

Analysis of the structural diversity of the microbial community in a paper-mill water system

Thea van der Merwe^{1*}, Karl-Heinz Riedel² and Francois Wolfaardt^{1,3}

¹Department of Microbiology and Biochemistry, University of the Free State, Bloemfontein 9300, South Africa

²School for Environmental Sciences and Development: Microbiology, Potchefstroom University for Christian Higher Education, Potchefstroom 2520, South Africa

³SAPPI Forest Products, Technology Centre, PO Box 3252, Springs 1560, South Africa

Abstract

Microbial populations in paper-mill water systems are usually enumerated using microbiological techniques such as plate counts and the most probable number technique. These conventional methods can only quantify a limited percentage of the microbial populations and the microbial numbers are, therefore, generally underestimated. One possible alternative to these methods involves the analysis of signature lipid biomarkers to study the structural diversity of microbial populations. The aim of this study was, therefore, to evaluate the applicability of signature lipid biomarker analysis in a paper-mill water system. Samples from the sessile and planktonic phases were collected over a period of one year at the Sappi Cape Kraft paper-mill. The samples were subjected to analysis of signature lipid biomarkers as well as conventional culturing. Analysis of the phospholipid fatty acids revealed the presence of a large diversity of micro-organisms. The same trends in the number of cultured cells and the counts obtained with signature lipid biomarker analysis were observed, although the numbers obtained with signature lipid biomarker analysis were at least 1 000 times higher. Profiles of signature lipid biomarkers reflected changes in production and water management practices, which could not be detected with culturing techniques. The analysis of signature lipid biomarkers provided more information for the characterisation of microbial communities in paper-mill water systems and has potential for application in other industrial water systems.

Introduction

Many problems are experienced with conventional microbiological techniques such as the standard plate count procedure, and studies have indicated that only 0.01% to 1% of all microbes are culturable on artificial media (Vestal and White, 1989; Palojarvi et al., 1997). Culturing methods are selective due to the media used and the interactions that exist between micro-organisms (Kerster et al., 1997). Furthermore, most sampling techniques applied in industrial and environmental microbiology result in microbial stress. The micro-organisms may subsequently become dehydrated and damaged, which result in their non-culturability (Macnaughton et al., 1997). In order to overcome the difficulties of cultivation, alternative assays have been developed. These assays include the application of molecular techniques, the evaluation of substrate utilisation profiles (Biolog) and the evaluation of signature lipid biomarkers (SLBs) (White and Macnaughton, 1997).

A major disadvantage of the application of substrate utilisation profiles and molecular techniques is the non-quantitative recovery of microorganisms from the environment. White and Macnaughton (1997) stressed that although several methods for DNA extraction have been developed, there is no guarantee that all the DNA is extracted. Most of the molecular techniques are also very time-consuming and complicated to perform. The Biolog assay is a relatively simple and rapid technique compared to other community level approaches such as DNA analysis, but does not provide as much information as SLB analysis (Buyer and Drinkwater, 1997).

The analysis of SLBs provide a method that is quantitative, independent of cell culturability and allows the identification of micro-organisms that have distinctive phospholipid fatty acid (PLFA) profiles (Macnaughton et al., 1997). Petersen and Klug (1994) proposed that phospholipids could be a fingerprint of the microbial community and could, therefore, provide a means to determine overall changes in the composition of the microbial community (Frostegard et al., 1997). It has also been shown that specific patterns of PLFAs are indicative of physiological stress, nutritional status as well as the viable biomass of the microbial population, none of which are reflected by any of the alternative techniques (Mandelbaum et al., 1997; Steward et al., 1996).

Valuable information can, therefore, be obtained using the SLB technique, which could assist in the development of successful microbial control programmes in paper mills. Microbial control is very important in the paper industry, since biofilm formation can lead to breakages, spotting, holes and discolouration of the paper, resulting in a loss of production and product quality (Robertson, 1993). Signature lipid biomarker analysis was, therefore, used to study the microbial populations in the water system of a paper-mill.

The Sappi Cape Kraft paper-mill, Cape Town, South Africa produces both fluting and linerboard from recycled fibre. Fluting is produced from pulp at pH 6.5 to pH 7.5 while linerboard is produced at pH 4.5 to pH 5.5. Fluting is produced without additives, while additives are included during the production of linerboard to improve the printing quality and to make storage of boxes under conditions of high humidity possible. A large percentage of the water at the mill is reused and the mill shuts down monthly for routine maintenance operations. The influence of these operations on microbial populations was evaluated using SLB profiles. To our knowledge no similar study has been conducted previously in paper mill water systems.

* To whom all correspondence should be addressed.

☎ 051 401- 3332; fax 051 444- 3219; e-mail: greyliid@sci.uovs.ac.za

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Materials and methods

Sampling

Duplicate water and biofilm samples were collected every two to three weeks from the paper machine over a period of one year. The samples for the SLB analysis were immediately frozen in liquid nitrogen and transported on dry ice while the samples for the plate counts were cooled to 4°C for transportation. Samples were processed within 24 h of sampling.

Lipid extraction and fractionation

The planktonic samples (approximately 10 ml) were filtered using Whatman 4 filter paper (20 µm) and the biomass was freeze-dried. Biomass from the sessile samples (approximately 10 g) was freeze dried without any further treatment. Total lipids were extracted overnight from lyophilised samples using a mixture of chloroform and methanol (2:1 v/v). The total lipids were fractionated on activated silicic acid columns using 1,1,1-trichloroethane (150 ml) for neutral lipids, acetone (100 ml) for glycolipids, and methanol (100 ml) for phospholipids (Kock and Ratledge, 1993). The phospholipid fraction was dissolved in chloroform (200 µl), methylated by the addition of 200 µl trimethyl sulphonium hydroxide (TMSH) (Jeffrey et al., 1997).

Signature lipid biomarker analysis

The lipid fractions were analysed using a Hewlett Packard 5890 series II gas chromatograph (GC) with a Supelcowax 10 column (30 m x 0.75 mm ID). The injection temperature was 180°C and the flame ionisation detector temperature was 300°C. The initial oven temperature was 145°C, which remained constant for 6 min before being increased by 3°C·min⁻¹ to a maximum of 245°C. Nitrogen was used as a carrier gas at a flow rate of 5 ml·min⁻¹. The identity of different compounds was verified by using a Hewlett Packard 5890 Series II GC, coupled to a Hewlett Packard 5972 Mass Selective (MS) Detector. A SPB 5 fused silica capillary column (60 m x 0.25 mm ID) with an inlet temperature of 180°C was used to obtain separation. The initial oven temperature was 130°C, which increased at a rate of 6°C·min⁻¹ to a maximum of 300°C. Helium was used as a carrier gas at a flow rate 1 ml·min⁻¹. Dodecanoic acid (C12:0 fatty acid) was used as internal standard.

The cell counts for PLFA analysis were determined by using a conversion factor of 2.5 x 10⁴ cells per pmol of PLFA. The conversion factor was derived from the analysis of rapidly growing cells and assuming 4.0 x 10⁻¹³ g of dry mass per bacterial cell and 1.0 x 10⁻⁴ mol PLFA per gram of dry cells (Peacock, 2000).

Statistical analysis

The SLB profiles were analysed using the NCSS 97 software (Statistical Solutions, Ireland). Stepwise variable selection was employed during discriminant analysis and differences between groups of fatty acids (terminally branched saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids) were considered to be significant at the 99.5 % confidence level.

Conventional counts

A serial dilution series (10⁻² to 10⁻⁸) of water or biofilm was prepared for every sample using distilled water. Viable cell counts were obtained using the pour plate technique with nutrient agar

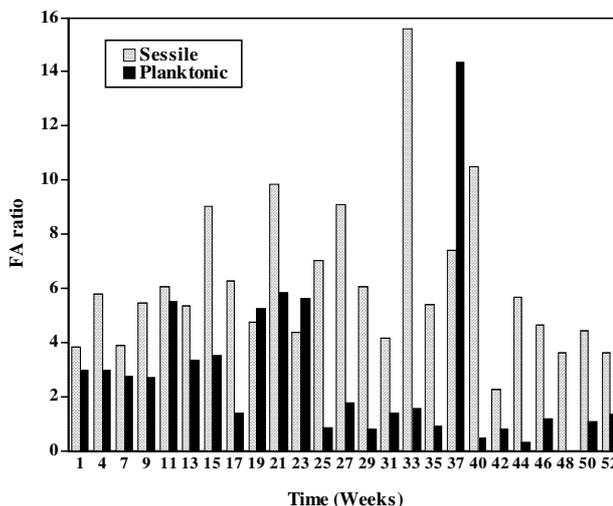


Figure 1

Ratio of saturated to unsaturated C18 fatty acids (FA) in sessile and planktonic phase collected over one year

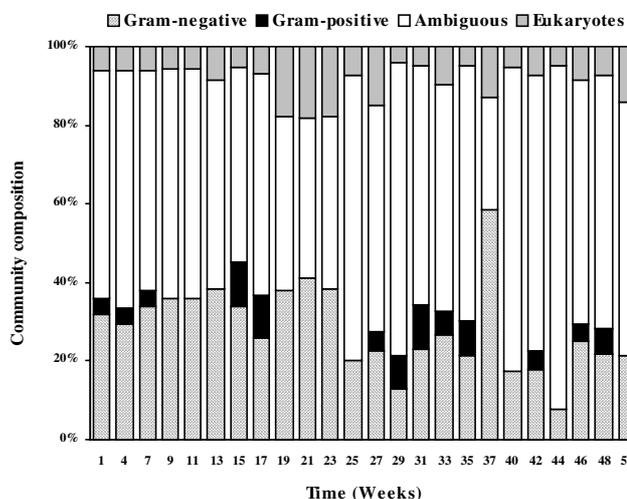


Figure 2

Microbial community composition in the planktonic phase based on different groups of fatty acids

(Walter, 1967). The plates were incubated at 30°C for 5 d and the colonies enumerated.

Results and discussion

White et al. (1996) reported that poly-unsaturated PLFAs are found almost exclusively in eukaryotes and Frostegard and Baath (1996) proposed that the C18:2 fatty acid should be used as a fungal biomarker. The number of fungi present (as reflected by the C18:2 fatty acid) was more abundant in both water and biofilm samples when linerboard was produced than when fluting was produced. The number of fungi in the sessile phase increased from 2.27 x 10⁶ cells·mL⁻¹ when fluting was produced to 2.47 x 10⁶ cells·mL⁻¹ when linerboard was made. The number of fungi in the planktonic phase increased from 1.96 x 10⁶ cells·mL⁻¹ when fluting was made to 4.80 x 10⁶ cells·mL⁻¹ when linerboard was produced. These results were expected, since fungi grow optimally at a pH of 2.0 to 6.0 (Hughes, 1993), which was the pH range of the water when linerboard was produced.

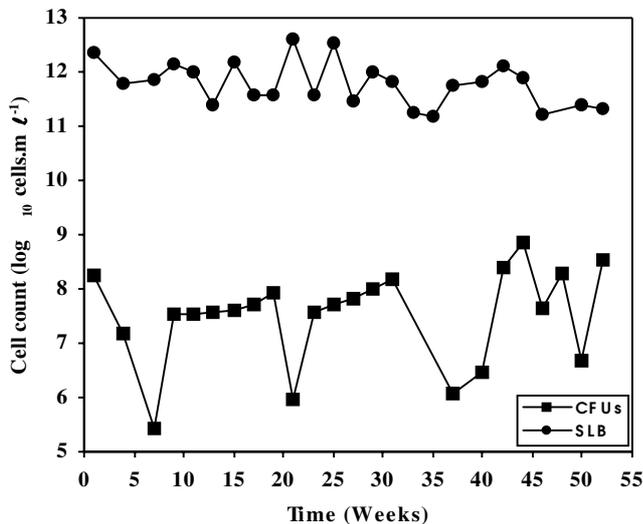


Figure 3

Comparison of numbers of cells obtained with signature lipid biomarker analysis (SLB) and plate counts (CFUs) for the sessile samples as obtained over a period of one year

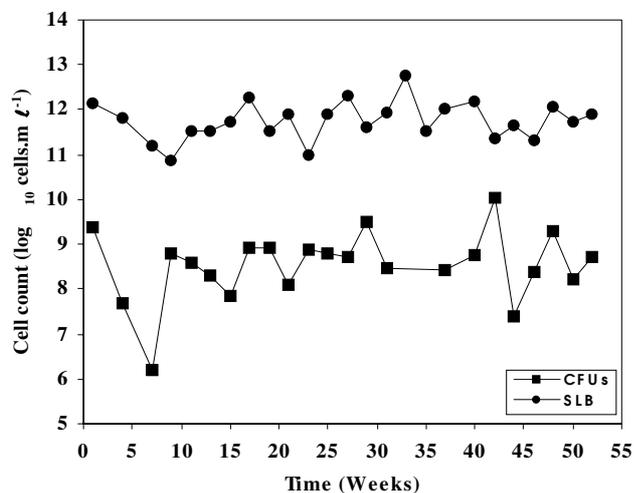


Figure 4

Comparison of numbers of cells obtained with signature lipid biomarker analysis (SLB) and plate counts (CFUs) for the planktonic samples as obtained over a period of one year

Terminally branched saturated fatty acids are generally considered to indicate the presence of Gram-positive bacteria (Zelles, 1999). The concentration of terminally branched saturated fatty acids indicated that the distribution of Gram-positive organisms varied substantially between the sessile and planktonic phases. The number of Gram-positive bacteria in the sessile phase increased from 6.34×10^6 cells·m⁻¹ when fluting was produced to 8.51×10^6 cells·m⁻¹ when linerboard was made. The number of Gram-positive bacteria in the planktonic phase increased from 1.09×10^6 cells·m⁻¹ when linerboard was produced to 1.63×10^6 cells·m⁻¹ when fluting was made. These results could have significant implications for the biocide programmes at the paper-mill and should, for example, be changed during the production of different board grades.

Mono-unsaturated fatty acids (e.g. C16:1, C17:1 fatty acids) have been used as indicators of Gram-negative bacteria (Ratledge

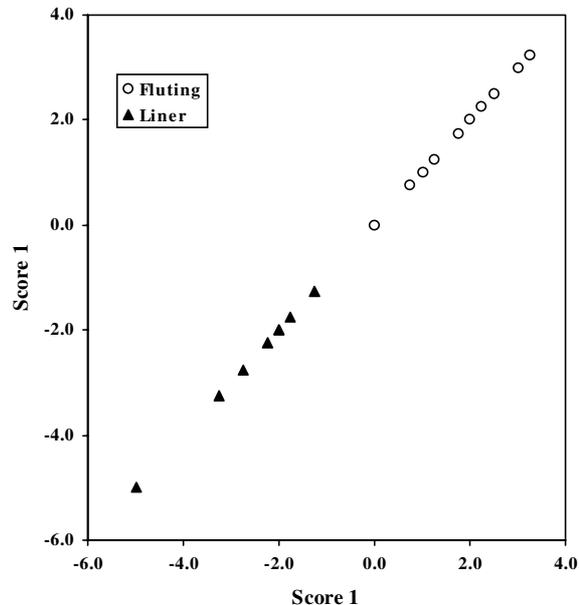


Figure 5

Ordination plot of the canonical-variate scores based on the combined effect of the different groups of fatty acids for the planktonic microbial community after 1 d of production of the specific paper grades

and Wilkinson, 1988). The concentration of mono-unsaturated fatty acids in the sessile phase increased from 9.16×10^6 cells·m⁻¹ when fluting was produced to 1.52×10^7 cells·m⁻¹ when linerboard was made, while an increase from 7.05×10^6 cells·m⁻¹ to 1.53×10^7 cells·m⁻¹ was obtained in the planktonic phase with a switch from fluting to linerboard. The Gram-negative bacteria were, therefore, more abundant in both the sessile and planktonic communities when linerboard was produced.

The profiles of 22 out of 25 water samples contained a larger ratio of C18 fatty acids than the associated biofilm samples (Fig. 1). Unsaturated fatty acids increase the elasticity of membranes (Madigan et al., 1997) and it could possibly increase the resistance of the planktonic community to shear stress leading to better survival. These results are similar to those reported by Valeur et al. (1988) who reported that planktonic bacteria had a lower ratio of saturated to unsaturated C18 fatty acids.

Analysis of the SLBs present in the planktonic (Fig. 2) and sessile phases revealed the presence of a large diversity of micro-organisms within the water system. During certain periods, the planktonic microbial community composition in the water samples changed notably (Fig. 2). During these periods (9th to 13th, 19th to 25th and 37th to the 40th week), Gram-positive bacteria, as reflected by terminally branched saturated fatty acids, were absent. The relative abundance of the eukaryotic community (reflected by poly-unsaturated fatty acids) was higher during these periods. We could not attribute these changes to a single operational parameter, but it could possibly be ascribed to continuous changes in the process parameters (paper grades, biocide applications and shutdowns).

The same trends in the number of cultured cells and the numbers calculated from PLFAs were observed for both the sessile and planktonic samples (Figs. 3 and 4). Cell counts, based on PLFA analysis, were more consistent (standard deviation = 0.78) in the sessile phase than in the planktonic phase (standard deviation = 0.87). However, the cell counts obtained with PLFA analysis were

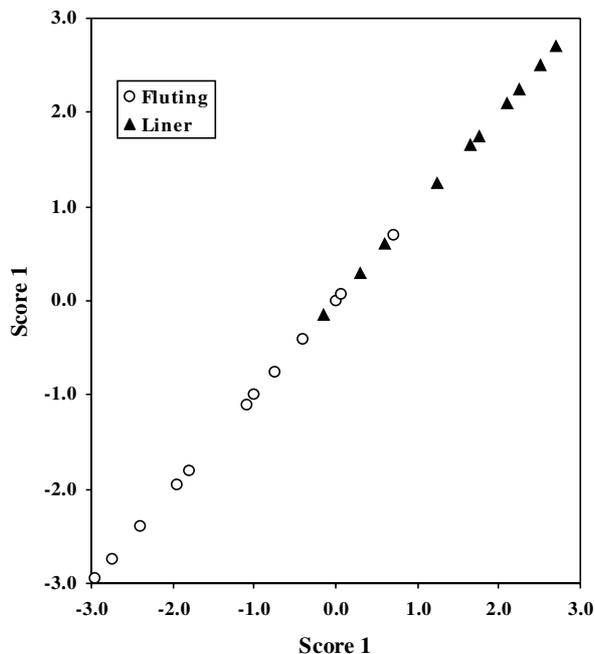


Figure 6
Ordination plot of the canonical variate scores based on the combined effect of the different groups of fatty acids for the sessile microbial community after 1 d of production of the specific paper grades

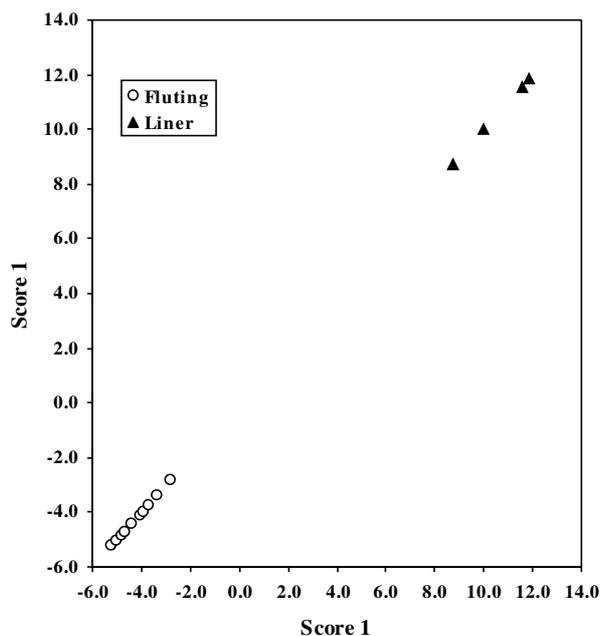


Figure 7
Ordination plot of the canonical variate scores based on the combined effect of the different groups of fatty acids for the sessile microbial community after 5 d of production of the specific paper grades

much higher (10^3 times higher) than the counts obtained with the conventional culturing technique. We regard these results as confirmation that less than 1% of all microbes are culturable on artificial media (Vestal and White, 1989; Palojarvi et al., 1997). Phospholipid fatty acid analysis, therefore, made it possible to

monitor a wider range of micro-organisms in the paper-mill water system than were possible with conventional methods.

The relative abundance of fatty acids differed when fluting and linerboard were produced. The diversity of the planktonic microbial communities differed significantly ($p \leq 0.005$) on the day of a change in production grade (Fig. 5) and might be ascribed to the microbial adaptation to the change in chemical composition of the water. Frostegard et al. (1993) also found that environmental parameters had a significant influence on the PLFA composition of the cell membranes. Factors such as temperature, pH, nitrogen source and salinity may also bring about a variation in the fatty acid profiles (Dowling et al., 1986). The diversity of the sessile microbial communities did not change as rapidly as the planktonic communities (Fig. 6). Signature lipid biomarker analysis indicated that two significantly ($p \leq 0.005$) different microbial communities developed in the sessile samples only after 5 d of production of a new board grade (Fig. 7). The rapid change in the fatty acid profiles of the planktonic community reflects the ability of this community to adapt faster than the sessile community, because it is more exposed to the environment.

Conclusions

Similar trends were observed in the cell counts obtained using conventional culturing techniques and PLFA analysis, although the values obtained with PLFA analysis were significantly higher. The higher cell counts obtained by PLFA analysis stressed the under-estimation of microbial numbers using conventional culturing methods.

Different fatty acid profiles indicated that the production of the different paper grades selected for different microbial communities in both the sessile and planktonic phases. The abundance of terminally branched saturated fatty acids, mono-unsaturated fatty acids and poly-unsaturated fatty acids varied with the change in paper-production parameters. The signature lipid biomarker approach was, therefore, effective in detecting shifts within the microbial community at the paper-mill as result of changes in operations. No significant differences could be detected as a result of a difference in biocide programmes or during routine shuts for maintenance and cleaning, but substrate utilisation profiles could differentiate between communities based on these parameters (Greyling, 2000).

Information on the different microbial communities in the water system and the interactions between the different guilds of micro-organisms will also assist in the selection of suitable biocides. A substantial amount of information concerning the structure of the microbial community could be obtained using the analysis of SLBs. Although this technique could be considered as too expensive for routine application, it could aid in the management of water systems, especially in systems with diverse microbial communities.

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