but it is clear that the ASE Bligh/Dyer extraction yielded the greatest amount of PHA per gram and is the most consistent extraction method. The large errors (as represented by the standard deviation) can be attributed to the fact that the amount of PHA extracted was quite low because of limited space in the ASE 200 extraction cells.

Lipopolysaccharides (LPS)

The lipopolysaccharide LPS is one of the most important lipid- types that can be used for analysis of air biocontamination. Lipopolysaccharides form part of the outer cell envelope of Gram negative bacteria and can be indicative of airborne endotoxins that cause a wide variety of clinical symptoms, including respiratory disorders. Usually, the hydroxy fatty acids present within LPS are extracted from the sample residue following a conventional Bligh/Dyer extraction. However, given the improved extraction efficiency of the ASE, it was necessary to determine whether or not extraction with the ASE resulted in any loss of LPS from the sample residue. Therefore, we compared the LPS extraction from filter borne air biomass following either ASE Bligh/ Dyer or conventional Bligh/Dyer extractions of PLFA. Airborne biomass was collected on 3 separate filters (to ensure homogeniety, samples were collected from outdoors). The filters were then cut in 2 and the conventional Bligh/Dyer extraction or the ASE Bligh/Dyer extractions were carried out. Following the conventional Bligh and Dyer extraction of filter bound airborne biomass, the organic layer (containing PLFA) was removed, and the aqueous layer (including the air filters) was filtered to collect the residue. After the ASE extraction, the

cells were opened and the filters removed. Acid hydrolysis at 80°C was performed on the ASE filters and the residue of the conventional Bligh/Dyer to extract the 3-OH fatty acids present in the lipopolysaccharide. These fatty acids were analyzed by GC/MS and the results are shown in Figure 18.

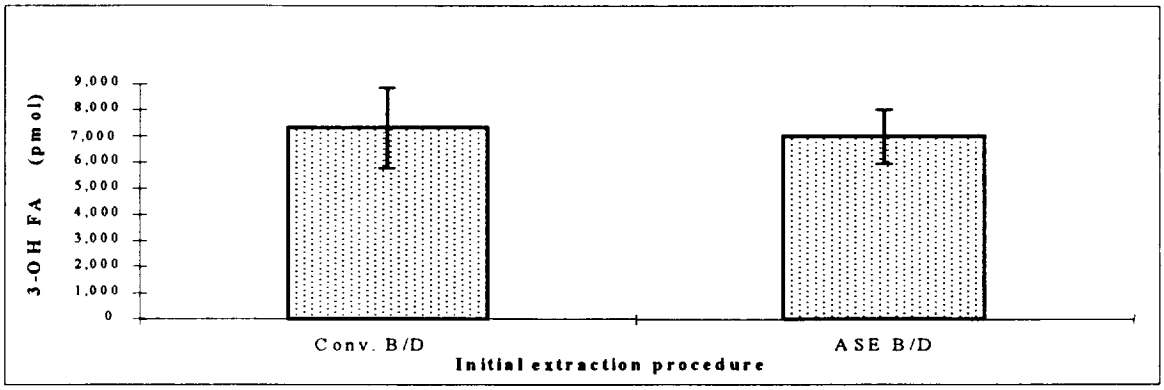

FIGURE 18: Mean recoveries of LPS fatty acids from biomass loaded onto filters following the conventional or the ASE Bligh/Dyer extraction procedures, n=3, error bars represent standard errors of the means.

There was no significant difference in extraction efficiency for the ASE Bligh/ Dyer and the conventional Bligh/Dyer, therefore this experiment indicated the ASE 200 does not interfere with the extraction of LPS. Consequently, ASE can be used as an integral part of any combined PLFA/LPS analysis.

Mycolic Acids

Certain lipid molecules are known to have potential as biomarkers for pathogens (many of which could be airborne). Mycolic acids are α-branched β-hydroxy long chain (60-90 carbons) carboxylic acids that are commonly found in, but not limited to, species of *Mycobacterium*. The rapid detection of mycolic acids could provide a means to detect pathogens such as *Mycobacterium tuberculosis*. Due to their size, all analysis of mycolic acids was done using high performance liquid chromatography. Unfortunately, the mycolic acids are not easily extractable and require a strong KOH saponification. The stainless steel fittings of the ASE prevent any use methanolic potassium hydroxide above 0.06% (w/v; pH = 12). As a result, using the ASE 200 did not improve extraction efficiency or time and was not suitable for the extraction of mycolic acids.

Aflatoxins

Aflatoxins are toxic secondary metabolites and carcinogens produced by a number of different fungi including *Aspergillus* spp. These toxins are widespread in food stuffs (*e.g.* grain, nuts) but have also been implicated in airborne biomass contamination. Rapid and sensitive extraction and analysis of these compounds may well be highly beneficial in any indoor air monitoring program. Sensitive high performance liquid chromatography (HPLC) detection methods have been developed (Dunne *et al.*, 1993 *J. Chromatography*, 629: 229-235), however, the initial extraction procedures most commonly used are still very rudimentary. Given the solvent based nature of the extraction procedures, we

investigated utilization of accelerated solvent extraction and have compared this aflatoxin extraction method with the standard procedures. All aflatoxin detection and identification was performed using HPLC. The principal four aflatoxins from *Aspergillus flavus* are aflatoxin B1, aflatoxin B2, aflatoxin G1, and aflatoxin G2, so named because of the color (blue or green) that they fluoresce. The focus of this study was the extraction and detection of aflatoxins B1, B2, G1, and G2 from a pure strain of *A. flavus* (ATCC 11498).

To determine whether the ASE extraction procedure would cause any deterioration of the aflatoxins, glass fiber filters were prepared that had been spiked with 50 μL each of standards of aflatoxins B1, B2, G1, and G2 of known concentration (1 mg/mL). The spiked filters were extracted using the conventional simple extraction into chloroform:water (10:1 v/v) (Leitao *et al.*, J. Chromatography, 1988, 435:229); the conventional Bligh/Dyer extraction; and finally, two ASE based procedures (80°C, 2 static heat cycles with 1200 psi) in methanol and in the conventional Bligh/Dyer solvent system. Extracts were then analyzed by HPLC. Extraction efficiencies are shown below. Detection limits were: B1, 100 ng; B2, 100 pg; G1, 1 ng; and G2, 200 pg.

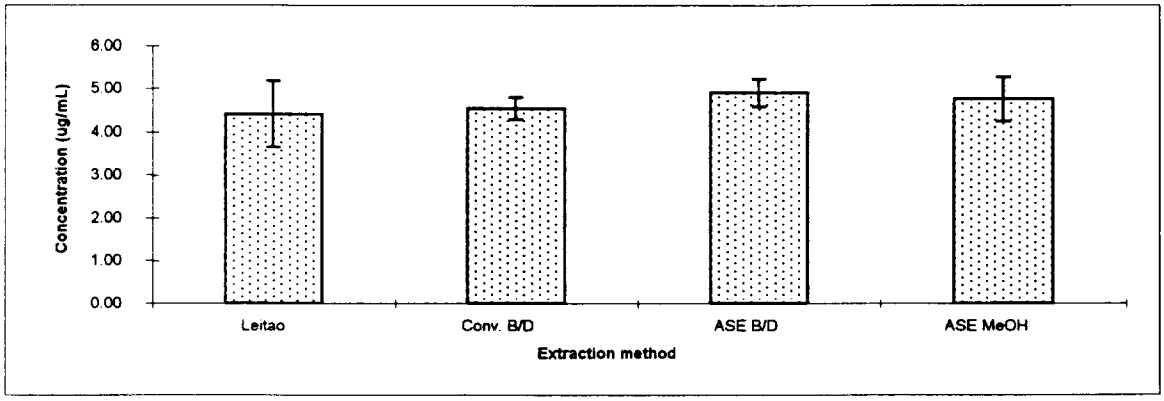

Figure 20: Extraction of aflatoxin standards from spiked glass fiber filters (n=3, error bars represent standard errors of means).

It was clear that the ASE extraction procedures (high temperature and pressure) did not cause any breakdown of the aflatoxins. To determine the extraction efficiency of the ASE procedures compared to the conventional procedure, the extractions were repeated using pure fungal biomass rather than standards. A known aflatoxin producing strain of *Aspergillus flavus* was used, with *A. niger* as the negative control. Using the conventional procedure, pure biomass (spores) were covered with 10 mL chloroform and 1 mL nanopure water in a test tube. The test tubes were sealed with PTFE caps and sonicated thirty minutes. The chloroform layer was removed and 2 additional 1 mL washes of chloroform were combined with the initial extract. The chloroform was then evaporated off under a stream of nitrogen. The samples were transferred to HPLC vials and analyzed as above. The parameters used for the ASE extractions were 2 static heat cycles at 80°C and both 1200 or 2000 psi. Solvents systems used were 100% methanol and methanol : chloroform : phosphate buffer (2:1:0.8, v/v/v; the conventional Bligh/Dyer solvents). Where methanol was used as the extractant, it was evaporated under nitrogen prior to analysis by HPLC. The methanol : chloroform : phosphate buffer solvents were

split by the addition of chloroform and water (1:1:0.9 v/v/v). The chloroform was removed and dried down under nitrogen prior to HPLC analysis. Following quantification by HPLC, only the conventional procedure {Leitao *et al.*, (1988)} yielded detectable aflatoxin. Neither the conventional Bligh/Dyer, ASE methanol, or ASE Bligh/Dyer extractions yielded aflatoxin from the pure biomass.

Although problems were encountered with aflatoxins and mycolic acids, the automation of lipid extraction methods was successfully accomplished for phospholipid fatty acids, polyenoic unsaturated fatty acids, isofucosterol, ergosterol, poly β-hydroxyalkanoic acids, and lipopolysaccharides from both pure biomass and environmental samples. As has been discussed, these chemicals can serve as biomarkers for potentially harmful airborne contaminants. Increasing the extraction efficiency and reducing the time spent for each of these analyses provides invaluable service, especially considering the concentration of some of these biomarkers in indoor air is often at the current limit of detection. In addition, the ability to perform multiple assays from the same sample filter using the ASE 200 with Bligh/Dyer solvents in combination with fractionation provides for a more thorough analysis of each sample. In conclusion, with increasing concerns over the quality of indoor air and "sick buildings", the advances made in this project to quantify and characterize the microbial community can assist in finding and correcting air-related problems in homes, offices, and industries.

Objective Three:

Previously, separate methods have been developed for the direct extraction and purification of lipids and DNA from environmental samples. Initial results from phase I of this study showed that DNA could be extracted from glass fiber filters and was suitable for PCR. Using supercritical fluid extraction (SFE) procedures, the extraction of DNA from pure biomass has been developed (D.C. White, Personal communication). However, successful lipid extraction was not possible using SFE. Following development of the ASE 200 methodologies for lipid analysis, we applied the same technique to the extraction of DNA, the aim being a combined process that would enable simultaneous extraction of lipids and DNA.

Using the methanol : chloroform : phosphate buffer (2:1:0.8 v/v/v) solvent system, we attempted to extract DNA from *E. coli* loaded glass fiber filters. Following DNA precipitation, no DNA was visible on the agarose gel (Figure 21). This was in comparison to the normal mechanical DNA extraction that resulted in clearly visible DNA (Figure 21). In order to maximize recovery of any DNA present following accelerated solvent extraction, we decided to use a single solvent phase (phosphate buffer; 0.1 M, pH 8) for all further experiments regarding extraction of DNA. In this study, *Bacillus subtilis* was the test bacteria. However, in comparison with the conventional mechanical extraction, this also proved unsuccessful (Figure 22). The most likely reasons for the low extraction recovery were unsuccessful cell lysis which, given the success of lipid extraction was unlikely, or the large solvent volume required for any ASE extraction. Given that no extraction cells that used smaller solvent volumes were available, the accelerated solvent extraction of DNA was abandoned.

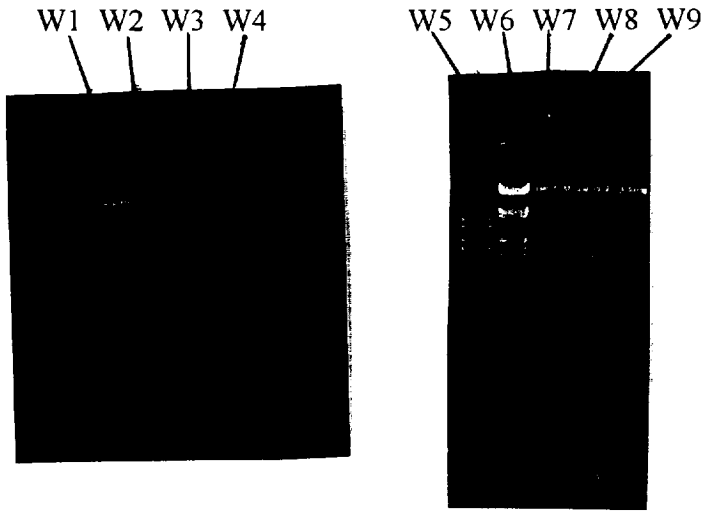

Figure 21: Agarose gels showing DNA extracted from *E. coli* using the ASE 200 (wells 2-4) or the standard mechanical extraction (wells 7-9). Wells 1 and 6 contain the λ *Hind III* DNA size marker, and well 5 contains the 1 Kb size ladder.

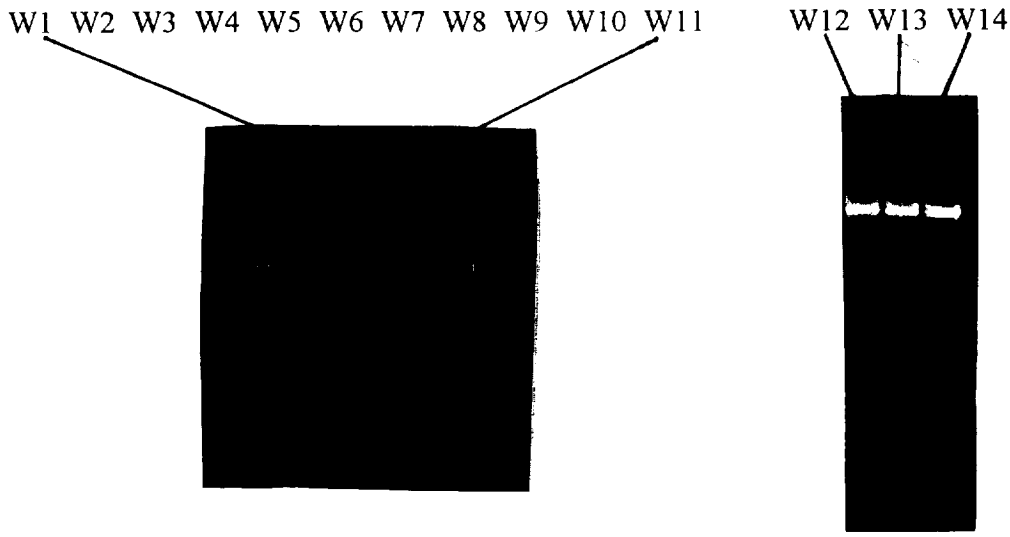

Figure 22: Agarose gels showing DNA extracted from *B. subtilis* spores using the ASE 200 (wells 2-10) or the standard mechanical extraction (wells 12-14). Cells extracted using the ASE without a phenol:chloroform purification are represented in wells 2-4, cells extracted using the ASE with a phenol:chloroform purification are shown in cells 5-7, cells extracted directly into phenol:chloroform using the ASE are seen in wells 8-10, and wells 12-14 show DNA extracted using the standard mechanical method. Wells 1 and 11 contain the λ *Hind III* DNA size marker.

Technical merit and feasibility assessment

Using the PASD, it has been shown that it is possible to characterize the microbial community of breathable air. With an ever-increasing awareness of illnesses associated with airborne biocontaminants, the fact that the PASD works effectively and efficiently makes it ideal for indoor air testing at virtually any location. The most notable drawback to the PASD is that, even though wheels have been added for ease of mobility, it is still awkward and quite heavy. Travelling great distances with it can be impractical, and thus, the Quick Form sampler (Figure 1B) was developed. The primary limitation of the Quick Form sampler is the fact that even an industrial strength vacuum cleaner does not have as great a capacity for air flow as the PASD. This restriction means the sampling time required must be increased to acquire sufficient biomass per filter, and because of the noise level of an industrial vacuum cleaner, this is inconvenient. Even with these limitations, however, the air sampling technology resulting from this Phase II project has potential for widespread use.

In addition, the incorporation of the ASE 200 to perform the traditional time-consuming lipid extraction provides a more efficient process with faster turn-around time that increases the potential for commercial success. Multiple assays can also be performed on each sample, providing a more complete understanding of the components of indoor air. Moreover, the ASE 200 is commercially available and simple to use. The primary limitation to this technology is that extraction efficiency is dependent on a variety of different parameters that must be defined for each type of analysis. In this study, the best extraction method for all primary types of lipid analysis were investigated in detail and final parameters set for all future work, but any new extractions to be performed will have to be thoroughly examined prior to sample analysis.

Research Findings

Using Companies X,Y, and Z as models, it has been shown that the Portable Air Sampling Device can be used to determine important information concerning the airborne microbial community. Phospholipid fatty acid analysis of samples acquired with the PASD revealed levels of biomass 1-3 orders of magnitude higher than those obtained using traditional methods of counting culturable organisms. As such, this method of sampling and subsequent analysis provides a more accurate representation of the actual indoor air composition.

The subsequent automation of the extraction and identification process simplified the steps required to proceed from sample acquisition to report generation. The importance of this lies not only in speed of analysis, but also in reproducibility. Although the identification process must be monitored, many simple errors are eliminated by the repetitive processes of the instrumentation. Using the parameters set forth in this work, the ASE 200 is now capable of efficiently extracting phospholipid fatty acids from any environmental sample thus far analysed (including air samples); poly b-hydroxyalkanoates from soil; and polyunsaturated fatty acids, certain sterols, and lipopolysaccharides from different types of pure biomass and environmental samples.

It was also discovered that the ASE 200 did not enable extraction of DNA. The most likely reason for this is that the solvent volume was too large for adequate analysis and any subsequent concentration is not practical when there are currently numerous simple, effective mechanical extraction procedures available.

Potential applications for the project results in a Phase III for NASA purposes and for commercial purposes

The successful results of the Phase II work have provided a means to develop Phase III applications for both NASA and commercial services leading to the rapid characterization of the entire indoor air microbial community. The habitat experiments that NASA is conducting to determine the effects of humans and plants co-existing for long periods could directly benefit from monitoring of the indoor air microbial communities. Already this program is utilizing lipid analysis to characterize microorganisms in the water system.

The contractor has begun to market the developed system to industrial hygienists for the characterization of the total indoor air microbial communities in sick buildings. To help promote wide spread utilization of this system, the contractor has also developed a sampling attachment that fits on the end of a household or industrial vacuum cleaner (Figure 1B). With this portable attachment, the contractor can quickly send calibrated sampling supplies that are easy to use for the industrial hygienist.

The initial service will be targeted at members of the industrial hygiene society who will find this service beneficial, as it is the only way to characterize the total indoor air microbial community. It will have a rapid turnaround (5-10 days) and a low cost (under $150 per sample). We expect the demand to grow to 5,000 samples a year by the second year of our mass marketing effort, generating revenues of one half to three quarters of a million dollars per year.

Funding for this Phase III commercialization will come initially from the sale of the indoor air microbial analysis as a service. The contractor has three years experience marketing a similar service to the environmental field to characterize microbial communities in water and soil. The contractor has specific expertise at developing the methods and then streamlining the process to provide a rapid, high value, low cost analysis. The capital requirements for the expanded marketing efforts to promote this system as a service will be financed entirely through the contractor's existing/expanding cash flow.

Papers Resulting from Work Performed During Phase II

Macnaughton, S.J., Jenkins, T.L., Wimpee, M.H., Cormier, M.R., and White, D.C. "Rapid extraction of lipid biomarkers from pure culture and environmental samples using pressurized accelerated hot solvent extraction." *Journal of Microbiological Methods.* **31**: 19-27 (1997).

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." *American Industrial Hygiene Association Journal.* **58**: 270-277 (1997).

Macnaughton, S.J., Jenkins, T.L., and White, D.C. "Definitive localization of airborne biocontamination by quantitative polar-lipid analysis of metal working machine aerosols not definable by classical culture techniques." Submitted to *American Industrial Hygiene Association Journal.* 1998.

Macnaughton, S.J., Jenkins, T.L., Cormier, M.R., Davis, G.D., and White, D.C. "Lipid biomarkers as a measure of microbial contamination in indoor air: a large scale database." In preparation. 1998.

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Bioaerosols have been linked to a wide range of different allergies and respiratory illnesses. Currently, microorganism culture is the most commonly used method for exposure assessment. Such culture techniques, however, generally fail to detect between 90-99% of the actual viable biomass. Consequently, an unbiased technique for detecting airborne microorganisms is essential. In this Phase II proposal, a portable air sampling device has been developed for the collection of airborne microbial biomass from indoor (and outdoor) environments. Methods were evaluated for extracting and identifying lipids that provide information on indoor air microbial biomass, and automation of these procedures was investigated. Also, techniques to automate the extraction of DNA were explored

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