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# Seasonal changes in the microbial community of a salt marsh, measured by phospholipid fatty acid analysis

M.J. KEITH-ROACH[1,3,*], N.D. BRYAN[1], R.D. BARDGETT[2,4] and F.R. LIVENS[1]

*[1]Department of Chemistry, University of Manchester, Manchester, M13 9PL, UK; [2]School of Biological Sciences, University of Manchester, Manchester, M13 9PT, UK; [3]Current address: Department of Nuclear Safety Research, Risø National Laboratory, P.O. Box 49, DK-4000 Roskilde, Denmark; [4]Current address: Institute of Environmental and Natural Sciences, Department of Biological Sciences, University of Lancaster, Lancaster, LA1 4QY, UK; *Author for correspondence (e-mail: Miranda.Keith-Roach@Risoe.dk; phone +45 4677 4911; fax: +45 4677 4913)*

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**Abstract.** Microbial activity within the environment can have distinct geochemical effects, and so changes in a microbial community structure can result in geochemical change. We examined seasonal changes in both the microbial community and the geochemistry of an inter-tidal salt marsh in north-west England to characterise biogeochemical processes occurring at this site. Phospholipid fatty acid (PLFA) analysis of sediment samples collected at monthly intervals was used to measure seasonal changes in microbial biomass and community structure. The PLFA data were analysed using multivariate techniques (Ward's method and the Mahalanobis distance metric), and we show that the use of the Mahalanobis distance metric improves the statistical analysis by providing detailed information on the reasons samples cluster together and identifying the distinguishing features between the separate clusters. Five clusters of like samples were defined, showing differences in the community structure over the course of a year. At all times, the microbial community was dominated by PLFA associated with aerobic bacteria, but this was most pronounced in summer (August). The abundance of branched fatty acids, a measure of the biomass of anaerobes, started to increase later in the year than did those associated with aerobes and the fungal biomarker 18:2$\omega$6 showed a brief late-summer peak. The salt marsh remained mildly oxic throughout the year despite the increase in microbial respiration, suggested by the large increases in the abundance of PLFA, in the warmer months. The conditions therefore remained most favourable for aerobic species throughout the year, explaining their continual dominance at this site. However, as the abundance of PLFA synthesised by anaerobes increased, increases in dissolved Mn concentrations were observed, which we suggest were due to anaerobic respiration of Mn(IV) to Mn(II). Overall, the geochemical conditions were consistent with the microbial community structure and changes within it.

## Introduction

Microbial processes can affect both the chemical conditions of natural environments and the behaviour of elements within the system. This is exemplified by elements with more than one oxidation state because microbial processes can oxidise or reduce the element, changing its chemical behaviour significantly. The well known

cycling of iron and manganese (Kerner 1993; Lovley 1997) is a good example of this as the solubility of both increases on reduction to the + 2 state, resulting in dissolution of Fe/Mn precipitates. Reduction can occur either directly, through their use as a terminal electron acceptor in anaerobic respiration, or indirectly, through microbial alteration of the redox potential (Eh)/pH of the local environment. Less common metals can sometimes be utilised as an electron acceptor in anaerobic respiration, for example, iron-reducing bacteria, *Geobacter metallireducens* and *Shewanella putrefaciens*, and some sulfate-reducing microorganisms, are known to be able to utilise U(VI) (Lovley 1997).

Oxidation can occur through the reduced metal being used specifically as an electron donor in chemolithotrophic respiration (Stolp 1988) or through the system becoming more oxidising. Metals may also be solubilised through complexation by microbial metabolites or siderophores (Wildung and Garland 1980), and microbes can reduce metal solubility through biosorption or bioaccumulation. Microbes therefore have an important role in the cycling of major and trace elements (Malcolm et al. 1990) and so, when a microbial community changes in either size or structure, there may be associated geochemical changes within the system (Findlay et al. 1990; Rajendran et al. 1994).

The characterisation of a microbial community over time and the changes within it, mapped to changes in the chemistry of the system, may therefore provide information which could help in understanding these often complex processes. Until the development of phospholipid fatty acid (PLFA) analysis (Bobbie and White 1980) it was difficult to assess microbial community structure accurately in environmental samples. PLFA are biochemical components of cell membranes which are characteristic of different types of microorganisms and hence, provide 'signatures' of different microbial groups (Tunlid and White 1992). This technique delivers relatively sensitive and reliable information through a reasonably straightforward methodology (Findlay et al. 1989; Rajendran et al. 1992; Bååth et al. 1998). A few studies have used PLFA to examine differences in microbial community structure between seasons. The sampling strategies of these vary, for example Rajendran and Nagatomo (1999) sampled in spring and autumn, Bardgett et al. (1999) sampled 4 times in a year and Findlay and Watling (1998) conducted monthly samplings over 15 months. All of these studies demonstrated that microbial communities do vary over the course of a year in response to changing conditions.

PLFA data sets are generally very large, with many variables within each individual sample as a result of the different PLFA present. Statistical tools are required to assess the similarities and differences between the individual samples, as it is very difficult to extract detailed information on this by examining the raw data. Ludvigsen et al. (1997) have shown that the methods of statistical analysis employed to interpret these large data sets influence the information derived. They tested 4 common methods – principal component analysis (PCA), correspondence analysis (CA), partial least squares (PLS) and discriminant analysis (DA) – and showed that, while all the techniques contributed to the analysis, none provided a full interpretation. Indeed, they suggest that further techniques may have provided additional information. Therefore, when studying the differences in entire PLFA

profiles, a statistical tool that uses an unbiased approach may provide a fuller analysis and improve the quality of the data interpretation. Cluster analysis of a complete, normalised data set is one way of achieving this, as used by Findlay et al. (1990).

A previous study (Morris 1996) has shown that the 30–35 cm zone of a salt marsh in north-west England is geochemically active, displaying seasonal cycling of Fe, Mn and Pu. Therefore, in this study, our intention was to test the hypothesis that the microbial community at this depth changes sufficiently in structure or size throughout the course of a year to drive the observed geochemical changes. PLFA analysis was selected to characterise the community over time and sampling was synchronised with interstitial water collection to obtain a concurrent assessment of the microbial community and geochemical data. In order to obtain more information from the statistical analysis of the data we have included Mahalanobis analyses (Mahalanobis 1936) in the cluster analysis to calculate the distances between the mean abundances of each PLFA in each cluster. This gives a great deal of information on why samples cluster together and the differences between different clusters, and so improves the level of information available. The Mahalanobis treatment of the PLFA data was compared with a standard cluster analysis program to determine how the results depend on the technique utilised, since this is a novel application of the Mahalanobis approach.

## Methods

### *Sampling*

Three replicate sediment samples for PLFA analysis were collected monthly over a year between August 1996 and August 1997 from random locations within 3 m of a reference point on an inter-tidal salt marsh in the Esk Estuary, west Cumbria (UK National Grid Reference SD 089947). The sedimentological and hydrological processes at the marsh have been studied extensively by Carr and Blackley (1986). Briefly, the sediments are composed mainly of silt and fine sand (2 to 125 $\mu$m in diameter: 89%) and clay (< 2 $\mu$m in diameter: 7%) on a gravel bed. The sediment is covered in relatively salt-tolerant vegetation dominated by *Halimione portulacoides* in the upper zone and *Puccinellia maritima, Suaeda maritima* and *Salicornia spp* in the lower. During the tidal cycle, seawater flows upwards through the sediment, so although the marsh is only inundated during spring tides, the sub-surface sediment is re-wetted with oxic seawater each high tide.

The 2 cm diameter cores were collected using a plastic borer, and the section of 30–35 cm depth was bagged and stored in ice for transport to the laboratory. The samples were then stored at 4 °C for a maximum of 18 h before PLFA extraction. The 30–35 cm depth section of the sediment was chosen to allow comparability with dissolved trace element data, obtained from interstitial water samplers emplaced at that depth [a full description of the samplers is given in Keith-Roach et

al. (2000)]. A previous study has shown that Pu and trace element cycling occurs in this zone (Morris 1996), making this a region of biogeochemical interest. The Eh, pH and temperature of each interstitial water sample were measured in the field using a hand-held meter (Hanna water test meter). The sample was then transferred to a pre-weighed storage bottle containing a known amount of AnalaR 16 M $HNO_3$, sufficient to reduce the pH to < 1 and prevent nuclide sorption to the surface of the bottle. The sample volume was determined by weighing. Fifty millilitre sub-samples of each sample were retained for stable element analysis. Iron, Mn and Na were determined in these by inductively coupled plasma-optical emission spectrometry, and dissolved organic carbon (DOC) by UV absorption. The remainder of the sample ( $\sim$ 800 ml) was used in radiochemical analysis (Keith-Roach et al. 2000).

*Phospholipid fatty acid analysis*

PLFAs were extracted using the methodology described by Bardgett et al. (1996) which is based on the method of Bligh and Dyer (1959) as modified by White et al. (1979). A 19:0 fatty acid internal standard (238.5 nmol $ml^{-1}$, 10 $\mu$l in methanol – nomenclature is described later), was added to the sample prior to the mild alkaline methanolysis stage.

The samples were dissolved in dichloromethane (100 $\mu$l) and analysed on a HP 5890 gas chromatograph (GC) fitted with a mass selective detector. The GC was used with a DB5 phase silica column (15 m in length, 0.32 mm internal diameter and film thickness 0.25 $\mu$m). The samples were run from 150–250 °C with a 5 °C $min^{-1}$ ramp. Analysis was carried out using the Micromass MassLynx software package. Standard PLFA methyl ester mixtures (Sigma) were run to calibrate retention times, and the software library used to aid peak identification. Absolute amounts of the PLFAs were determined from peak intensities by calculating ratios to the 19:0 internal standard.

*Interpretation of the PLFA analysis*

The PLFA nomenclature used here (Rajendran et al. 1992) designates each fatty acid as nx:y$\omega$d, where n is the position and length of a branch in the chain; x is the number of carbon atoms in the fatty acid chain, counting from the acid group; y is the number of double bonds; and d is the position of the double bond. Thus, 16:1$\omega$7 designates a 16 carbon chain with no branching and a double bond between the seventh and eighth carbon atoms, and 10Me16:0 designates a saturated 16 carbon chain with a methyl branch on the tenth carbon atom.

Different biomarkers are used in different studies (Rajendran et al. 1992; Haldeman et al. 1995; Bardgett et al. 1996), and are present in different microbial communities, so comparisons between studies or locations must be made with caution. Ten PLFA were resolvable in our GC spectra, and these biomarkers were grouped as follows:

1. ubiquitous to all species – 16:0, 18:0 (Findlay et al. 1990);

2. aerobic bacteria – 16:1$\omega$9, 19:1 (Rajendran et al. 1992), 16:1$\omega$7, 18:1$\omega$9 (Findlay et al. 1990);
3. fungi/microalgae – 18:2$\omega$6 (Findlay et al. 1990; Rajendran et al. 1992); and
4. anaerobic, Gram-positive, and sulphate reducing bacteria – 16:0 branched (Findlay et al. 1990; Rajendran et al. 1992).
5. It has been reported that 16:1$\omega$9 and 18:1$\omega$9 fatty acids can be produced by microalgae, as well as by aerobic bacteria (Findlay et al. 1985; Rajendran et al. 1992). Since fungal/ microalgal PLFA (18:2$\omega$6) was observed in every sample, there is some uncertainty in the origin of the 16:1$\omega$9 and 18:1$\omega$9 fatty acids. However, this is only a minor problem as the 18:2$\omega$6 fatty acid has a much lower abundance in these samples than the PLFA indicative of aerobic bacteria. Also, although 16:0br PLFA are indicative of a group of anaerobic bacteria which includes sulphate-reducing bacteria (SRB), no PLFA indicative of the common SRB *Desulfobacter* (10Me16:0) or *Desulfovibrio* (17:1$\omega$7) were observed. This suggests that the PLFA of this type measured at this site did not arise from SRB.

*Statistical analysis*

To clarify the description of the techniques below we define a *sample* as being one of the individual 'replicate' sediments taken each month. Since ten different PLFA were analysed in each sample, there are ten *components* in each sample which can be used to differentiate the sample as a whole from other samples, *i.e.* there are 10 variables determined for each sample. Multivariate statistical analysis is a way of looking at all 10 variables simultaneously and is used to group samples into *clusters* of the most similar samples on the basis of all 10 PLFA components.

*Clustan analysis*

The Clustan multivariate statistical analysis programme (Wishart 1987) was used to perform cluster analysis of the data. The components of each sample were firstly normalised (z-scored) to give each the same weight in the analysis, regardless of magnitude. Comparison of the data is an extension of the approach used in scatter plots for the comparison of 2 variables. One dimension is required for each variable and the distance between data points can be calculated in each dimension. In this case, with ten variables, ten dimensional hyperspace is used. Two independent clustering techniques were applied, a conventional analysis using Ward's clustering algorithm, and reloc, a relocating method.

Cluster analysis using Ward's method is a standard hierarchical technique, where samples and groups are successively combined (fused) to provide final clusters. During clustering, fusion takes place such that the "error sum of squares" is minimised. The error sum of squares is defined as the sum of squares of the distances between a sample and the centre of its group. In this case, with ten PLFA being compared, there are 10 distances in the error sum of squares calculation for each sample. The "centre" of the group comprises 10 mean positions, one for each PLFA, so that the distances are calculated from the central position of the group for each PLFA. In Ward's method, once a sample has been assigned to a group, it may not

move to another group even if it would fit better in that group. However, the results can be presented as a "dendrogram" which traces the evolution of the clustering process, and shows the degree of similarity between different groups.

In the reloc method, fusion also proceeds such that the error sum of squares is a minimum. The difference is that, at each fusion stage, samples are allowed to relocate to other groups if they belong better in that group. Following relocation, group centres are recalculated, and samples given the opportunity to move again. This procedure is repeated until a stable configuration is achieved. The method then proceeds to the next fusion stage, and so on. Unfortunately, because of its complexity, the results of the reloc procedure cannot be easily represented in a diagram.

*Mahalanobis analysis*

The groups obtained from the reloc procedure were then examined using the Mahalanobis distance metric (Mahalanobis 1936). The distance between an individual PLFA value and the mean value for a group is defined here as the difference in concentration between the sample and group mean, divided by the standard deviation of that PLFA in that group. Since the PLFA concentrations within a group will be normally distributed then, by definition, if a given sample is a good member of a group, 2/3 of its PLFA concentrations should lie within one standard deviation of the group mean concentrations, and no more than 5% of those concentrations should lie more than two standard deviations from the means. That is, for each sample > 66% of its Mahalanobis distances should be less than one and no more than 5% greater than 2. This provides a useful test of the 'correctness' of the assignment of a given sample to a given group. For a sample set of 10 PLFA concentrations, there should be a minimum of 6 or 7 Mahalanobis distances < 1, and a maximum of 1 Mahalanobis distance > 2.

To provide a further test of the group assignments, a total or combined Mahalanobis distance between each sample and each group is calculated. Two different metrics are used. The city block total Mahalanobis distance, $C_{i,j}$, between a group, i, and sample, j, will be given by

$$C_{i,j} = \sum_{l=1}^{N} (x)$$

where N is the number of PLFA concentrations and x is $|xM_{i,j}(l)|$, the Mahalanobis distance between sample j and group i for PLFA l. The Euclidian total Mahalanobis distance, $E_{i,j}$, is given by

$$E_{i,j} = \sqrt{\sum_{l=1}^{N} (x)^2}$$

A combined Mahalanobis distance is thus calculated between each sample and the centre of every cluster, with the shortest distance identifying the cluster in which the sample fits best. The results from both metrics should agree with the original Mahalanobis assignments if the samples truly belong in a cluster.

A computer program based on these ideas (Bryan 1991) was used in this work to calculate the number of PLFAs for each sample which have a Mahalanobis distance of < 1 and > 2 in relation to each group. The criteria outlined earlier are used to decide whether a sample genuinely lies within a group or not, and hence to assign the samples to the 'correct group'. This approach is especially effective when there are two or more groups that are very similar.

The distances between each group centre and the other group centres were also calculated in the program. For a given PLFA, a large distance shows that the PLFA differs greatly between the two groups, and a small distance that the PLFA is very similar in both. Thus, the key components which discriminate between groups can be identified. When comparing two groups there will of course be two Mahalanobis distances depending upon which group's standard deviation is used. Statistical dilution factors, essentially a measure of the ratio of the magnitudes of two groups, are also calculated.

The data from these analyses therefore show how well each sample fits into a group, and how each group differs from other groups. Both the abundance concentration data, which included dilution effects and the proportion, expressed as a percentage of total measured PLFA, of each PLFA in each sample were used in separate analyses. The second approach had the advantage of excluding concentration effects, such as variations in moisture content or losses in the separation, from the analysis.

## Results

*PLFA data*

The 3 'replicate' samples from each month varied considerably in their total PLFA concentrations, as shown in Figure 1. Figure 1 shows only a small part of the raw data, since 10 PLFA variables were monitored in each sample, but illustrates the importance of using statistical analysis to establish the similarities and differences between samples when there is not only a large number of variables, but also a high degree of dispersion within 'replicates'.

In the statistical analysis, samples are labelled with the month in which they were collected and a "replicate" number of 1 – 3. The 13 month sampling time meant that August samples were collected in two consecutive years, and so the year is denoted a or b. Cluster numbers are those given in the computer output. The results from the Ward's hierarchical analysis of the abundance data are presented, followed by a confirmatory analysis using the reloc procedure, a hierarchical analysis of the percentage data and finally the results of the Mahalanobis analysis of the abundance data.

The results from Ward's method analysis of the abundance data are shown in Figure 2. The outliers, Aug a1, Aug a2, and Aug b3, were identified in a preliminary analysis and removed from the data set.

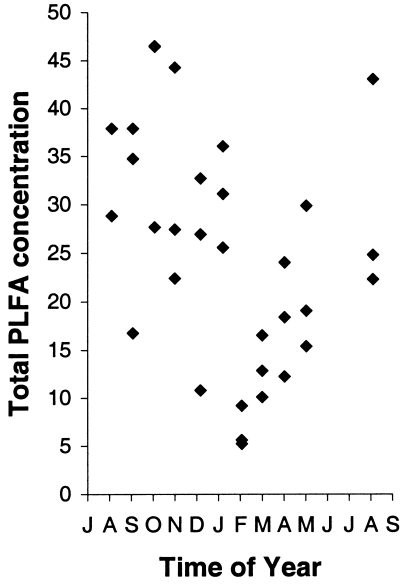


*Figure 1.* Total PLFA Concentration of each Sample. The total PLFA concentration (nmol $g^{-1}$ dry weight sediment) of each sample plotted against month of sampling. Three sediment samples were analysed each month, to account for the spatial variability in microbial abundance.

The dendrogram shows the formation of 6 clusters of like samples, based on the 10 PLFA components. Each generally contains samples which are from a similar time of year to the others in that cluster, with the exceptions of cluster 3, which contains a number of samples from both winter months and summer months, and cluster 4, which contains only 3 samples. At this level of analysis there is no information about why they cluster in this way, and what are the main driving forces behind the clustering. The groupings obtained by Ward's method were confirmed by the reloc procedure.

The dendrogram arising from the percentage data resulted in two major clusters, generally representing the winter and summer months. The more detailed clustering is quite fragmented, and difficult to interpret usefully. The reloc procedure resulted in the formation of 7 clusters and these are shown in Table 1.

In the reloc procedure, many samples fitted into a second cluster nearly as well as the cluster they were actually assigned to. This is particularly true of members of cluster 6, which are close to cluster 3, and members of cluster 3, which are close to cluster 1. This suggests that the community structure is changing gradually, giving a slight merging of clusters from the summer to the autumn and the autumn to the winter. In addition, May a1, May a2, Apr a3 and Mar a1 would fit quite well in cluster 9. So although the groupings here are rather mixed and poorly separated, the reloc analysis gives a useful overview of the shifts in the community.

The percentage data were not analysed beyond this stage because of the splintered and ill-defined clustering, and because they contain less information than the

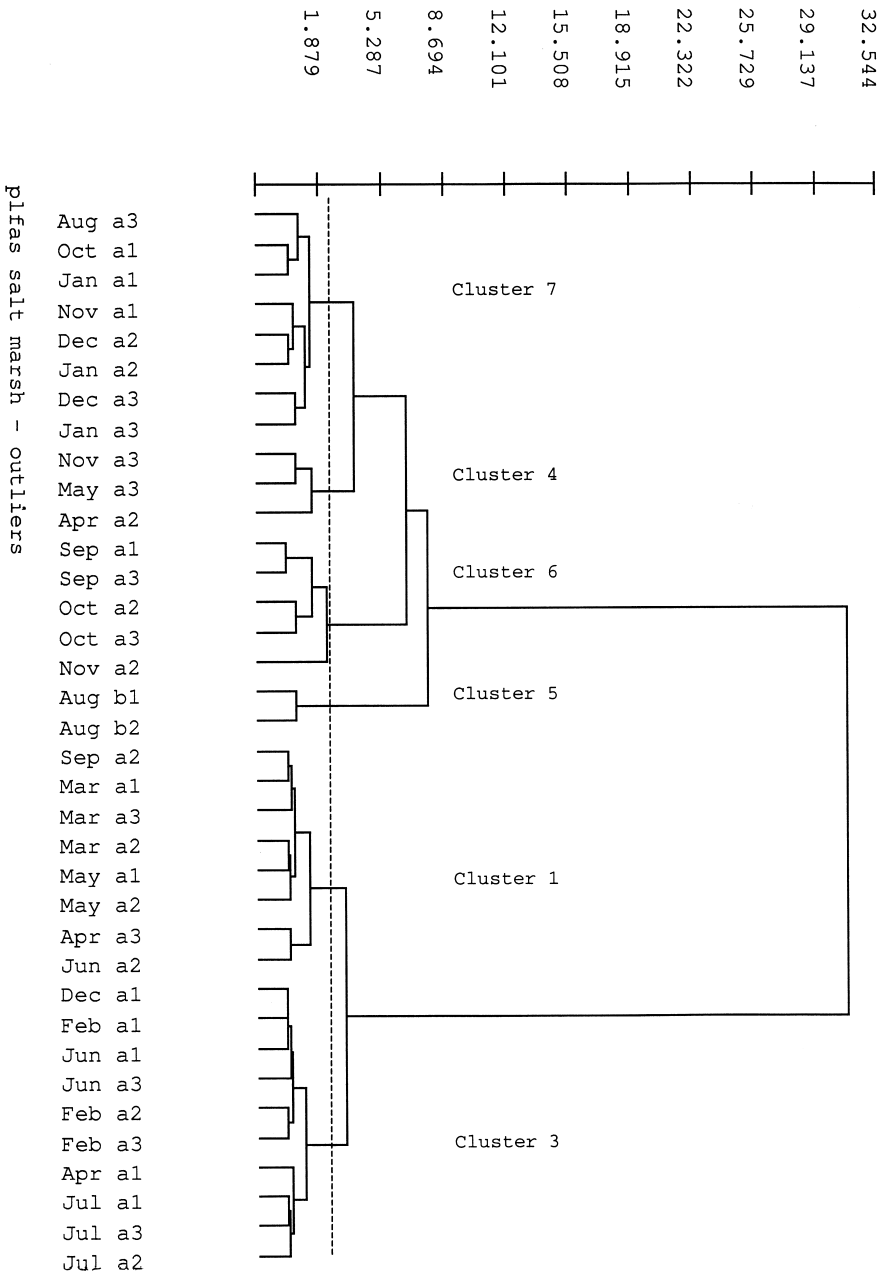


*Figure 2.* Dendrogram from the Ward's Analysis. Dendrogram mapping the hierarchical clustering of samples during the Ward's analysis of the abundance data.

abundance data. The result is useful though, in explaining the anomalous cluster 3 found in the analysis of the abundance data. Here, the June and July samples lie in

*Table 1.* The results from the reloc procedure performed on the percentage data

| Cluster 1 | Cluster 3 | Cluster 4 | Cluster 5 | Cluster 6 | Cluster 8 | Cluster 9 |
|---|---|---|---|---|---|---|
| Aug b1 | Sep a1 | May a3 | Aug a2 | Oct a1 | Jun a1 | Jul a1 |
| Aug b3 | Sep a3 | Jun a3 | May a1 | Oct a3 | Aug a3 | Jul a2 |
| | Nov a3 | | Feb a2 | Jan a2 | Oct a2 | Jul a3 |
| | Mar a1 | | Sep a2 | Dec a1 | Jun a2 | |
| | | | Feb a3 | Dec a2 | | |
| | | | Jan a1 | Jan a3 | | |
| | | | May a2 | Dec a3 | | |
| | | | | Nov a2 | | |
| | | | | Apr a3 | | |
| | | | | Nov a1 | | |

separate clusters from the December and February samples, with which they are grouped in the abundance data dendrogram. The community structure defined by this analysis is more reasonable, with the June and July samples grouping with other summer samples. This suggests another factor must be causing the abundance data results for June and July to fall in cluster 3. The PLFA concentrations in these samples are atypically low, possibly due to a poor extraction efficiency. The Mahalanobis distances, discussed later, show that it is indeed a concentration effect that places these samples in cluster 3, again supporting the suggestion that this results from a poor extraction.

The Mahalanobis analysis of the abundance data yielded very similar results to the Ward's/Reloc analysis. The results are shown in Table 2. The clusters were determined by the number of PLFA which have Mahalanobis distances (distance/standard deviation) of $< 1$ and $> 2$ from the mean of the cluster, and confirmed by the total Mahalanobis distances of each sample from the centre of each cluster, using both of the metrics discussed earlier. The clusters formed were robust, as the total distance metrics were in agreement with the result from the original Mahalanobis clustering, and largely agree with the results of the Ward's analysis and reloc procedure. The real benefit of the Mahalanobis analysis over the Ward's/reloc is that it provides an assessment of how well each sample fits into a cluster, and a number of samples can be identified as fitting poorly, such as some of the samples in cluster 3. The removal of these leads to a general decrease in the standard deviations of the mean PLFA values for cluster 3.

The six clusters generally appear to be quite reasonable, given that three summer samples lie in cluster 3 due to a poor extraction efficiency. Cluster 4 cannot be related to any particular time of year, but has only three members. Cluster 1 generally contains spring samples, cluster 3 mid-winter and samples of poor extraction efficiency, cluster 5 summer samples, cluster 6 late summer samples and cluster 7 winter samples.

The Mahalanobis analysis gives the differences between the clusters in terms of the PLFA components. Again, these differences are given as Mahalanobis distances,

*Table 2.* The results from the Mahalanobis calculations.

| Cluster 1 | Cluster 3 | Cluster 4 | Cluster 5 | Cluster 6 | Cluster 7 | True Outliers | Poor Fit |
|---|---|---|---|---|---|---|---|
| Sep a2 | Dec a1 | Nov a3 | Aug b1 | Sep a1 | Aug a3 | Aug a1 | Nov a2 |
| Mar a1 | Feb a1 | May a3 | Aug b2 | Sep a3 | Oct a1 | Aug a2 | Jan a3 |
| Mar a3 | Jun a1 | Apr a2 | | Oct a2 | Jan a1 | Aug b3 | Feb a2 |
| Mar a2 | Feb a3 | | | Oct a3 | Nov a1 | | Jun a2 |
| May a1 | Apr a1 | | | | Dec a2 | | Jun a3 |
| May a2 | Jul a1 | | | | Jan a2 | | Jul a2 |
| Apr a3 | Jul a3 | | | | Dec a3 | | |

and are calculated relative to the standard deviation of the particular PLFA concentrations for the reference group. First, a group is used as the reference point and the other groups are compared to it, and then each of the other groups is used as the reference. Since each cluster is compared with all other clusters, two Mahalanobis distances are calculated which depend on the identity of the reference group.

The overall Mahalanobis distances between the clusters show that the total PLFA has a major part in distinguishing the clusters, giving statistical dilution factors between clusters up to 5.3. The highest dilution factor is between clusters 6 (late summer) and 3 (mid-winter), and corresponds to cluster 6 containing an actual average of 5.9 times more PLFA than cluster 3. Cluster 1, which contains spring samples, has the second lowest mean amount of PLFA, suggesting that the community is quite slow to develop in the spring, and that it declines quite slowly with the onset of winter. The Mahalanobis distances between the mean positions of the individual PLFA give more detailed diagnostic features between the groups, and the main differences are outlined in Table 3.

Table 3 shows the driving forces behind the clustering, other than total PLFA, and therefore identifies differences between the community structures of the clusters. First, the fungal PLFA (18:2$\omega$6) distinguishes cluster 6 (late summer) from all of the others, suggesting that fungal biomass is notably higher at this time of year than any other. Secondly, PLFAs indicative of aerobic bacteria are particularly important in distinguishing the summer and late summer clusters. Thirdly, we can observe the importance of the anaerobic PLFA (16:0 br) in distinguishing between all of the clusters. In some cases this is alongside a general decrease in all PLFA, *e.g.* cluster 5 (summer) from 3 (mid-winter), but equally it is the main discriminant between other clusters, *e.g.* cluster 7 (winter) from 1 (spring).

The changes in the abundance of the different groups of PLFA biomarkers over the 'seasons', as defined by the Mahalanobis clustering and the PLFA biomarkers detailed earlier, are shown in Figure 3. Note that the total PLFA exceeds the sum of the different types of PLFA due to the inclusion of PLFA ubiquitous to all microorganisms. The community structure and the differences between the clusters are shown clearly in this figure. The community was always dominated by aerobic species, but there were also significant amounts of PLFA present throughout the year that are indicative of both fungi and anaerobic bacteria. Aerobic bacteria start mul-

*Table 3.* Discriminant features between the clusters found through the Mahalanobis calculations. The reference groups are in the 1st column. Group 4 has been omitted. (1 > 3) denotes that the concentrations in Cluster 1 are greater than Cluster 3. Note that, since the discriminating features depend on the standard deviation of the PLFA for the reference group, comparison of Cluster 3 with Cluster 1 may not give the same result as comparison of Cluster 1 with Cluster 3.

| | Cluster 1 (Spring) | Cluster 3 (Mid-Winter) | Cluster 5 (Summer) | Cluster 6 (Late Summer) | Cluster 7 (Winter) |
|---|---|---|---|---|---|
| Cluster 1 (Spring) | | 16:0br (1 > 3) | 19:1 (5 > 1) | 18:1 (6 > 1) all different | 16:0br (7 > 1) |
| Cluster 3 (Mid-Winter) | 18:0, 16:0br, 16:1 (1 > 3) | | 16:0br, 16:1, 18:0, 19:1 (5 > 3) | 16:1, 18:2 (6 > 3) | 16:1, 16:0, 16:0br (7 > 3) |
| Cluster 5 (Summer) | 16:0br (1 > 5) | 16:0br, 18:1 (5 > 3) | | 16:0br, 18:2, 18:1 (6 > 5) | 16:0br (7 > 5) 19:1 (5 > 7) |
| Cluster 6 (Late Summer) | 16:0br | 16:0br, 18:2 (6 > 3) | 19:1 (5 > 6) 16:0br, 18:2 (6 > 5) | | all change by a small but significant amount |
| Cluster 7 (Winter) | 16:0br, 16:0 (7 > 1) | 16:0br, 16:0 (7 > 3) | 19:1 (5 > 7) | Generally small changes | |

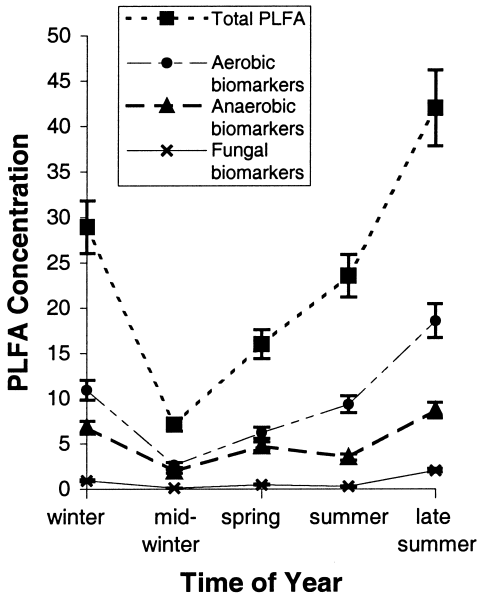


*Figure 3.* Seasonal Variations in the Concentrations of PLFA Biomarkers. The seasonal changes in the concentrations (nmol $g^{-1}$ dry weight sediment) of the different types of PLFA biomarker, where 16:0 branched PLFA are indicative of anaerobic bacteria, monounsaturated PLFA indicative of aerobic bacteria, and 18:2$\omega$6 PLFA indicative of fungi. The samples are clustered together as defined by the Mahalanobis analysis, and labelled with the 'season' representing the samples in that cluster. The PLFA concentrations are the mean concentrations of the samples in each cluster. Error bars are one standard deviation of the relevant PLFA concentrations.

tiplying earlier in the year than do anaerobes, as indicated by an increase in aerobic PLFA from spring onwards while the abundance of anaerobic biomarkers only starts to increase significantly from summer into late summer. Also, the amount of aerobic biomarker PLFA increased 7-fold between mid-winter and late summer, as opposed to 4.5 fold for the anaerobes over the same period. This results in the ratio of aerobic to anaerobic biomass varying between 1.3 in the mid-winter and 2.6 in the summer. The difference in this ratio may well be responsible for the 'winter' and 'summer' clusters forming during the analysis of the percentage data. Although the concentration of the fungal biomarker (18:2$\omega$6) is quite variable throughout the year, with a marked maximum in late summer, it is always low in comparison with the bacterial biomarkers. In this case, while the seasonal variations appear to be relatively large, the Mahalanobis analysis has shown that these differences are only of importance in distinguishing the late summer cluster from the others.

*Dissolved Fe and Mn concentrations*

The concentrations of dissolved Fe and Mn found in this study are illustrated in Figure 4. There was a rise in Mn porewater concentrations from June to September, the last data point collected. The Fe concentrations fluctuated slightly throughout the study, unrelated to the Mn concentrations, or any apparent seasonal cycle.

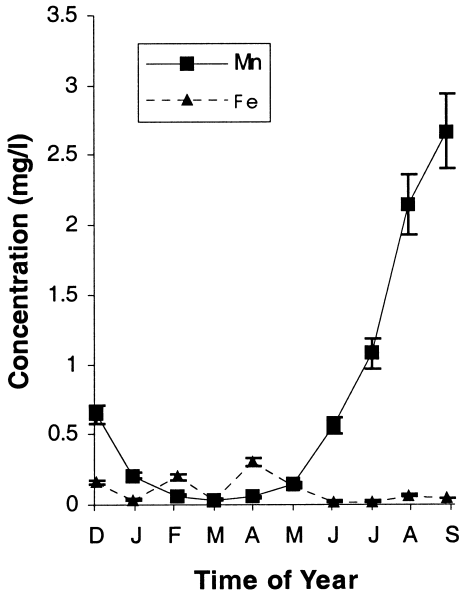


*Figure 4.* Seasonal Variations in the Concentrations of Dissolved Fe and Mn. Changes in the interstitial water dissolved Fe and Mn concentrations (mg $l^{-1}$) over time.

*Physical data*

Eh, pH and temperature measurements show that the marsh was always mildly oxic though the Eh was lower in May/June (range over measurement period = 145 – 284 mV), the pH was very stable (pH 6.3 – 6.8), and the only real variation over time at that depth was in temperature. The temperature was at its lowest in February and March of the first year, between 4 and 5 °C, then increased to a maximum of 17.5 °C in August. The temperature followed a similar pattern in the second year but was generally a little higher. Na concentration (an index of salinity) remained almost constant (8700 ± 800 mg $l^{-1}$), and the DOC concentration was below 10 mg $l^{-1}$ over the sampling time.

*Microbial response to temperature*

Figure 5 shows that there was a direct, but slightly lagged, relationship between total PLFA and temperature at 35 cm depth over the year. The correlation between temperature and total PLFA is best when the PLFA data are shifted backwards by 2 months from the central month of each cluster, giving $R^2 = 0.55$, $P = 0.15$. However, the PLFA indicative of aerobic bacteria were better correlated with temperature ($R^2 = 0.76$, $P = 0.05$ with a two month time-lag). PLFA indicative of anaerobic bacteria and fungi were not related to temperature ($R^2 = 0.18$ and 0.25 respectively).

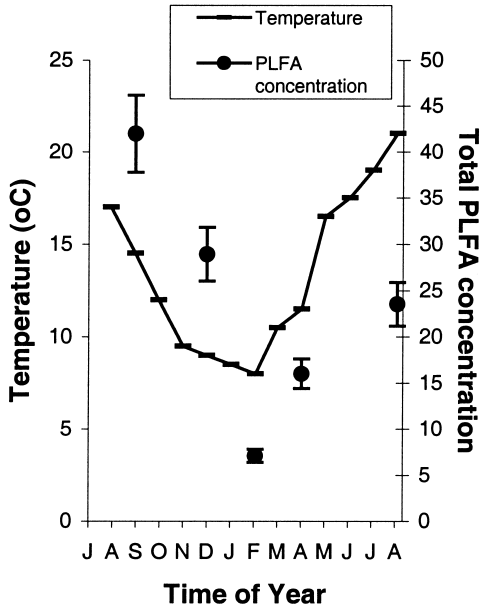


*Figure 5.* Seasonal Variations in Temperature and Total PLFA. Total PLFA (nmol $g^{-1}$ dry weight sediment) plotted using the mean PLFA value of the samples in each cluster against the central month of each cluster. Temperature is plotted for each month.

## Discussion

Although the Ward's/reloc and Mahalanobis analyses of the abundance data gave very similar clusters, the Mahalanobis analysis gave much more detailed information on the differences between the groups of like samples. Importantly, the differences can be quantified in terms of each of the 10 fatty acids analysed. Similar, but qualitative, information may be derived by plotting the mean PLFA values of each cluster, as in Figure 3, but quantification gives increased confidence in the conclusions. For example, the fungal variations throughout the year are proportionately large, but the Mahalanobis distances show that they are only diagnostic of the late summer cluster. Also, it is difficult to identify the features separating clusters 7 (winter) and 5 (summer) with confidence from Figure 3. These two clusters have total PLFA values within 1 of each other, and it is therefore extremely important to have quantitative information on the distinguishing characteristics. The Mahalanobis analysis showed that cluster 5 (summer) had a large amount of 19:1 PLFA, indicative of aerobic bacteria (Rajendran et al. 1992), whereas cluster 7 (winter) had a greater amount of 16:0 branched PLFA, indicative of anaerobic bacteria. This difference underlies the separation of summer and winter type clusters. Again, the Ward's analysis of the percentage data separated the data into a summer and winter type cluster, but without explanation. The Mahalanobis analysis allows quantifica-

tion of the important differences between clusters, reducing uncertainty in the interpretation.

The three "replicate" samples taken each month were generally not assigned to the same cluster in any of the analyses, suggesting that natural sediments are very heterogeneous and that the microbial community is affected by local sediment properties. High variability between samples taken at the same time is common (Rajendran and Nagatomo 1999; Findlay and Watling 1998). Nevertheless, at a coarser level of discrimination, the clusters appear to represent *times* of the year, showing that PLFA analysis is successfully mapping seasonal patterns in the microbial community structure in the salt marsh.

The five distinct clusters formed by the seasonal PLFA abundance data show that there were variations in both the size and structure of the microbial community over time, with the overall size, defined by total PLFA, changing the most markedly. PLFA indicative of aerobic bacteria, anaerobic bacteria and fungi were detectable throughout the year and the amounts and proportions of these markers varied systematically between seasons but no PLFA indicative of specific SRBs (*Desulfobacter* or *Desulfovibrio*) were detected, suggesting the anaerobes present were not SRB species. Aerobes were dominant at the marsh throughout the year, particularly in the summer months. The total PLFA in each sample was diagnostic of different clusters, with the ratio of one cluster to another ranging up to 5.9, but the PLFA profile also played an important part in distinguishing clusters, with different types of species multiplying and declining at different times of the year. However, since there was no great interchange between dominant groups of microbes and no appearance of previously absent groups, such as SRB, the microbial community structure was apparently relatively steady throughout the year.

The prevailing conditions at the site were reasonably constant, with the main variation being in temperature. Mildly oxic conditions persisted throughout the year (Eh = 145 – 284 mV) and the pH varied little (pH 6.3 – 6.8). The general dominance of the aerobic bacteria and the lack of specific SRB indicator PLFA are consistent with these Eh/pH conditions. However, the detection of PLFA characteristic of anaerobic species demonstrates either that conditions, at least in microhabitats, were anaerobic or that facultative anaerobes were present.

Our results are consistent with those of Pye et al. (1997), who found that aerobic bacteria dominate the microbial communities of a salt marsh in Warham, UK to a depth of $\sim$75–80 cm. Their results from the lower reaches of the salt marsh are particularly in agreement with ours, with branched saturates being the second most abundant PLFA. Although PLFA indicative of SRB were observed at their site, they only made up a relatively small percentage of total PLFA. The microbial biomass decreased with depth, with an order of magnitude difference in PLFA concentrations between the surficial sediments and 30–35 cm depth, where there was $\sim$22 nmol of PLFA per gram dry weight sediment. They sampled in the spring, and their result compares well with our spring cluster which had a mean total PLFA concentration of 16 nmol $g^{-1}$ dry weight sediment.

Rajendran and Nagatomo (1999) reported a 6.5-fold increase in total PLFA concentrations between samples collected in spring (13.8 °C) and autumn (22.4 °C)

from Hiroshima Bay. As in our study, the majority of the PLFA detected were the branched and the monounsaturated PLFA, but they saw a switch in the dominant PLFA type from monounsaturated (aerobes) in spring to branched (anaerobes) in autumn, coinciding with an Eh change from 183 mV to 62 mV. The concentrations of PLFA in the Hiroshima Bay sediments (Rajendran and Nagatomo 1999) were about 30 times higher per gram dry-weight sediment than observed in our study. This is consistent with surficial sediments having larger microbial populations than sediments at lower depths, as noted earlier. However, the change over time we observed in total PLFA, by a factor of 5.9, is comparable to that seen by Rajendran and Nagatomo (1999), and is almost twice that seen by Findlay and Watling (1998) in marine sediments. This shows that, despite the microbial biomass being smaller at a 30–35 cm depth, it is still dynamic, changing in a similar fashion to the communities of other sediment systems.

The microbial biomass at our site responded to the warmer conditions in the spring and summer, and declined in the colder winter temperatures. The best correlation found was between temperature and aerobic biomass with a 2 month timelag, suggesting that aerobic bacteria are best suited to the field conditions, but take some time to respond to the change. The amount of aerobic biomarker PLFA increased 7-fold between mid-winter and late summer, as opposed to 4.5-fold for PLFA synthesised by anaerobes. This is consistent with their being better suited to the field conditions at this site, and so having a more marked response as the conditions become more conducive to microbial growth.

The main increase in anaerobic biomass occurred after the aerobes had already begun to bloom, and so although the conditions were still generally more favourable for the aerobes, there were perhaps improved conditions for the anaerobes in microhabitats due to oxygen depletion as the aerobic biomass increases. The anaerobic biomass also declined more slowly in winter than did the aerobic biomass, making the ratio of aerobes to anaerobes closest to unity in the winter months. Fungal biomass, measured as the abundance of 18:2$\omega$6, was at a maximum in the late summer months, perhaps due to an increased input of plant detrital material towards the end of the growing season. This finding is consistent with studies of soil microbial communities which often reveal late summer peaks in fungal growth (Bardgett et al. 1993; Doetsch and Cook 1973).

The geochemical conditions were relatively constant throughout the year, with the exceptions of temperature and dissolved Mn concentrations. The regular upflush of oxic sea-water (Carr and Blackley 1986) through the sediment each tidal cycle, replacing the oxygen supplies, will help to counteract the microbial removal of oxygen. This may explain both why the conditions stayed oxic and the community was dominated by aerobes most significantly in the summer, rather than switching to more anaerobic conditions, as seen by Rajendran and Nagatomo (1999). Generally, the most marked microbially-driven geochemical changes result from changes in redox conditions, metabolic use of terminal electron acceptors other than oxygen, and the resultant dissolution or formation of chemical substances. The lack of a ‘redox-switch’ here, and the stability of the geochemical parameters, are consistent with the microbial groups observed.

The increase in the amount of anaerobic biomarker PLFA between summer and late summer suggests that these microorganisms did multiply rapidly over this time. This increase in growth of anaerobes corresponds with an increase in dissolved Mn concentrations through the summer, with the highest concentrations occurring in September. The increasing Mn concentrations do not coincide with unusually low Eh measurements for this salt marsh, or an increase in Fe concentrations. However, Kerner (1993) has observed that microbial Mn(IV) reduction can occur in oxic sediments, while Fe(III) reduction requires sub-oxic conditions. The increase in growth of anaerobic microorganisms at the salt marsh is therefore consistent with the use of Mn(IV) as a terminal electron acceptor. The lack of significant Fe dissolution, with Fe concentrations fluctuating almost continually, but remaining low, is also consistent with this finding. These data demonstrate how dissolved Fe/Mn concentrations can be a sensitive measure of changing biogeochemical conditions. This is particularly useful when changes in Eh/pH are difficult to quantify beyond the normal measured variation. Additionally, it is possible to use nitrate and sulfide concentrations to examine the terminal electron accepting processes (nitrate- and sulfate-reduction) occurring before the onset of Mn-reduction and after Fe-reduction, respectively.

This study set out to test the hypothesis that the PLFA technique could be used to examine temporal patterns of microbially-driven geochemical change in a salt marsh in north-west England. We have shown that: (1) the Mahalanobis analysis of the data largely agreed with a standard clustering technique, demonstrating the robustness of both approaches; (2) the Mahalanobis analysis provides detailed, quantitative information on the differences between clusters, aiding interpretation of the results; (3) the microbial community of the salt marsh at a 30–35 cm depth shows marked seasonal variation in both its size and structure, and; (4) that seasonal changes in microbial community structure can be related to the limited geochemical change.

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