MICROBIAL COMMUNITY STRUCTURE BY FATTY ACID ANALYSIS DURING POLYCYCLIC AROMATIC HYDROCARBON DEGRADATION IN RIVER SEDIMENT AUGMENTED WITH *PLEUROTUS OSTREATUS*

By

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Microbial Community Structure by Fatty Acid Analysis during Polycyclic Aromatic Hydrocarbon Degradation in River Sediment Augmented with *Pleurotus ostreatus*

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Abstract:

This research was conducted to determine changes in the microbial community in contaminated sediment during fungal remediation of polycyclic aromatic hydrocarbon (PAH) contaminated Mahoning River sediment. The fungus used for remediation was *Pleurotus ostreatus*, white rot fungi which is capable of degrading a wide range of organic contaminants including PAHs. Microbial community structure was determined using fatty acid profiles from microbial lipids extracted directly from the sediment. Contaminated sediment was collected from Lowellville, OH and was incubated at 25 ^{o}C. There were 4 treatments (1 liter of contaminated river sediment) done in duplicate runs as follows: 1) untreated sediment, 2) sediment amended with sawdust, 3) sediment amended with sawdust and augmented with *Pleurotus ostreatus* and, 4) sediment amended with sawdust, augmented with *Pleurotus ostreatus* and amended with extra nitrogen after 21 days. At day 0, 21, and 42, lipids were extracted from each treatment (in triplicate). Microbial fatty acids were purified from the lipid extract, methylated and analyzed by GC-MS. The sediment microbial community structure showed great heterogeneity shown as high variability within triplicate samples and as differences between duplicate treatments. Groups of anaerobic bacteria (sulfate reducers and methanogens) persisted throughout the treatments, even though they were exposed to oxygen during mixing and from the surface during the incubation. The abundance of gram negative bacteria, a group of bacteria associated with PAH degradation, showed highest relative abundance on day 42. Even though the microbial structure changed, the microbial biomass (measured as lipid phosphate) remained consistent between triplicate samples and duplicate runs, and changed little during the incubation.

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Chapter 1: Introduction

1.1 Mahoning River History

The Mahoning River starts at Winona, Ohio, extends towards to Leavittsburg and flows south easterly into Pennsylvania through the cities of Warren, Niles, Girard, Youngstown, Struthers, and Lowellville (http://en.wikipedia.org/wiki/Mahoning_River). It ultimately joins the Ohio River by first joining with the Shenango River to form the Beaver River, which meets with the Ohio River (figure 1). The lower branch of the Mahoning River is the most polluted site.

During the 19th century, raw sewage and industrial waste were released into the river. In the 20th century steel mills along the banks of the river poured tons of industrial waste into the river. The list of contaminants includes heavy metals, grease, oil, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and other carcinogens. The steel mills were shut down in the late 1970s. Many of the toxic substances were flushed down stream. Due to this, the quality of water improved but the quality of the sediment has not. PAHs from the industrial waste are found as 'black mayonnaise' in the sediment (Mahoning River Education Project).

Due to the contamination there is a marked reduction in the number of river animals and plant life. There are widespread deformities in the fish at the river bottom. The Ohio Department of Health has issued an advisory against contact with the sediment and eating the fish because of the carcinogenic nature of these toxins. (Mahoning River Education project).

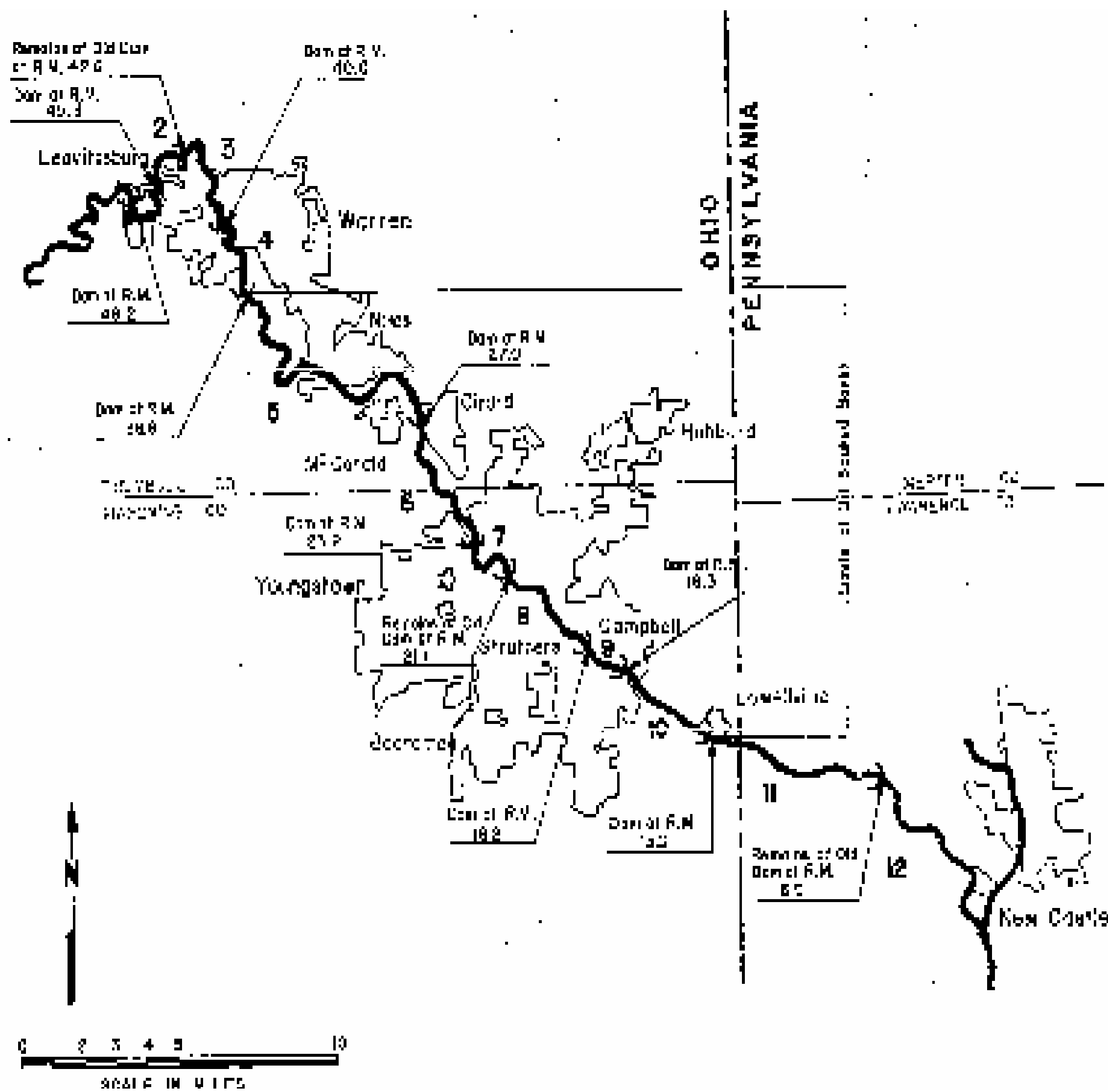


Fig. 1 Map of the lower Mahoning River.

1.2 Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic Aromatic Hydrocarbons (PAHs) are a group of organic compounds that contain two or more fused aromatic rings. PAHs are formed by incomplete combustion of organic materials during industrial and other human activities which include processing of coal, crude oil, combustion of natural gas. (Wikipedia-Polycyclic aromatic hydrocarbon). PAHs are lipophilic in nature and have low water solubility, due to which they tend to adsorb to the sediment. The solubility in water decreases approximately by one order of magnitude for each additional ring. Degradation of PAHs is very slow and these compounds are persistent, which can be attributed to site specific reasons like nutrients, adsorption to particles, temperature, oxygen and presence of indigenous PAH degrading microorganisms in the sediment (Air quality guidelines-second edition, 2000).

PAHs are highly toxic and many of the PAHs including phenanthrene, fluorene and fluoranthene are listed by the US Environmental Protection Agency (USEPA) as priority pollutants (USEPA, 1985). PAHs are carcinogenic, mutagenic and teratogenic in nature (Fang et. al, 1996). PAHs can accumulate in the food chain and cause long term effects to the aquatic and human life. PAH contamination can be remediated by either chemical or biological methods. Bioremediation has been shown to be effective and does not generate secondary contamination (Mueller et al., 1991a).

Biodegradation studies using *Pleurotus ostreatus* have shown the potential of the white-rot fungi to degrade PAHs. These microorganisms can decompose lignin to obtain cellulose from wood fiber using an enzymatic complex. This capability enables the white-rot fungi to degrade PAHs too. These fungi when incubated in the sediment will

release the enzymes to the extracellular medium, allowing the fungi to degrade large molecules like PAHs with high molecular weight (R. Canet et.al., 1999).

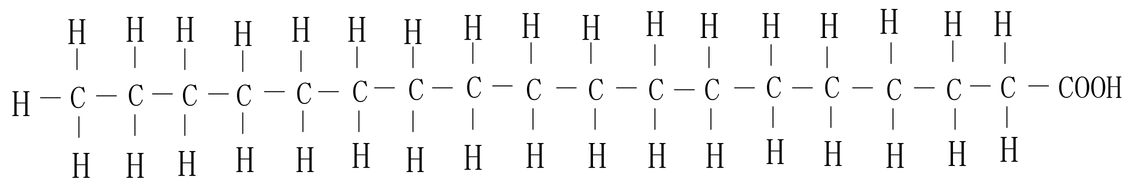
1.3 Fatty Acids

The structure of fatty acids constitutes a hydrocarbon chain with a carboxylic acid group. The long hydrocarbon chain is hydrophobic in nature and the carboxylic acid end is hydrophilic in nature.

Fatty acids are important constituents of microbial cells, plasmalemma and cell organelle membranes and act as storage material. The major fatty acids that typically occur in membrane phospholipids and storage triacyl glycerides of fungi are palmitic and stearic acids (Suutari 1995). The composition of mycelial fatty acids depends on conditions such as nutritional factors, oxygen and temperature (Suutari 1995). Microbial community structure can be determined by extracting phospholipid fatty acids (PLFAs) as lipids are present in all living cells and are important biomarkers.

By extracting the phospholipids from the cell membrane, the fatty acid tail can be extracted and analyzed as Fatty acid methyl esters by GC/MS. A methyl group is added to the fatty acid to increase volatility for GC analysis (Langworthy et. al., 2002). It has been proven to be relatively effective for assessing community structure. The method is reproducible and uses small sample sizes to simultaneously recover PAHs, FAMEs, and biomass in the same sample from a direct soil extraction (Fang and Findlay, 1996). However, disadvantages of this method are that individual fatty acids cannot be used to represent individual species. This is because individual species may contain numerous fatty acids and those same fatty acids can occur in more than one species (Kirk et al., 2003).

Fatty acids are named based on the total number of carbon atoms present, the double bonds and the position of the first double bond from the methyl end (ω) of the molecule (Langworthy et al., 2002). For example, 18:0 would indicate that there are 18 carbon atoms in the chain and no double bonds (Figure 2). 20:5ω3 would indicate 20 carbon atoms, 5 double bonds and the first double bond occurring at the number three carbon. Different prefixes can be used in naming of fatty acids (Table 1). Based on the modified Findlay classification, fatty acid methyl ester (FAME) biomarkers can be classified into different groups (Table 2).



ω end

Fig. 2 Structure of Stearic acid C18:0

Table 1. Fatty acid prefix designations (Langworthy et. al., 2002)

"i"	iso-branched (methyl branch on the 2nd carbon from the methyl end)
"a"	anteiso-branched (methyl branch on the 3rd carbon from the methyl end)
"10Me"	methyl branch on the 10th carbon from the carboxylate end
"cy"	cyclopropyl
"br"	Methyl branching at undetermined positions
"c" and "t"	*cis* and *trans* geometric isomers

Table 2. Common fatty acid methyl esters (FAME) biomarkers (modified from Findlay 2004)

Microbial Group	Fatty Acid Biomarkers
Green algae & higher plants	16:1ω13t, 18:1ω9, 18:3ω3, 18:2ω6
Heterotrophic micro eukaryotes	18:1ω9, 18:2ω6, 18:3ω6, 20:2ω6, 20:3ω6, 20:4ω6
Fungi	16:0, 18:1ω9, 18:2ω6, 18:3ω6, 18:3ω3
Bacteria	15:0, i15:0, a15:0, 16:1ω5, 16:1ω9, i17:0, a17:0, cy17:0, 17:0, 18:1ω5, i19:0, a19:0
Gram-positive bacteria	Mid-branched fatty acids
Gram-negative bacteria	16:1ω7, 18:1ω7, cy17:0, cy19:0
Methanotrophs	Type I: 16:1ω8c, 16:1ω5c , 16:1ω6 Type II: 18:1ω8c, 18:1ω8t
Sulfate reducing bacteria and other anaerobic bacteria	16:0, 10Me16:0, a17:0, i17:0, 17:0, 18:0, cy17:0, cy19:0

1.4 Microbial biomass

The viable biomass of a microbial community is an estimate of the amount of active microorganisms in the sediment. The microbial biomass can give an estimate of the capability for microbial transformation in that sediment. All the cells in the membranes contain phospholipids which are not stored, but are turned over rapidly during metabolism (White et al., 1979). The extraction and analysis of the phospholipid components of a community is one of the ways to measure the biomass (White et al. 1979).

1.5 Objectives

The objective of this research is to study the relationships between concentrations of PAHs, microbial community structure and microbial biomass in the sediment augmented with *Pleurotus ostreatus*. As microorganisms respond to modifications in the environment and under stress, the structure of the community may change. Therefore, by analyzing the microbial ecology, it may be possible to offer a biomonitoring tool to measure environmental stress.

Chapter 2: Methods

2.1 Experimental Design

Sediment was collected in plastic tubes, from the Lowellville site of the Mahoning River. The sediment was stored at 4 ^{o}C until use. One liter of the sediment was taken in 2 liter glass containers ("fish bowls"). Four experimental treatments were tested in triplicate in the laboratory during a 6 week period. The first treatment contained only sediment which was used as control. To the second treatment sawdust was added (60% by volume). To the third treatment *Pleurotus ostreatus* (10% by volume) was added. The fungus was grown as described in Bosiljcic (2008). After 21 days, to half of the sediment in the third treatment, nitrogen (10% by volume) was added. The incubation was set up in a 25 ^{o}C incubator. Sampling was done on days 0, 21, and 42. Two runs (Run 1 and Run 2) were done in triplicate. The samples were collected and then the PAHs, lipids and biomass, extracted.

2.2 Procedure For PAH and Lipid extraction

Lipids were extracted using a modified extraction method for organic pollutants and microbial lipids (Fang and Findlay, 1996) based on the Bligh and Dyer method (figure 3). Sediment was taken from the treatments described above. A mixture of dichloromethane (DCM), methanol, phosphate buffer (pH 7.4) and a surrogate solution were added to the samples, mixed well and extracted (Detailed procedure in Appendix 1 and 3).

The organic phase (DCM) which contains the PAHs and the lipids was collected and purified by passing through sodium sulfate columns and was concentrated by

evaporating the solvent. The samples were used at this stage for the biomass analysis and also for the PAH and lipid extraction.

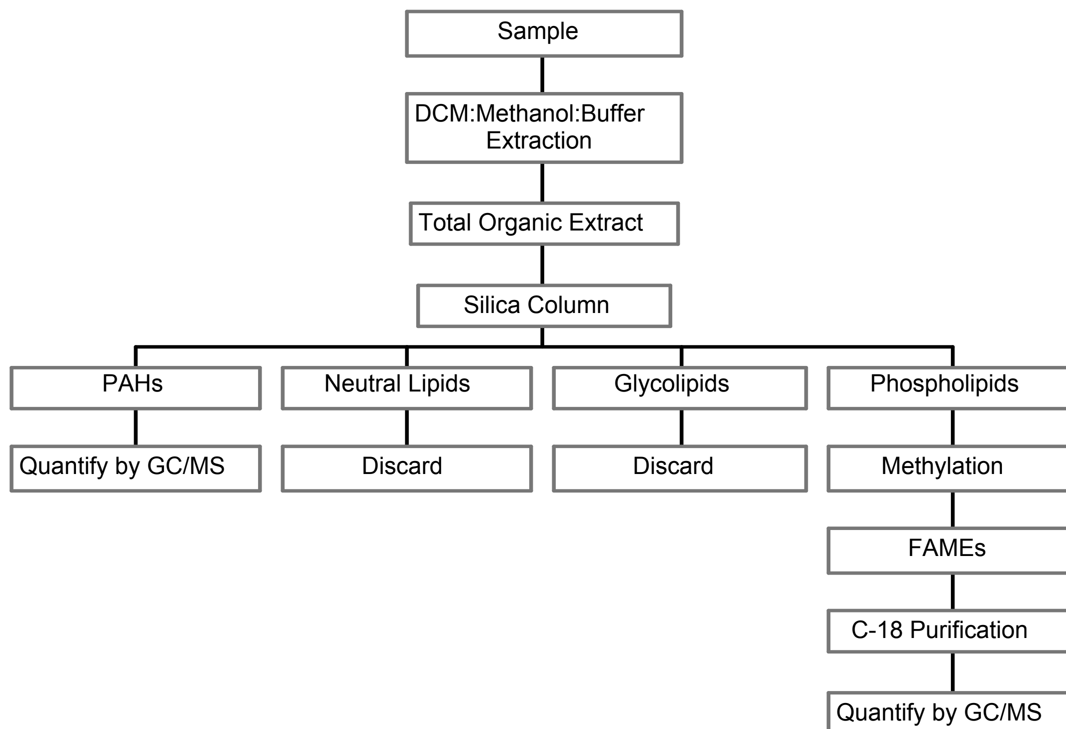


Fig. 3 Simultaneous PAH and lipid extraction procedure flow chart (Fang and Findlay (1996)

2.3 Biomass Analysis

Microbial biomass was determined by measuring the lipid-bound phosphates from microbial cells (Vestal and White, 1989). The phosphates were extracted from phospholipids by organic solvents following the same extraction procedure used for the FAME analysis. The phosphate was removed from the lipids by a potassium persulfate digestion and oxidized to orthophosphate, which reacts colorimetrically with malachite green. The absorption was measured on a spectrophotometer at 610 nm (Findlay, 2004).

2.4 PAH Fraction

The sample solution in chloroform was concentrated and separated by passing through silica columns and further purified twice on aminopropyl columns. The PAHs were then analyzed by GC-MS.

For further separation of the sample into neutral, glyco, and phospholipid fractions, a series of solvents were passed through the silica column.

2.5 Lipid extraction

Neutral lipid and glycol lipid fractions were obtained by passing chloroform and acetone consecutively through the silica columns. A combination of chloroform: methanol: DI water (5:5:1) was passed through the columns to collect the phospholipid fraction.

2.6 Conversion of phospholipids to fatty acid methyl esters (FAMEs)

For the phospholipid to FAME conversion, the dry lipid was dissolved in methanol: toluene (1:1) and then KOH in methanol was added which transmethylates the PLFAs (phospholipid fatty acids), forming FAMEs. Then acetic acid was added followed by chloroform and DI water. The bottom phase (chloroform layer) containing the FAMEs was collected and the internal ethyl ester standard (C22:0). It was then concentrated under nitrogen at 37 ^{o}C. The concentrated sample was stored at -20 ^{o}C until purification.

2.7 Purification of FAMEs

FAMEs were purified by passing through octadecyl (C-18) columns. These columns were initially saturated by passing a series of solvents to enhance the purification and recovery. Finally the FAMEs were collected by passing hexane: chloroform (95:5) solution through the column. The elevate was concentrated and analyzed by GC-MS.

2.8 Analysis of PAHs and FAMEs

The PAHs and FAMEs were analyzed on a Hewlett Packard 5890 Gas Chromatograph/5970B Mass Spectrometer. A DB-5 column with 30 M length, 0.32 mm ID, and 0.25 µm film thickness was used.

Temperature program on GC-MS for PAH analysis

The injector and the detector temperatures were set at 250 ^{o}C and 300 ^{o}C, respectively. The oven temperature was held at 45 °C for 2 minutes, and then ramped at 20 ^{o}C per minute to 310 °C. The final temperature was held for 5.5 minutes.

Temperature program on GC-MS for FAME analysis

The injector and detector temperatures were set at 250 ^{o}C and 300 ^{o}C, respectively. The oven temperature was set at 80 ^{o}C and then ramped at 4 ^{o}C per min to 250 ^{o}C, where it was held for 20 minutes.

Chapter 3: Results and Discussion

3.1 PAH Analyses

A total of eleven PAHs were detected in the Mahoning River sediment using the modified Fang and Findlay lipid extraction procedure (figure 4). Six of them are low molecular weight PAHs, namely naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene and anthracene. Five high molecular weight PAHs were detected which include Fluoranthene, benzo[a]anthracene, pyrene, chrysene and benzo[b,k]fluoranthene. The contaminated sediment had an initial PAH concentration of 342.0 µg/gm dry weight. Individual concentration of PAHs ranged from 1.32 to 85.1 µg/gm dry weight. Of the detected PAHs, fluoranthene was of the highest concentration with 85.10 µg/gm dry weight, followed by pyrene with 69.14 µg/gm dry weight (table 3). These results are in agreement with Lee (2005). She measured the PAH concentrations of the river bottom sediment taken from Lowellville.

Table 3 PAHs detected in Mahoning river sediment by lipid extraction procedure

S.No	PAHs	RT*	Conc#
1	Naphthalene	9.05	13.03
2	Acenaphthylene	11.26	1.23
3	Acenaphthene	11.49	3.03
4	Fluorene	12.19	11.76
5	Phenanthrene	13.55	39.37
6	Anthracene	13.55	46.64
7	Fluoranthene	15.20	85.10
8	Pyrene	15.53	69.14
9	Benzo(a)anthracene	17.42	17.48
10	Chrysene	17.42	15.10
11	Benzo(b&k)fluoranthene	19.79	40.19
12	Benzo(a)pyrene	0	N.D**
13	Dibenz(ah)anthracene	0	N.D**
14	Indeno(1,2,3-cd)pyrene	0	N.D**
15	Benzo(ghi)perylene	0	N.D**

*RT – retention time in mins
#Concentration - µg/gm dry weight sediment
**N.D – Not detected

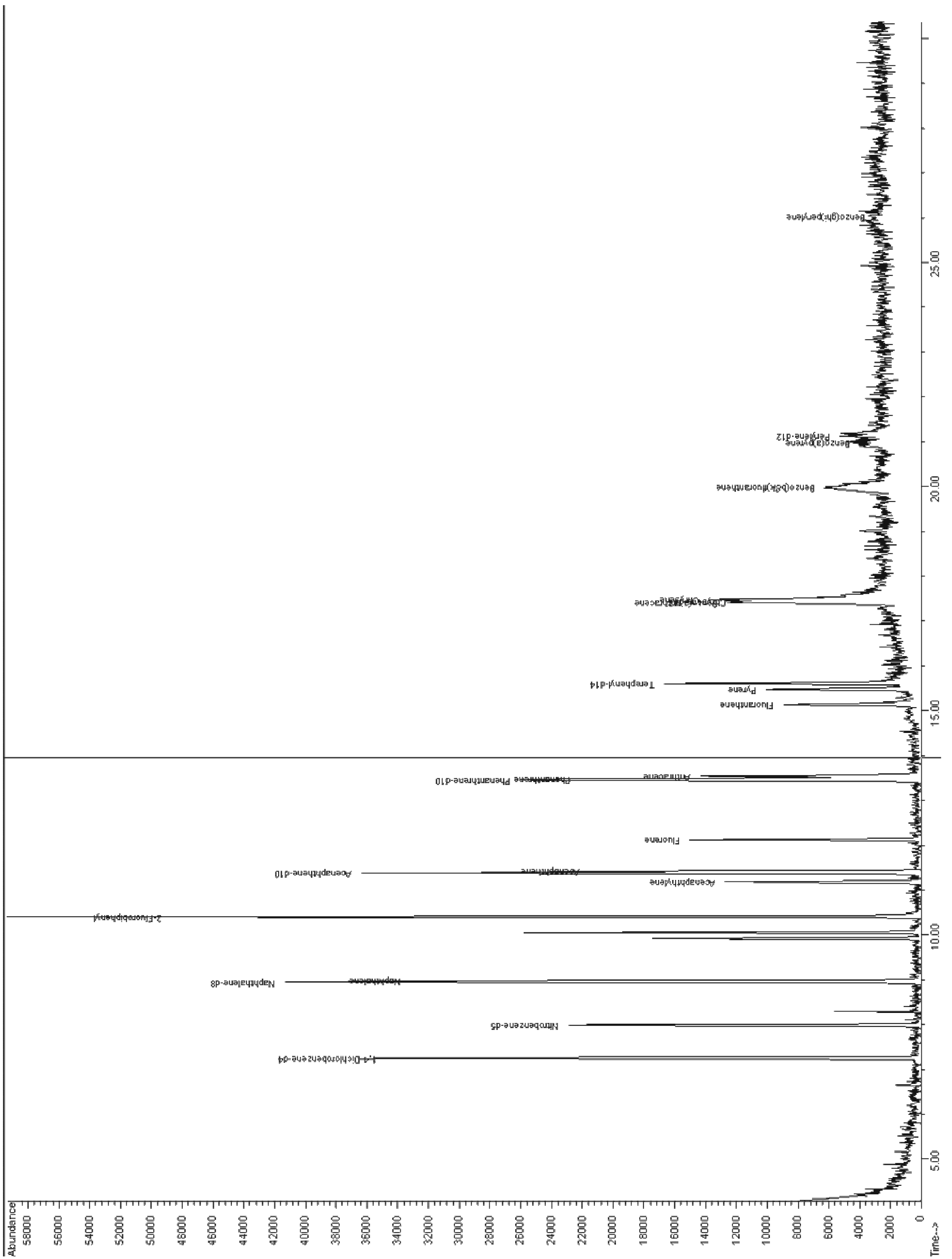


Fig. 4 Chromatogram of PAHs from sediment sample

Triplicate samples were extracted, but due to the malfunction of GC-MS, they were not analyzed in time for this thesis. So the interpretations were done based on results from Pabba (2008). The PAHs extraction was based on a modified USEPA 3550 procedure, using dichloromethane as a solvent (Pabba, 2008). Four treatments were done: (1) Control (2) Sawdust (3) Sawdust + fungi (4) Sawdust + fungi + nitrogen. By the end of the experiment (42 days) nine of the eleven PAHs detected, showed degradation in the sample with *Pleurotus ostreatus* + sawdust (figure 5). The total PAH concentration was reduced by 58.6% from 253 µg/gm of dry weight on day 0 of incubation to 106 µg/gm of dry weight after 42 days.

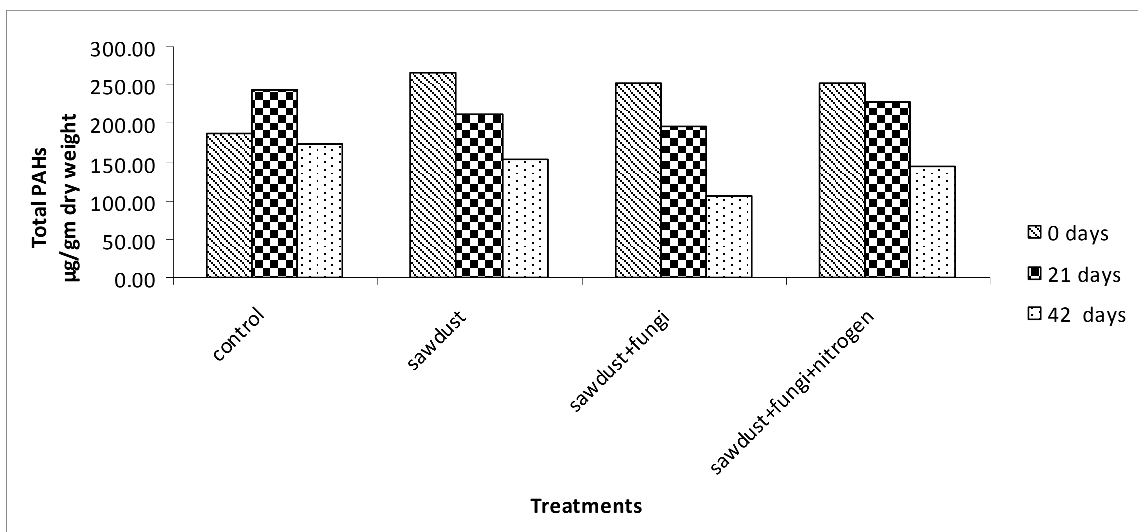


Fig.5 Total PAH degradation in all the treatments (Pabba thesis 2008)

3.2 Fatty acid methyl esters (FAMEs)

A total of fourteen fatty acid methyl esters (FAMEs) were detected in the sediment (i15:0, a15:0, i16:0, 16:1ω7, 16:1ω6, 16:0, i17:0, cy17:0, 18:2ω6, 18:1ω9, 18:1ω7, 18:0, a19:0, cy19:0). Based on modified Fang and Findlay classification, the lipids detected in the River sediment can be classified into different microbial groups (table 4). All groups represented and the results are in agreement with Lee (2005). She

detected the same FAMEs in the Lowellville bottom sediments. In addition, she also detected 10Me16:0, br17:0b and br17:0a. The possible factors contributing to the differences are soil heterogeneity, time gap and sampling procedure.

Table 4 Microbial biomarkers detected in Mahoning River sediment

Group	Fatty Acid
Fungi	18:1ω9 18:2ω6 16:0
Gram negative bacteria	16:1ω7 18:1ω7 cy17:0 cy19:0
Gram positive bacteria	a15:0 i15:0 i16:0 a19:0
Sulfate reducing bacteria	i17:0 cy17:0 cy19:0 18:0
Methanotrophs	16:1ω6

3.3 Gram negative bacteria biomarkers

The FAMEs cy17:0, cy19:0, 16:1ω7 and 18:1ω7 that were identified in the samples are characteristic biomarkers for gram-negative bacteria (Zelles 1999). The use of biomarkers to indicate specific taxonomic or functional groups is however limited due to overlapping (Bossio et al., 1998).

The gram negative bacteria showed a consistent increase in the relative abundance in the control in both runs. In Run 1 the total relative abundance of gram negative bacteria increased from 14.10% (± 7.04%) on day 0, to 58.70% (± 11.83%) on 42nd day, and in Run 2 from 18.16% (± 9.07%) to 38.74% (± 8.57%) with an exception of 18:1ω7

which was the only fatty acid which showed a slight decrease from 18.15% (± 11.15%) to 16.35% (± 5.81%). %). This kind of decrease was observed in work done by Gramss et al., (1999) where the fungi delayed the net degradation of PAHs due to the fact that mycelia of *Pleurotus ostreatus* killed most of the indigenous soil bacteria expected to take part in the degradation of PAHs In the treatment sawdust + fungi there was an increase in cy17:0 from 0 to 2.47% (± 0.79%) and 18:1ω7 from 4.07% (± 7.05%) to 13.58% (± 0.94).

An overall increased response of cyclo propyl fatty acids in all the treatments can be explained by the work done by Denich T.J. et al., (2003). It was based on the observation that environmental factors such as temperature, nutrients, chemicals and stress can change the shift in microorganisms' lipids composition. This can lead to the conversion of monoenoic PLFAs (16:1ω7, 18:1ω7) to cyclopropane PLFAs (cy17:0, cy19:0).

In the treatment with sawdust, Run 1, cy17:0 and cy19:0 increased in relative abundance from 0 to 11.88% (± 2.11%), 18:1ω7 remained constant at 14.45% (± 2.67%), and 16:1ω7 decreased slightly from 19.90% (± 1.16%) to 18.59% (± 0.25%) by the 42nd day (figure 6(a)). In Run 2, cy17:0 showed an increase from 0 to 4.10% (± 3.57%), cy19:0 was not detected, and 16:1ω7 decreased in the relative abundance, from 20.95% (± 3.95%) to 15.52% (± 4.58%).

In the treatment with sawdust + fungi, Run 1, the fatty acid biomarkers, cy17:0, cy19:0 and 18:1ω7 increased from 16.54% (± 0.45%) to 25.0% (± 0.71%) in relative abundance. While 16:1ω7 showed a decrease from 11.6% (± 0.63%) to 8.6% (± 1.24%).

In Run 2, all the fatty acid biomarkers for gram negative bacteria showed a decrease on the 42nd day when compared to their initial values on day 0.

In the treatment with sawdust + fungi + nitrogen, cy17:0, cy19:0 and 18:1ω7 showed a total increase from 4.08% (± 2.35%) to 17.20% (± 4.31%) in relative abundance while 16:1ω7 decreased from 18.13% (± 7.63%) to 4.36% (± 0.16%). Similar trends were observed in Run 2 (figure 6(b)).

3.4 Sulfate reducing bacteria biomarkers

Even though sulfate reducing bacteria are anaerobes, the extraction was mostly done in aerobic conditions. There was no overall trend even though some of the bacteria seemed to show an increase in relative abundance by the 42nd day. This variation in the trends could be explained by the fact that sulfate reducing bacteria possess some kind of a protective mechanism against accidental exposure to oxygen (Novozhilova and Berezina, 1968). They also play an important role in aerobic environments if they can proliferate in anaerobic zones. It is believed that sulfate reduction accounts to a majority of the mineralization of organic matter in marine sediments and in aerobic wastewater treatments (Jørgensen, 1982). It is also believed to stimulate microbially enhanced corrosion of metals (Kühl, 1992), (Lens et al., 1995).

The PLFA biomarkers cy17:0 and cy19:0 represent not only sulfate reducing bacteria but also gram negative bacteria. These biomarkers showed an increase in both runs in all the treatments except for sawdust + fungi, wherein cy17:0 showed a slight decrease. C18:0, which is a typical biomarker for sulfate reducing anaerobic bacteria, decreased in all the treatments in both runs. In Run 1, i17:0 which is also a fatty acid biomarker for gram positive bacteria but typical for sulfate reducing bacteria, showed

variable trends between treatments and also runs. It appeared on 21st day and was not detected by 42nd day in the control (figure 7(a)). In all the other treatments, i17:0 showed

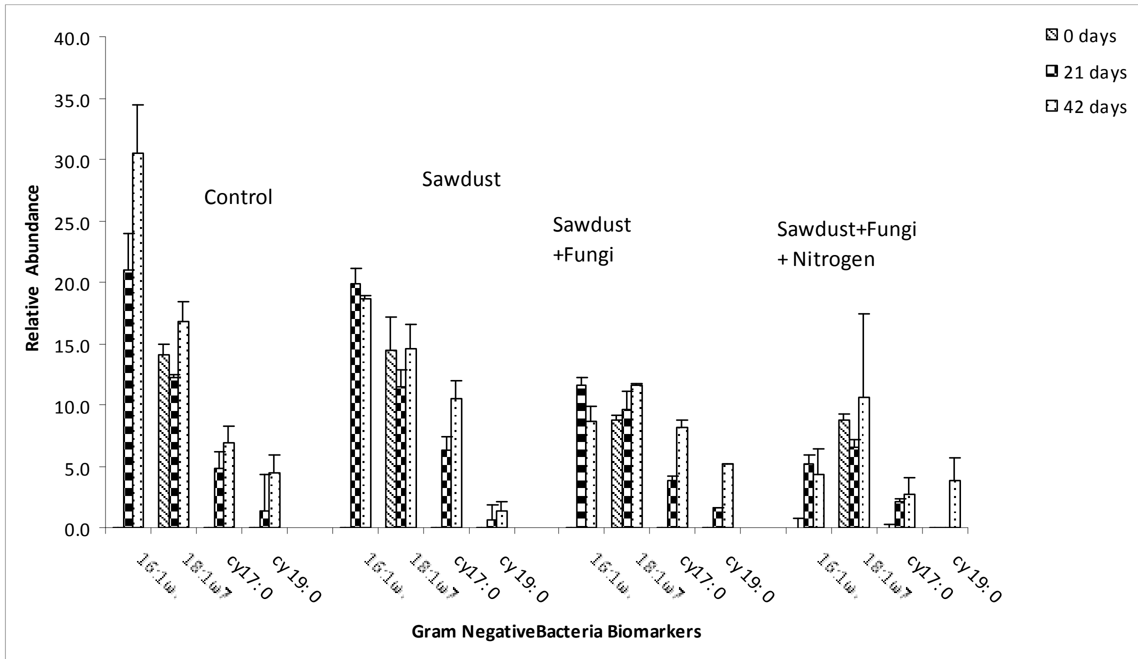


Fig. 6(a) Gram negative bacteria biomarkers, Run 1

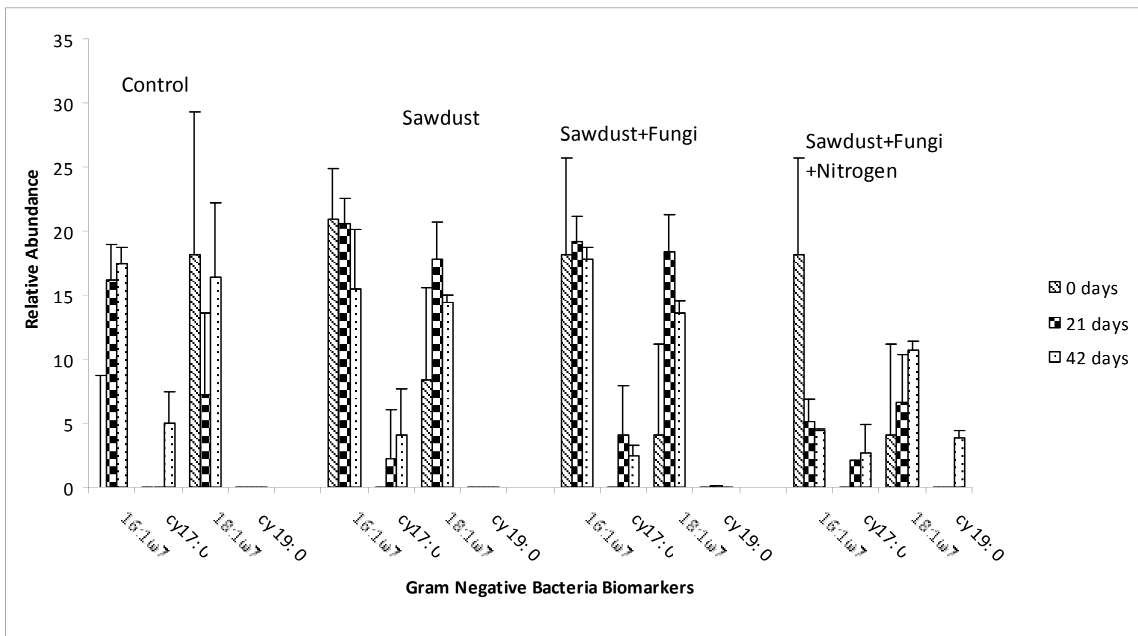


Fig. 6(b) Gram negative bacteria biomarkers, Run 2

an increase in the relative abundance by the 42nd day. In Run 2, i17:0 was not detected in the control, but in the treatment with sawdust + fungi, it appeared on the 21st day. The relative abundance was 0.57% (± 0.01%) and dropped below the detection limits by the 42nd day (figure 7(b)). In the treatment with sawdust + fungi + nitrogen, i 17:0 was found in detectable concentrations on the 42nd day only. Detected by 42nd day in the control (figure 7(a)). In all the other treatments, i17:0 showed an increase in the relative abundance by the 42nd day. In Run 2, i17:0 was not detected in the control, but in the treatment with sawdust + fungi, it appeared on the 21st day. The relative abundance was 0.57% (± 0.01%) and dropped below the detection limits by the 42nd day (figure 7(b)). In the treatment with sawdust + fungi + nitrogen, i17:0 was found in detectable concentrations on the 42nd day only.

3.5 Gram positive bacteria biomarkers

Mid branched fatty acids (i15:0, a15:0, i16:0, a19:0) are the characteristic biomarkers for gram positive bacteria but are also part of other classes of bacteria. Biduad et al., (1997) observed that gram positive bacteria can grow in the presence of fluoranthene, pyrene and anthracene. In Run 1, i15:0 increased in the control from 5.15% (± 0.32%) to 18.02% (± 1.53%) by the 42nd day. However, it showed an increase on the 21st day and then decreased slightly by the 42nd day in the treatment with sawdust and sawdust + fungi. The biomarker i16:0 showed a drastic decrease from day 0 to the 42nd day in the control, a trend which is observed in other treatments as well. The biomarker a19:0 was not detected in the control, but showed up on the 42nd day in treatment with

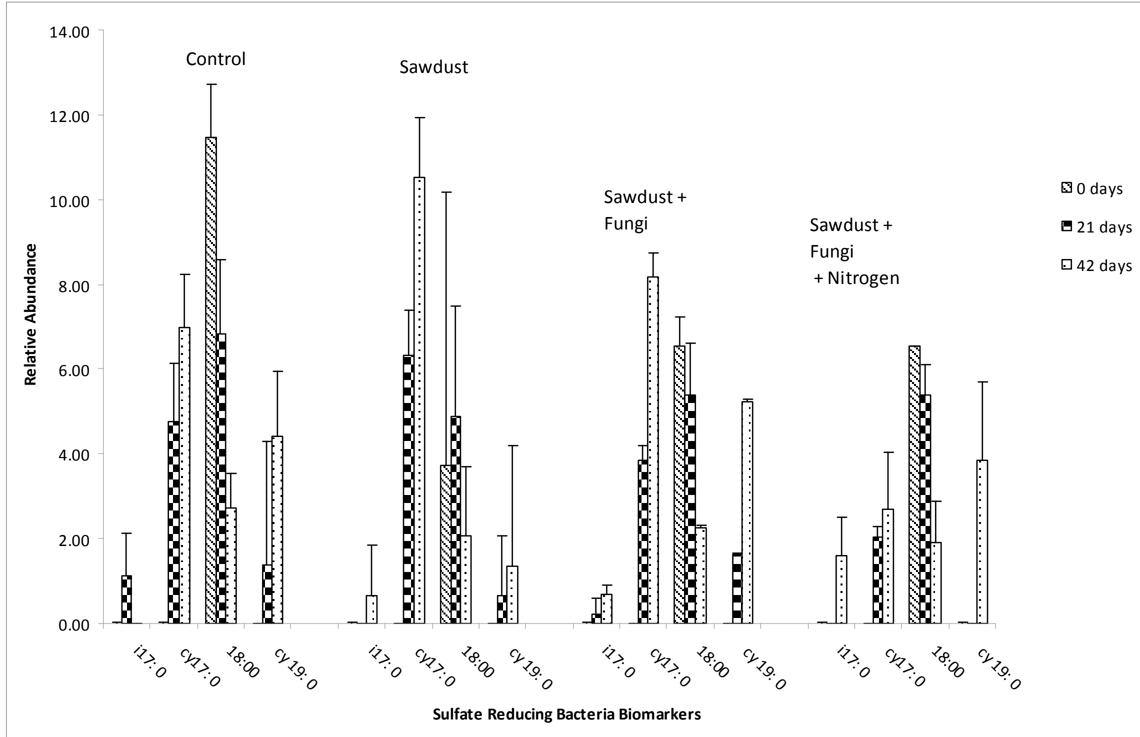


Fig. 7(a) Sulfate reducing bacteria biomarkers, Run 1

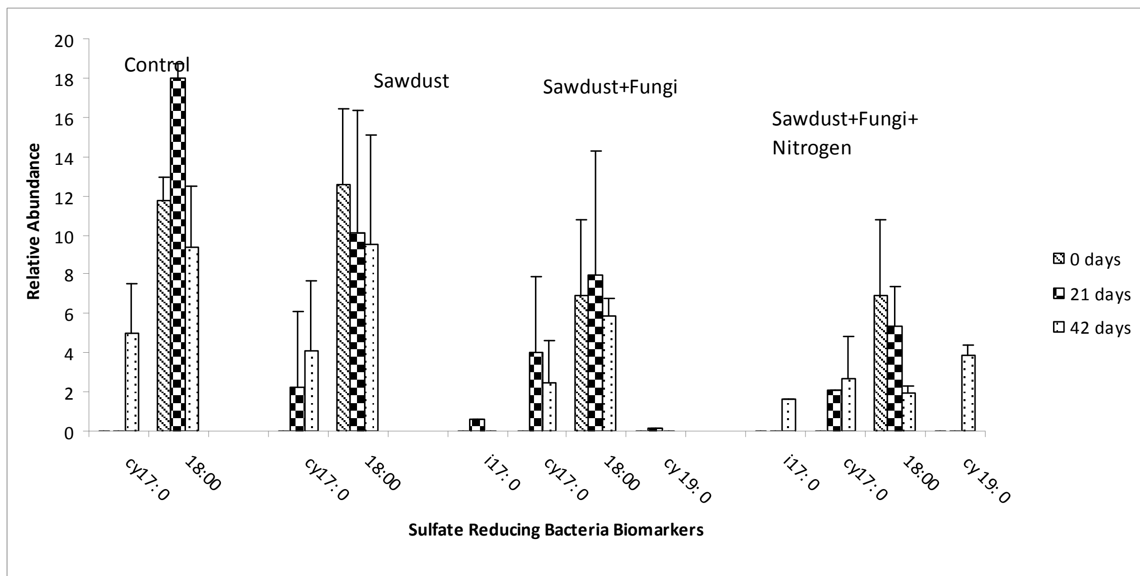


Fig. 7(b) Sulfate reducing bacteria biomarkers, Run 2

sawdust and sawdust + fungi. It showed up on the 21st day in the treatment with sawdust + fungi + nitrogen, and decreased by the 42nd day. Overall, a particular trend could not be detected for the biomarkers (figure 8(a)).

In Run 2, i15:0 showed an increase from 10.5% (± 1.43%) to 16.14% (± 3.0%) on the 42nd day in all the treatments except the sample with sawdust, in which the biomarker was not detected on the 42nd day. The other biomarker a15:0 showed an increase from 2.48% (± 4.28%) to 8.04% (± 2.59%) in treatments sawdust + fungi and sawdust + fungi + nitrogen. It showed a decrease in the relative abundance from 0 to 42nd day in the control, but showed up only on the 21st day in the treatment with sawdust. Overall phospholipids showed an increase in treatments sawdust + fungi and sawdust + fungi + nitrogen but a particular trend could not be detected for these biomarkers (figure 8(b)).

3.6 Methanotroph biomarkers

Methanotrophs serve as biofilters for the oxidation of methane produced in anaerobic environments. They generally use methane from the anaerobic soils, but in the absence of anaerobic environment, they oxidize atmospheric methane (Hanson et al., 1996). It could be due to this reason, the methanotrophs in the sediment exhibited variations.

16:1ω6 was the only fatty acid biomarker for methanotrophs that was detected in the sediment. In Run 1, it showed a decrease in the control from 7.9% (± 1.24%) to 2.0% (± 1.74%) (figure 9(a)). It decreased from day 0 to 42 in the treatments with sawdust and sawdust + fungi. It was not detected before the 42nd day in the treatment with sawdust + fungi + nitrogen.

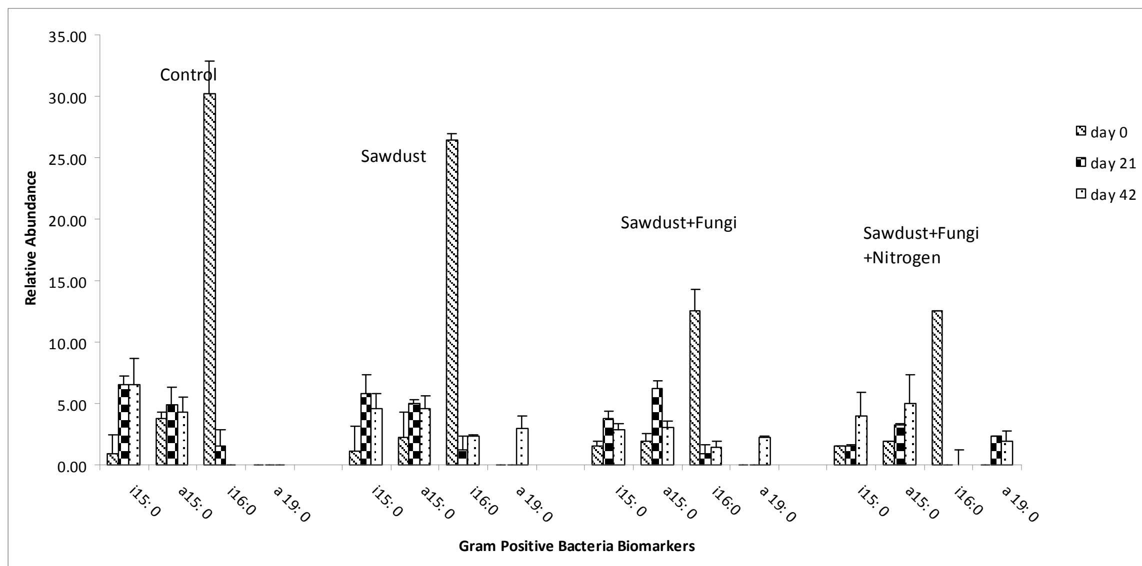


Fig. 8(a) Gram positive bacteria biomarkers, Run 1

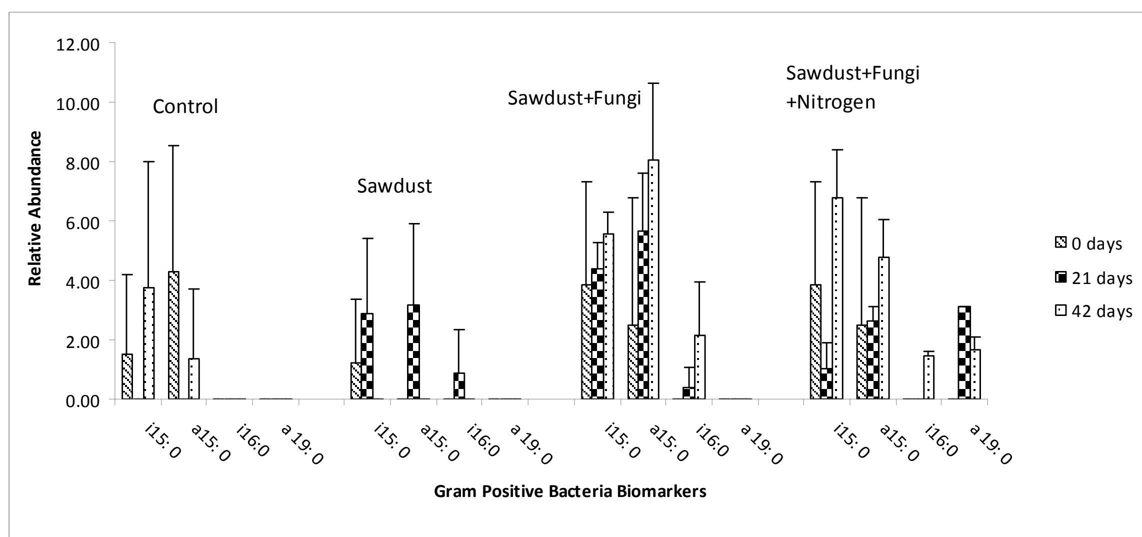


Fig. 8(b) Gram positive bacteria biomarkers, Run 2

On the contrary, in Run 2, 16:1ω6 showed an increase from 0 to 2.57% (± 2.28%) in the control, in sawdust + fungi from 0 to 4.21% (± 2.01%) and sawdust + fungi + nitrogen from 0 to 1.33% (± 0.12%) (figure (9b)). It was not detected in the sample with sawdust.

3.7 Fungal Biomarkers

A pure culture of *Pleurotus ostreatus* was taken and extracted to characterize the biomarkers present in the fungus. 18:2ω6, 18:1ω9 and 16:0 were the main fatty acids contributing 85% to the lipids in the fungi. Other fatty acids which were detectable but not quantifiable contributed 15% of the fatty acids (table 5).

The fatty acid characterization was also done on the grain used as a growth medium for the fungi. The fatty acids found were the same as in the fungi**,** namely18:2ω6, 18:1ω9 and 16:0. Sawdust and the nitrogen source used in the experiment were also tested. They did not contain any lipids that would interfere with the results.

Table 5. Biomarkers in fungi and grain from pure samples

Fungi	Lipids	Relative Abundance
	18:2ω6	52
	18:1ω9	18
	16:00	15
Grain		
	18:2ω6	54
	18:1ω9	16
	16:00	30
Sawdust	Not detected	-
Nitrogen source	Not detected	-

The biomarkers characteristic of fungi are 18:2ω6, 18:1ω9, 16:0. In Run 1, all the four treatments studied showed similar trends in the increase or decrease of the PLFAs which are representatives of fungi. But it is interesting to note that the fungal biomarker

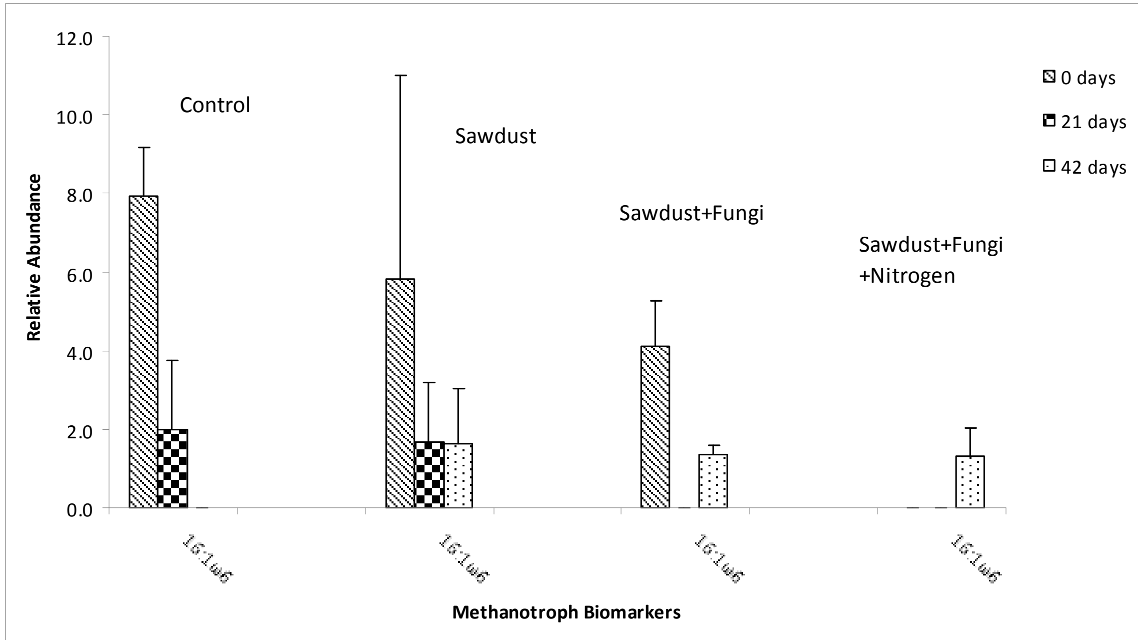


Fig. 9(a) Methanotroph biomarkers, Run 1

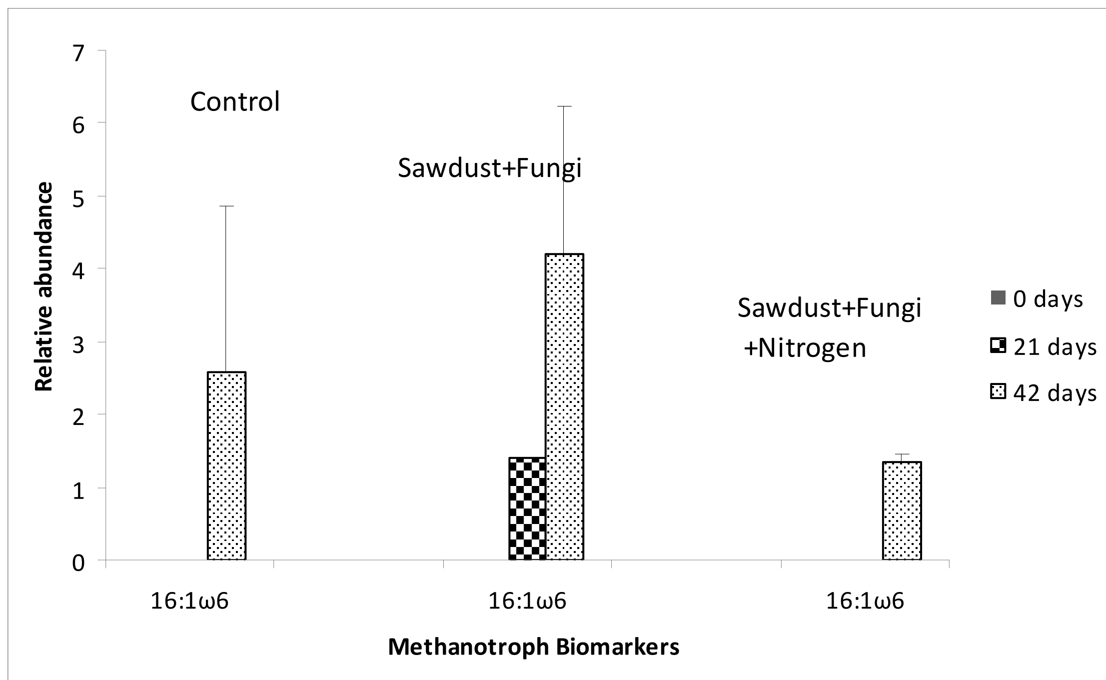


Fig. 9(b) Methanotroph biomarkers, Run 2

18:2ω6 (linoleic acid) decreased in all the samples. By the 42nd day, the relative abundance of 18:2ω6 was 0 in the control. In the sample with sawdust + fungi the relative abundance of the 18:2ω6 increased from 32.10% (± 4.7%) to 17.7% (± 1.34%). On the other hand, in the treatment with sawdust + fungi + nitrogen, the relative abundance of 18:2ω6 decreased from 32.1% (± 4.7%) to 6.0% (± 2.21%) (figure (10a)).

In the control, the fungal biomarker 18:1ω9 (oleic acid) showed a decrease from 10.5% (± 1.2%) to 5.8% (± 1.19%). The relative abundance of 18:1ω9 in the sample sawdust + fungi increased slightly from 12.0% (± 0.4%) to 12.2% (± 1.25%). In the treatment with sawdust + fungi + nitrogen, the final concentration of 18:1ω9 increased from 12.0% (± 0.4%) to 16.84% (± 11.49%)

The other fungal biomarker (16:0-palmitic acid) showed an increase in the final concentration in all the treatments. The fungal biomarkers in Run 2 showed different trends than those observed in Run 1. The decrease in unsaturation cannot be noticed in this run. The biomarkers 18:2ω6, 18:1ω9 and 16:0 in the control showed an increase on the 21st day and then decreased on the 42nd day. However, the highest relative abundance of the fungal biomarkers can be noticed in the treatment with sawdust + fungi + nitrogen on the 42nd day (figure (10b)).

There are several factors that might have contributed to these changes in the fatty acid composition of the fungi. These include temperature, nutrition and oxygen (Suutari 1995). The high concentration of the PAHs can also act as a stressor and can cause change in the fatty acid composition of the fungi. In general, fatty acid unsaturation decreases with an increase in temperature (Karine P et al., 2007). Our experimental results from Run 1 have shown that there is a decrease in the unsaturation at 25 ^{o}C and

that there is a decrease in 18:2ω6 in all the treatments and a relative increase in 18:1ω9 and 16:0. Initially, all the sediment samples were collected and stored at 4 ^{o}C until use and the incubation was set up at 25 ^{o}C. This change in temperature might have also contributed to this increase in saturated fatty acid concentration.

The characteristic lipids of fungi not only represent fungi but also other groups like green algae, higher plants and heterotrophic micro eukaryotes. The grain which was used as a nutrient, also contains the same biomarkers, so similar changes might have also occurred in the grain lipids. However, it is difficult to confirm that the changes in fatty acids are due to the temperature change alone and needs further investigation.

The exposure of the microorganisms to PAHs can cause an increase in the relative abundance of the degenerative genes including those of gram negative bacteria. The increase in PAH degradation, at least in part, may be due to the increased abundance of gram negative bacteria. The observed increase in the abundance of heterotrophic micro eukaryotes also suggests that a food web based on PAH concentration has developed (D.E Langworthy et al., 2002). The results confirm the existing microbial community response to PAHs.

3.8 Biomass results

The phosphate biomass level in the control, which was initially 0.45(± 0.01) nmol/gm dry weight of the sediment, increased to 0.53(± 0.01) nmol/gm of dry weight on the 21st day. But by the end of the experiment the biomass dropped to 0.48 (± 0.01) nmol/gm dry weight. The indigenous bacteria provided with favorable temperature and oxygen conditions might have contributed to the initial increase in lipid phosphate biomass (Canet, et al., 2001). The phosphate biomass in the treatment with fungi showed

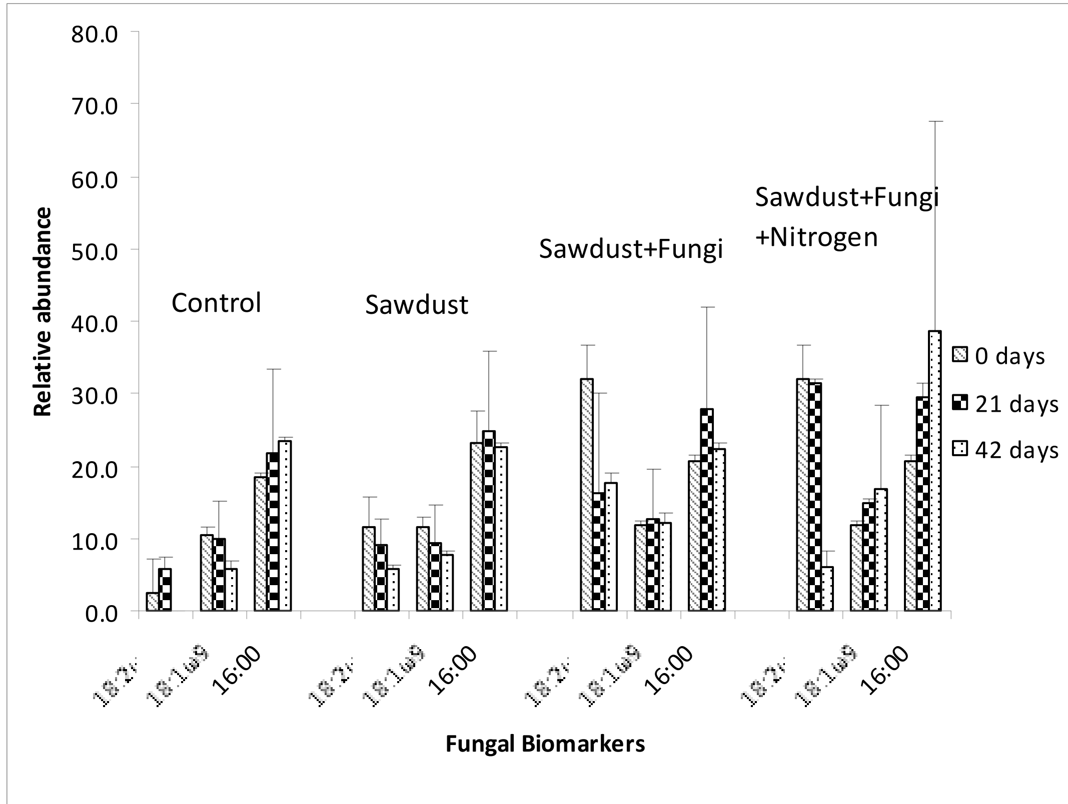


Fig. 10(a) Fungal biomarkers, Run 1

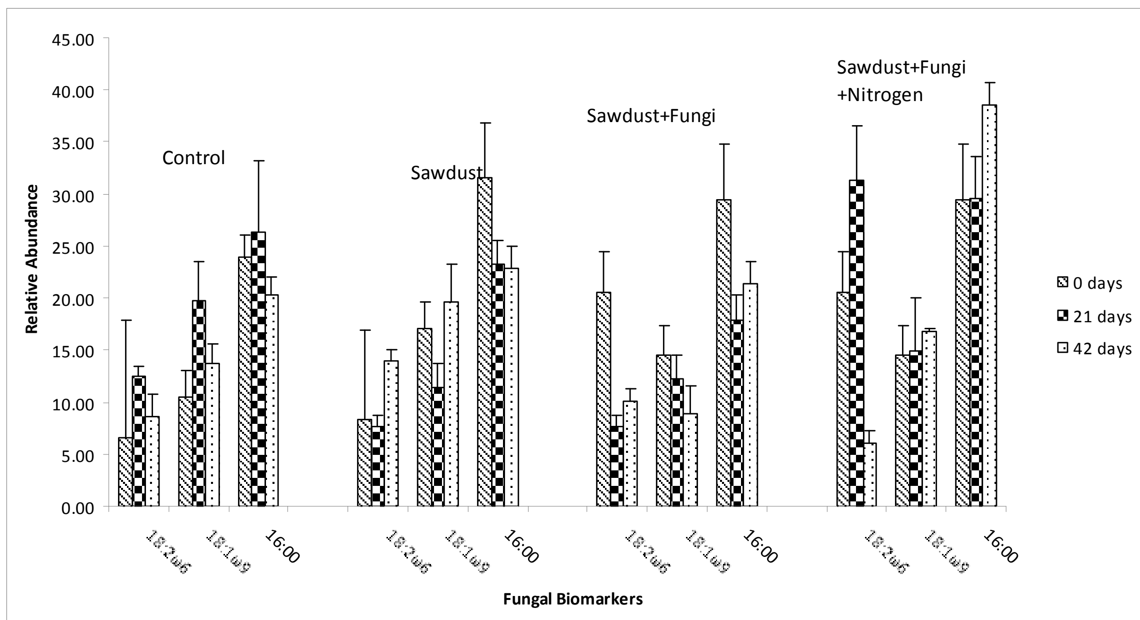


Fig. 10(b) Fungal biomarkers, Run 2

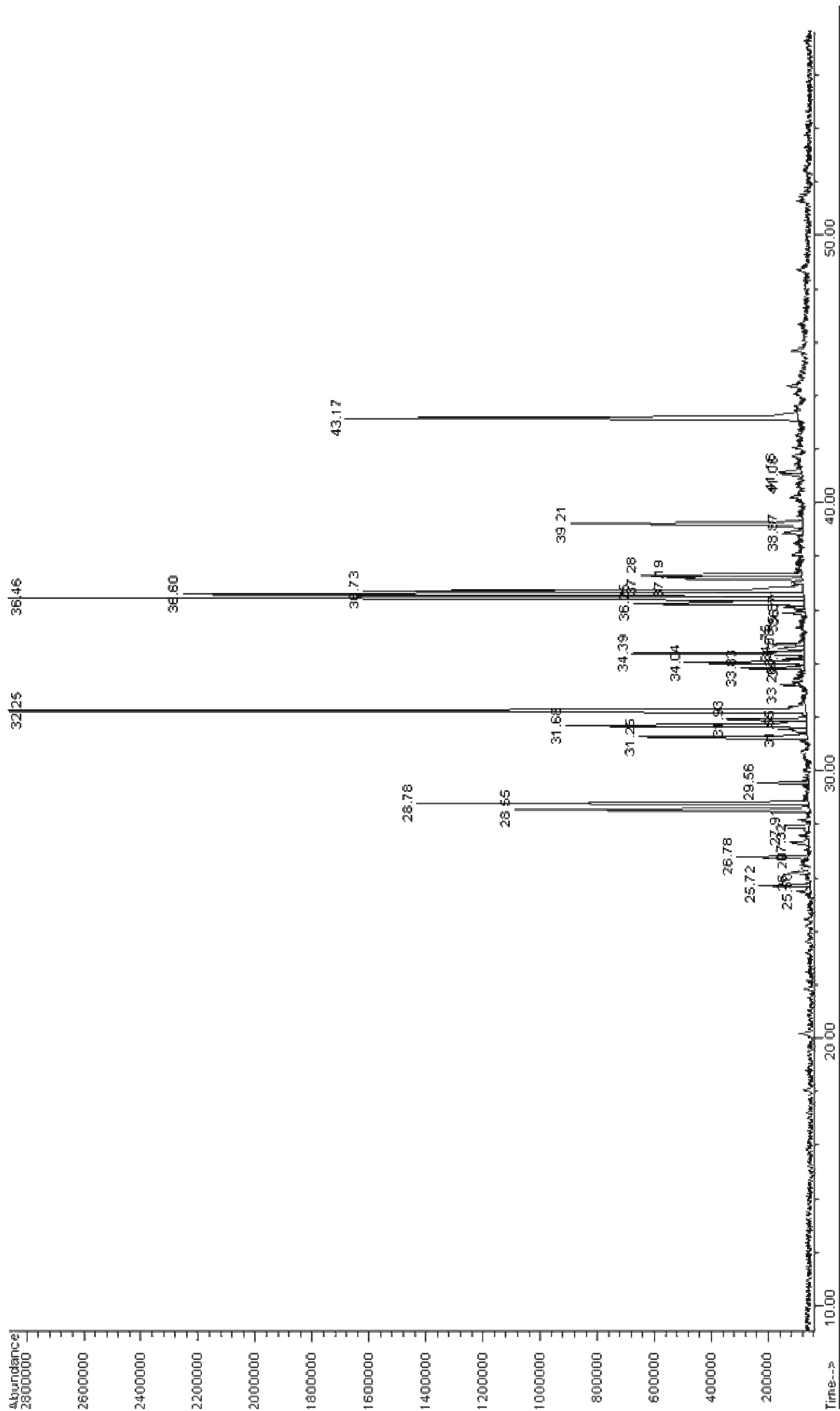


Fig.11 Chromatogram of FAMEs for sediment augmented with fungi

a slight increase. The initial value, which was 0.48(± 0.01) nmol/gm of dry weight, increased to 0.58(± 0.01) by the end of the experiment. Bacteria, fungi and sawdust might have contributed to the increased biomass.

The treatment with nitrogen, fungi and sawdust showed the highest concentration (0.65 (± 0.01) nmol/gm dry weight) of phosphate biomass at the end of the experiment. Biomass results showed consistency between runs (figures 12(a) and 12(b)).

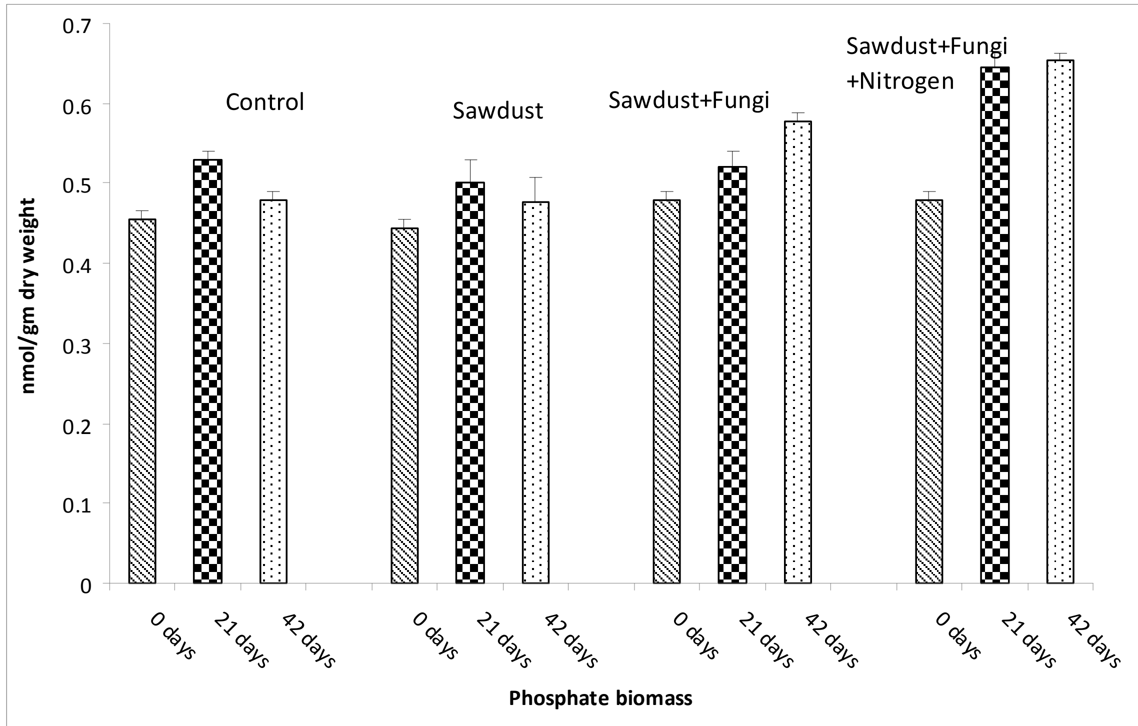


Fig. 12(a) Comparison of phosphate biomass values, Run 1

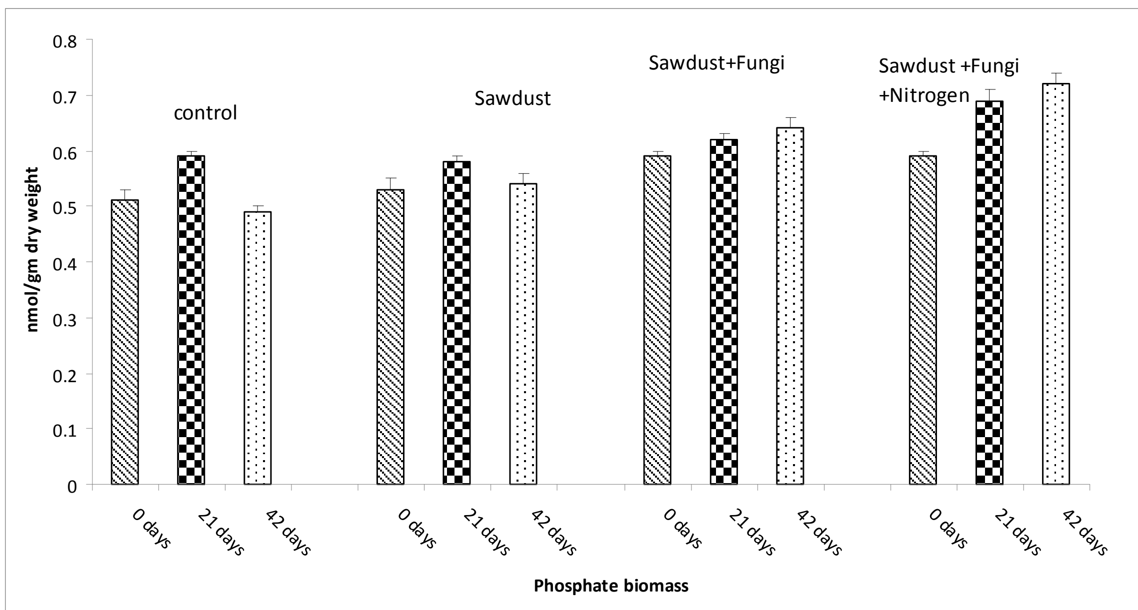


Fig. 12(b) Comparison of phosphate biomass values, Run 2

Chapter 4: Conclusion

PAH data obtained through sonication showed that the addition of *P.ostreatus* has a positive effect on PAH degradation and that it can be used as an effective tool in bioremediation. However, these results cannot be correlated with microbial community structure due to differences in the amounts of sediments taken in two procedures which would result in many variations.

There was high sample variability (within run, between runs) in microbial community structure by fatty acid analysis.

The microbial community has shown a change in its structure resulting in an increase in cyclo propyl fatty acids which indicate stressful conditions presented by PAH and/or metal contamination and nutrient enrichment. Sulfate reducing and gram positive bacteria and methanotrophs exhibited different trends.

The fungal biomarkers were higher in fungi-augmented samples when compared to the control and they were also found in grain fungal substrate. The fungal biomarkers in Run 1 showed a decrease in unsaturation which is an indicator of temperature adaptation.

The biomass results were consistent between runs and the highest values were observed in the treatment with sawdust + fungi + nitrogen.

Chapter 5: Future work

More research is needed to evaluate the relationship between microbial community structure and PAH contamination. Although triplicate samples were run for the extraction of PAHs, lipids and biomass, the PAH data were not obtained due to the technical problems with GC-MS. So comparing the PAH data from the same set samples with the lipid results, would give a better idea of the relation between PAH contamination and microbial community structure.

Use of a different fungal biomarker like ergosterol or a different substrate for fungal growth (other than grain) to prevent the overlap with biomarkers in the grain could give more coherent results.

More replicates (within a run) or more incubation runs (triplicate runs or more) should be done and analysis of sample sets should be performed on a single type of sediment taken from a particular site to minimize further heterogeneity.

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Appendices

Appendix 1: PAH extraction procedure

A) Reagents for PAH extraction

1. Dichloromethane(DCM) optima grade
2. Methanol optima grade
3. 0.25 N hydrochloric acid (HCl) (take 4 ml of HCl and make up to 200 ml with milli-Q water)
4. 50 mM phosphate buffer (add 8.7 g of KH_2PO_4 to 700 ml milli-Q water,stir and adjust he pH to 7.4 with 1 N HCl, complete to 1000 ml with milli-Q water)
5. Sodium chloride
6. Chloroform optima grade preserved with 0.75% ethanol.
7. Sodium sulfate for preparation of sodium sulfate columns
8. Unisil (activated silicilic acid, 100-200 mesh size) for construction of silica columns.
9. Aminopropyl columns
10. Copper filings

B) Preparation of Sodium Sulfate columns

1. Use 6 ml glass columns with Teflon frits in the bottom.
2. Prepare the columns just before the run, so that the DCM does not dry out.
3. Rinse columns with DCM
4. Load columns with frits
5. Load columns with 1 g of sodium sulfate
6. Add 2 ml of DCM to the assembled columns

7. Allow DCM to drip through, stopping when the meniscus is just above the Na_2SO_4.
8. Discard the DCM and the waste collection tubes.
9. Replace the waste tubes with clean round bottom evaporating flasks.

C) Preparation of silica columns.

1. weigh 0.5 g of Unisil in the glass columns with frits
2. Heat the columns with Unisil at 100 ^{o}C for 2 hours to activate Unisil
3. Place glass columns in Visiprep apparatus and close valves
4. Add 4 ml of chloroform to unisil in the glass columns
5. Open valves and let chloroform drip through at 1 drop per second, do not let the column dry
6. Rinse the glass column with 2 ml of chloroform
7. Stop the flow with chloroform reaches frit
8. Add copper filings(20-30) per column(cleaned in 2 rinses of 1N HCl, 2 rinses methanol, 2 rinses of DCM and dried under nitrogen

D) Preparation of Aminopropyl Columns

1. Use 3 ml aminopropyl columns (prepacked)
2. Rinse columns with 1 ml chloroform, rinse again with 2 ml and pull through with vacuum one drop per second
3. Rinse with 2 ml of hexane pull through with vacuum 1 drop per second but do not let the column dry.

E) Extraction of Polycyclic Aromatic Hydrocarbons

1. Take 0.65g of sediment and 0.5 ml milli-Q water in a 50 ml glass tube.

2. At this point a treatment can be done.
3. Add 7.5 ml of dichloromethane(DCM) and 15 ml of methanol
4. Add about 5.3 ml of phosphate buffer.
5. Mix the contents by shaking and venting, and check for any leaks.
6. Place samples on platform shaker for about 2 hours at 320 rpm. Cover with an aluminum foil in order to prevent light penetration.
7. Remove the samples from the shaker and add 7.5 ml of DCM and 7.5 ml phosphate buffer.
8. Shake and vent again.
9. Add a pinch of sodium chloride, shake and vent again.
10. The samples should be placed in the dark at 4 ^{o}C for 24 hours.

After 24 hours,

11. The sample shows 2 distinct phases.
12. Remove the upper water/methanol phase with a pipette and discard.
13. Remove the lower phase using another clean pipette to a 15 ml conical tube.
14. The amount of sample recovered should be recorded at this point (useful for biomass calculations).
15. To recover more sample add 1 ml of DCM to original tube, vortex and wait for 5 minutes, see if any organic phase can be recovered.
16. Repeat 2 more times with out vortex.(if sample cannot be seen in organic phase discard it)
17. Transfer all organic phase from the conical tubes to sodium sulfate columns on the Supelco visiprep apparatus.

18. Samples should be collected in 50/100 ml round bottom evaporating flasks under the Supelco visiprep apparatus.
19. Rinse conical tubes three times using 3 ml of DCM and transfer it to the sodium sulfate columns.
20. Rinse column with two1 ml aliquots of DCM, then pull to dryness using vacuum.
21. Take evaporating flasks to rotovap and concentrate to around 1 ml.
22. Transfer sample from evaporating flask to conical tube using a clean pipette.
23. Rinse evaporating flask with two 1 ml aliquots of DCM and add to the conical tube.
24. Concentrate the sample to one drop under nitrogen at 37 ^{o}C, but don't let the sample dry.
25. Bring volume of the sample to 1 ml- 1.5 ml using chloroform.
26. Record the amount of sample
27. Samples can be stored at -20 ^{o}C for short term storage or at -70 ^{o}C for long term storage. The sample at this stage can be used for the phosphate analysis and for the extraction of PAHs, neutral, glycol and phospholipids.
28. Biomass can be analyzed at this point or skip to PAH fraction

F) PAH Fraction

1. Silica columns should be prepared as outlined in appendix 1 (c)
2. If biomass is not run bring total volume of the sample.
3. Transfer the sample (1.0 – 1.5 ml) in chloroform to 200 µl hexane using solvent exchange.(do not let the sample dry as this will reduce PAH recovery), after this

the neutral, glycol and phospho lipids can be extracted by passing chloroform, acetone and CMDI solution respectively through the silica column.

4. Concentrate sample to 100 µl under nitrogen evaporator, then add 1 ml hexane. Concentrate sample to 100 µl again. Dope sample with one drop of chloroform, vortex and transfer to silica column. Draw sample through, but do not let column dry.
5. Repeat step 4 two more times using two aliquots 100 µl hexane
6. Rinse conical tube with 1 ml, 2ml and 2ml aliquots of hexane to recover more of the sample. Use this hexane to rinse sides of the silica column after rinsing conical tubes. Draw between each aliquot but do not let the column dry.
7. Rinse silica column once more with 100 µl hexane to finish recovery of PAH fraction.
8. Store PAH fraction in hexane without drying at -20 ^{o}C for short term storage or at -70 ^{o}C for long term storage until ready to clean with aminopropyl columns

G) Cleanup of PAH Fraction on Aminopropyl Columns

1. Aminopropyl columns should be prepared prior to cleanup.
2. Concentrate PAH fraction to one drop under nitrogen evaporator.
3. Bring volume to 200 µl with hexane.
4. If water is present in the sample add methanol until clear. Pipette off top PAH fraction and discard bottom methanol fraction.
5. Dope with one drop of chloroform, vortex sample and add to column.
6. rinse conical tube three more times using 300 µl hexane in three aliquots of 100 µl, dope with one drop of chloroform, vortex and add to column each time.

7. Draw sample through column
8. Wash PAH fraction from column using 5 ml hexane in three aliquots: 1 ml, 2ml, 3 ml and let go to dryness.
9. The sample is then concentrated to 1.0 ml and then transferred to an auto sampler vial
10. Add 20 µl internal standard before being read on the GC-MS.

Appendix 2: Phosphate Biomass procedure

A) Glassware for Phosphate Biomass

1. Rinse all glassware with phosphate free soap , followed by acid soaking for 10-15 minutes, rinse with DI water 3-4 times dry and finally rinse with DCM or methanol

B) Reagents for Phosphate Biomass

2. 0.1mM glycerol phosphate: add 0.216 g glycerol phosphate to 50 ml milli-Q water. Mix thoroughly and complete to 100 ml with milli-Q water. Pipette 1 ml of this solution in 50 ml milli-Q water, shake and complete to 100 ml with milli-Q water. Refrigerate until use.
3. Saturated potassium persulfate solution: use 200 ml volumetric flask, add 10 g $K_2S_2O_8$ to 150 ml milli-Q water. Stir and immediately add 2 ml of concentrated sulfuric acid. Complete to 200 ml with milli-Q water. Light sensitive use amber colored bottle and refrigerate, warm to room temperature before use.
4. 2.5 % ammonium molybdate solution: use 100 ml volumetric flask. Add 2.5 g $(NH_4)Mo_7O_{24}$ in 84 ml milli-Q water. Stir and add immediately 16 ml concentrated sulfuric acid. Light sensitive use amber color bottle.

5. Malachite green: add 1.11 g polyvinyl alcohol to 800 ml milli-Q water in 1000 ml beaker. Heat to 80 ^{o}C while stirring**.** Cool down and complete to 1000 ml with milli-Q water. Add 0.11 g of malachite green and stir. Store in dark place.

C) Standard curve for biomass analysis:

1. Take Glycerol phosphate in the concentrations mentioned in the table 6 in ampoules. Add 450 ul of saturated potassium persulfate
2. Flame seal the ampoules and let stand overnight at 105 ^{o}C

Table 6 Concentrations for Standard curve - biomass analysis

0.1 mM Glycerol Phosphate	Nmol
0 ul	0
20 ul	2
40 ul	4
60 ul	6
80 ul	8
100 ul	10

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D) Sample preparation for biomass

1. Take 0.1 ml of each sample in an ampoule.
2. Record the amounts used and the amounts left over
3. Samples should be brought to complete dryness under nitrogen and can be stored covered with a foil for later use.(or the following procedure can be continue)
4. Add 450 µl saturated potassium persulfate to each ampoule.
5. Flame seal ampoules and digest at 105 ^{o}C for 24 hours

6. After 24 hour period remove samples from oven and cool to room temperature
7. Open ampoules and add 100 µl of 2.5 % ammonium molybdate and let stand for 10 mins

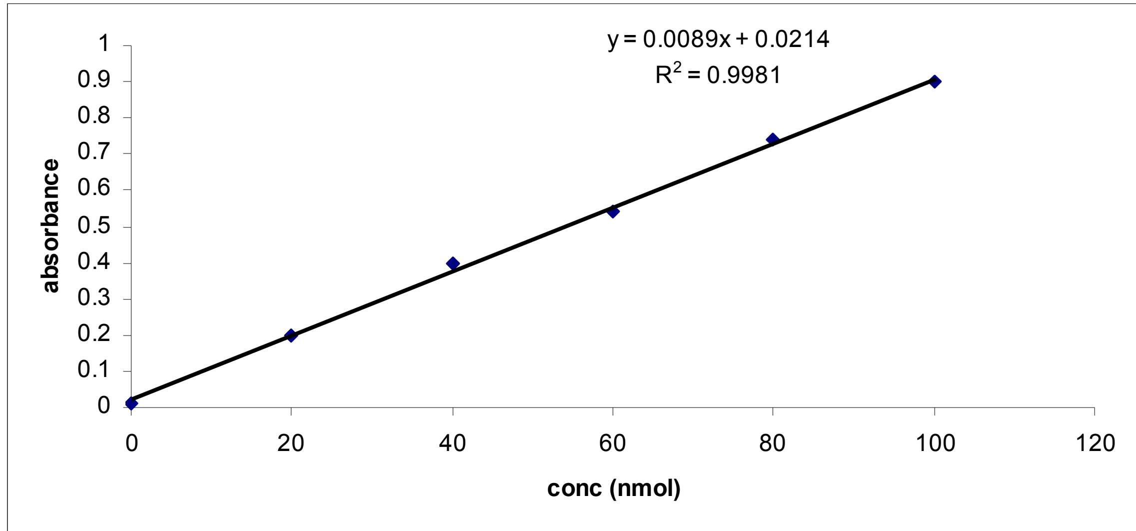

Fig. 13 Standard curve for biomass

8. After 10 mins add 450 µl malachite green and let stand for 20-30 mins.
9. Transfer the mixture to disposable cuvettes to be read on spectrophotometer at 610 nm.
10. To calculate the phosphate biomass, the triplicate absorbencies were averaged.

The linear equation created from the standard curve was used to calculate nmol of phosphate per ampule. To get nmol of phosphate per gram of dry weight, the following equation was used:

$$\frac{\text{Total amount of DCM (15 ml)}}{\text{Total amount of DCM recovered (13.5 ml)}} \times \frac{\text{Amount of chloroform added (1 ml)}}{\text{Amount taken out (0.1 ml)}} \times \text{nmol phosphate/ampule}$$

This number was then divided by the dry weight of the corresponding sample to get nmol of phosphate per gram of dry weight.

Appendix – 3: Lipid extraction procedure

A) Reagents for separation of lipids

1. DCM
2. Acetone optima grade
3. CMDI solution (chloroform: methanol: DI water – 5:5:1 v/v/v, made fresh daily
4. chloroform

B) Partition of lipids into neutral, glyco and phospholipid fractions

1. After the extraction of PAHs remove the conical tubes and place waste collection tubes in the visiprep apparatus and then pass four 1 ml aliquots of chloroform through the column,
2. Stop flow as chloroform reaches frit do not let it dry.
3. Add 1 ml of acetone and let it drip, columns will change from clear to white as acetone goes through. Neural lipids are extracted, discard.
4. Add four ml (2ml, 2ml) to make sure all chloroform is gone. Collect acetone in waste tube. Pull acetone to frit surface and stop flow. In this step glycolipids are extracted, discard.
5. Add 1 ml of CMD solution and let it drip carefully. Stop flow when CMDI reaches frit. Discard this.
6. Place clean conical tubes in the apparatus to collect phospholipids.
7. Add four ml (2ml, 2ml) CMDI solution and let it drip.
8. Use vacuum to pull remaining CMDI. This solution contains the phospholipids.
9. Dry samples in the nitrogen evaporator at 37 ^{o}C and store them in 1 ml DCM at -20 ^{o}C for short term storage or at -70 ^{o}C for long term storage.

10. If not, immediately methanolysis can be performed.

C) Reagents for formation of FAMEs (fatty acid methyl esters)

1. 0.2 N KOH solution :Weigh 1 pellet of KOH place in test tube, immediately add 5 ml methanol. Multiply pellets weight by 89.29 (this amount equals total volume of methanol in ml) make fresh daily.
2. Methanol : toluene solution (1:1) make fresh daily
3. Ethyl ester standards: weigh 10 mg of C22:0 acid and add it to 100 ml of hexane. This solution gives a concentration of 0.1 mg/ml
4. Chloroform
5. DCM

D) Formation of Fatty Acid Methyl Esters

1. Dissolve the dry phospholipids in 0.5 ml of methanol : toluene solution
2. Add 0.5 ml of 0.2 N KOH in methanol. Close the conical tubes, vortex and heat for 15 minutes at 37 ^{o}C.
3. This reaction transmethylates the PLFAs forming FAMEs.
4. cool the test tubes to room temperature and add 0.5 ml of 0.2 N acetic acid, vortex.

   Immediately add 2 ml of chloroform and 2 ml of deionized water and vortex for approximately 30 sec.
5. To separate the aqueous and lipid phases, centrifuge the mixture at 1500 rpm for 5 minutes.

6. The lipid phase (bottom) now contains the FAMEs. Transfer the lipid phase to a clean conical tube using Pasteur pipette. Avoid transfer of any of the aqueous phase. This may necessitate leaving a small drop of chloroform in the reaction conical tube.
7. Add 1 ml of chloroform to the reaction conical tube vortex and repeat steps 6 and 7
8. Repeat step 8
9. Add 1ml of chloroform to the reaction conical tube, but do not vortex or centrifuge
10. Transfer the chloroform to the clean conical tube. The clean tube should now contain a total of 5 ml of chloroform.
11. Add 50 µl of ethyl ester standards to sample tubes and to four clean tubes.(for calibration of the GC detector)
12. Dry samples and standards under nitrogen at 37 ^{o}C
13. Add 1 ml of fresh chloroform or DCM and store them at -20 ^{o}C for short term storage or at -70 ^{o}C for long term storage.

Purification of FAMEs:

Chemicals for purification of FAMEs

1. C_{18} reagent
2. Methanol
3. Chloroform
4. Acetonitrile:water (1:1)
5. Hexane

6. Hexane : chloroform (95:05)

E) Procedure

1. Add 1g of dry C_{18} packing to a 3 mL glass column fitted with a Teflon
2. Condition the column by passing a series of solvents through the packing. First hydrate the packing by adding 2 mL of deionised water to the column and initiate flow either by pulling a vacuum on the column or by pressurizing the head space of the column. Continue the flow until the column appears dry. Except where noted, the flow should be approxly. 1 drop s^{-1} and columns should not be allowed to run dry.
3. Wash the packing with 2 mL of methanol. Stop flow as the solvent-air interface reaches the head of the column. Wash the packing with 1 mL of chloroform. Add 2 mL of chloroform and close the column such that the solvent flow is inhibited. The column packing material will soon begin to effervesce and become translucent. Dislodge any air pockets and improve the column packing.
4. After the C_{18} packing has become translucent allow the chloroform to drip from the column, but do not let the column run dry. Wash the packing with 2 mL of acetonitrile:water (1:1) to complete the column conditioning. To prevent drying, leave approxly. 1 mL of acetonitrile : water wash over the packing and remove it just prior to the addition of the FAMEs to the column.
5. Add 250 µL of acetonitrile to the dry FAMEs. Vortexes 3 times over the next 10 mins.Then add 250 µL of deionised water, vortex, and transfer to the column and allow the solvent to drip through the column. To complete the transfer of FAMEs

to the column, repeat this process three times, omitting the 10-min wait, for a total of 4 transfers.

6. Wash the column with 1 mL of acetonitrile:water (1:1) and allow the column to run dry. Wash the column with 200 µL of hexane and allow the column to run dry. Dry the column completely either by fitting a drying attachment and establishing a flow of nitrogen for 5 min or by applying a vacuum to the bottom of the column for 15 min.
7. Hydrate the packing by adding 2 mL of deionised water to the column and allow it to run.
8. Replace the waste container with a clean 15 mL conical tubes close the column and add 750 µL of hexane: chloroform (95:5). Allow the column to stand for 2 mins prior to initiating the solvent flow.
9. Wash the column thrice with 0.5 mL of hexane: chloroform (95:5) and collect the FAMEs.
10. Evaporate the solvent in a nitrogen evaporator having a bath temp of 37^0 C.

Appendix – 4: Standards for PAHs

Surrogate Solution: Restek B/N surrogate mix

2-fluorobiphenyl
nitrobenzene-d5
p-terphenyl-d14

Calibration Mix: Restek SV Calibration Mix #5 / 610 PAH Mix

acenaphthene, acenaphthylene, anthracene, benzo(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, chrysene, dibenzo(a,h)anthracene, fluoranthene, fluorine, indeno(1,2,3-cd)pyrene, naphthalene, phenanthrene, pyrene

Internal Standards: Restek SV Internal Standard Mixes

acenaphthene-d10, chrysene-d12, 1,4-dichlorobenzene-d4, naphthalene-d8, perylene-d12, phenanthrene-d10
2,000 µg/ml each in methylene chloride, 1ml/ampul

Correlated Internal Standards with PAHs and Surrogates

Internal Standards:	Correlating PAHs and Surrogates
Napthalene-d8:	Nitrobenzene-d5 (surrogate), Napthalene
Acenaphthene-d10:	2-fluorobiphenyl (surrogate), Acenaphthylene, Acenaphthene, Fluorene
Phenanthrene-d10:	Phenanthrene, Anthracene, Fluoranthene, Pyrene,
Chrysene-d12:	Terephenyl-d14 (surrogate), Benzo(a)anthracene, Chrysene, Benzo(b,k)fluoranthene, Benzo(a)pyrene
Perylene-d12:	Dibenz(ah)anthracene, Ideno(1,2,3-cd)pyrene, Benzo(ghi)perylene

Standards for lipids

Arachidic Acid Ethyl Ester: Sigma

PUFA No. 1: Supelco
C14:0, C16:0, C16:1 ω7, C18:1 ω7, C18:1 ω9, C18:2 ω6, C18:4 ω3, C20:1 ω9, C20:2 ω6, C20:5 ω3, C22:1ω9, C22:1 ω11, C22:5 ω3, C22:6 ω3

PUFA No. 2: Supelco
C14:0, C16:0, C16:1 ω7, C18:0, C18:1 ω7, C18:1 ω9, C18:2 ω6, C18:3 ω3, C18:3 ω6, C20:3 ω6, C20:4ω6, C20:5 ω3, C22:4 ω6. C22:5 ω3, C22:6 ω3

PUFA No. 3: Supelco
C14:0, C16:0, C16:1 ω7, C16:2 ω4, C16:4 ω1, C18:0, C18:1 ω7, C18:1 ω9, C18:2 ω4, C18:2 ω6, C18:3ω3, C18:3 ω4, C18:4 ω3, C20:1 ω9, C20:4 ω3, C20:4 ω6, C20:5 ω3, C22:5 ω3, C22:6 ω3

BAME Standards: Supelco