01

ANALYSIS OF THE MICROBIAL COMMUNITY STRUCTURE AND FUNCTION IN A PAPER MILL WATER SYSTEM

by

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To my mother

"It is a mistake to believe that research is done in the laboratory. It is done in the head ~ the laboratory merely confirms or rejects what the mind conceives."

Sydney Harris

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PREFACE

More than three million tons of paper and board are produced annually in South Africa (Chabane, 2000). In 1999, corrugating materials contributed 913 000 tons of the total paper and board that were produced. The corrugating materials consisted of linerboard (695 000 tons) and fluting (218 000 tons). The export market consisted of 287 000 tons of linerboard and 31 000 tons of fluting (Chabane, 2000). It is, therefore, clear that the board industry plays a significant role in the economy of South Africa.

Predictions are that the demand for recycled fibre in the 1990s will grow six times as fast as the total paper demand (Anonymous, 1992). It is estimated that the demand for paper and board will increase at a rate of 2,1 % per year and the use of recycled fibre is expected to increase in all paper and board end products. One of the largest consumers of secondary fibre are mills that produce corrugated materials (Vendries & Pfromm, 1998).

Mills that use secondary fibre are very susceptible to microbial contamination of the water system, since the recycled fibre serves as a continuous inoculum for both bacterial and fungal contamination into the water system (Sorelle & Belgard, 1991).

Over the last few years, paper mills worldwide have made great efforts to reduce water pollution (Vendries & Pfromm, 1998). Due to the shortage of water in South Africa (O'Keeffe *et al.*, 1998), mills use less water than their counterparts in Europe and North America. Effluents are, therefore, treated or the water system is closed and the white water reused. The closure of water systems results in an increase in the dissolved substances and a lower dissolved oxygen content (Gudlauski, 1996). The temperatures in the water system are also frequently elevated upon closure of the water system (Vaisanen *et al.*, 1994). The recycling of water, therefore, also contributes substantially to the growth of microorganisms in the system (Vaatanen & Niemela, 1983).

An example of a mill that makes use of recycled fibre is the Sappi Cape Kraft paper mill in Milnerton, Cape Town, South Africa. The mill produces both fluting and linerboard from different sources of recycled fibre. Cape Kraft produces approximately 56 000 tons of corrugated board per annum. The corrugated board consists of 14 000 tons of linerboard and 42 000 tons of fluting. The mill uses 59 000 tons of recycled fibre during the production of the various paper grades. The mill has a relatively closed water system with low effluent discharge. Microbial contamination and fouling is consequently substantially enhanced due to the recycling of water and the use of recycled fibre.

The technical planning of the evaluation of the monitoring approaches included the following:

- **Analysis of the functional diversity of the microbial community in the water system.**

  During this study it was decided to evaluate the potential of substrate utilisation profiles as an alternative monitoring technique of microbial contamination in the mill (Chapter 2). This approach was used by Victorio *et al.* (1996) to characterize microbial communities in wastewater treatment systems and would, therefore, possibly be suitable for use in a paper mill water system. The carbon source utilisation approach yields a more sensitive and ecological relevant measure of heterotrophic community structure than conventional microbiological analysis (Schneider *et al.*, 1998).

- **Analysis of the structural diversity of the microbial community in the water system.**

  Recent studies (Palojarvi *et al.*, 1997; Vestal & White, 1989) have indicated that less than 1 % of all microbes can be cultured on artificial media. Since microbial numbers in industry are generally determined using conventional culturing methods, the potential of signature lipid biomarker analysis was also evaluated during this study. This method is independent of culturing and allows the analysis of the whole microbial community. The evaluation of the signature lipid biomarker approach is discussed in Chapter 3.

- **Microbiological audits of the Cape Kraft paper mill water system.**
  Based on the positive results that were obtained during the evaluation of the alternative monitoring techniques, it was decided to apply the substrate utilisation profile analysis as well as signature lipid biomarker analysis in the paper mill water system. Two microbial audits were performed at the mill in collaboration with South African Paper Chemicals. The results obtained during the audits are discussed in Chapter 4. The first audit was performed to assess the current biocide programme at the mill and the second audit was performed to evaluate the effect of the changes made to the microbial control programme.

Appendices have been included to provide the raw data where this data did not make an essential contribution to the chapters, but the relevant data from the appendices have been included in condensed form in the chapters.

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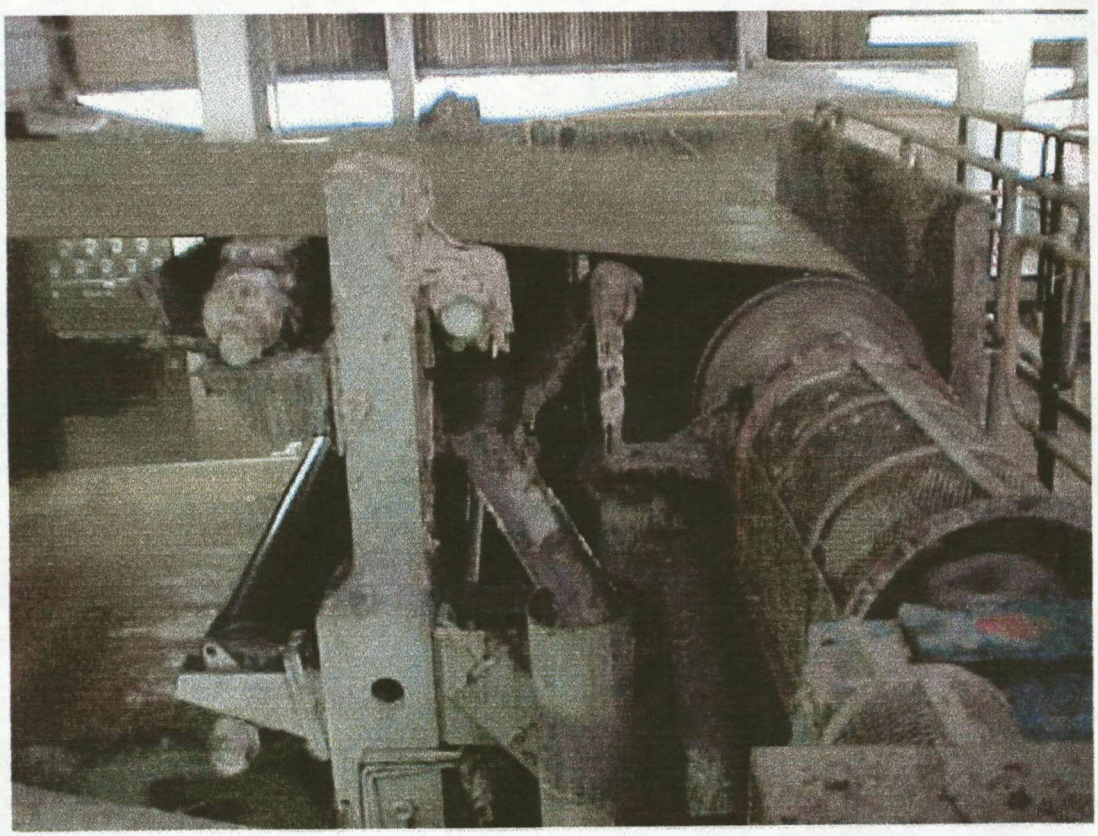
CHAPTER 1

EVALUATION AND CONTROL OF MICROBIAL CONTAMINATION IN PAPER MILL WATER SYSTEMS: A LITERATURE REVIEW



ABSTRACT

Microbial growth can result in the production of biofilm, which contribute substantially to microbial induced corrosion. Problems associated with microbial contamination subsequently result in substantial economic losses in the paper industry. Microbial growth is generally controlled by the addition of biocides that are specific for certain organisms or environmental conditions. Biocides can be divided into three major groups: oxidising biocides, non-oxidising biocides and biodispersants. The biocides are added to the process water and the efficacy of the biocide applications are monitored using various conventional culturing techniques as well as indirect methods. Previous research has, however, indicated that less than 1 % of microorganisms can be cultured on artificial media. Furthermore, culturing methods do not provide accurate information on the number of microorganisms or the groups of microorganisms present. An alternative approach used to monitor microbial numbers is the measurement of adenosine triphosphate (ATP). However, the concentration of ATP in microorganisms is not always constant. The most probable number method is based on estimations and is, therefore, not very accurate. Furthermore, with biochemical and microscopical methods it is also difficult to determine the viability of the community being examined. Due to the limitations of conventional culturing, numerous 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. These assays are independent of cell culturability and consequently circumvent many of the problems typically associated with conventional microbiological techniques. The aim of this review was to evaluate different monitoring techniques for application in the pulp and paper industry, with emphasis on the evaluation of substrate utilisation profiles as well as signature lipid biomarkers.

INTRODUCTION

The microbiologically associated problems that frequently occur in paper mills depend primarily on the degree of closure of the water system. Upon closure of the water system, the temperatures are frequently elevated and the nutrient concentrations generally increase (Gudlauski, 1996). As a result of the increased recycling of process water, nutrient salts and degradable carbon content increase, further contributing to the microbiologically associated problems in waste water systems (Vaisanen *et al.*, 1994). These factors generally increase the degree of biofilm formation and subsequent microbial induced corrosion (Bennett, 1985). Problems in the papermaking industry are frequently associated with the production of biofilms. When biofilms break loose, the deposits may result in paper breakages, spotting, holes and discolouration of the paper resulting in a loss of production and product quality (Martin, 1988; Robertson, 1993; Robertson, 1994; Robertson & Taylor, 1993; Stoner & King, 1994). Biofilms can also contribute to the production of odours in the produced paper, primarily due to the production of volatile fatty acids (Stoner & King, 1994; Vaisanen *et al.*, 1994). Microbial biofilms also play a significant role in microbiologically induced corrosion. These problems lead to poor runnability and lower production rates of the plant that have severe economic implications for a paper mill (Sorelle & Belgard, 1991).

BIOFILMS

In all water systems, microorganisms can be divided into two primary classes: the planktonic (free floating) and the sessile (attached) microorganisms (Costerton *et al.*, 1987; Robertson, 1993; Stoner & King, 1994). Costerton *et al.* (1987) and Johnsrud (1997) stated that the cell concentration in biofilms is generally three to four orders of magnitude higher than that within the planktonic phase. Biofilms are formed when planktonic microorganisms attach to a surface (Martin, 1988). When suitable environmental and growth conditions prevail, a biofilm will form as a result of the continuous adsorption and growth of the attached cells (Mueller, 1994) (Figure 1).

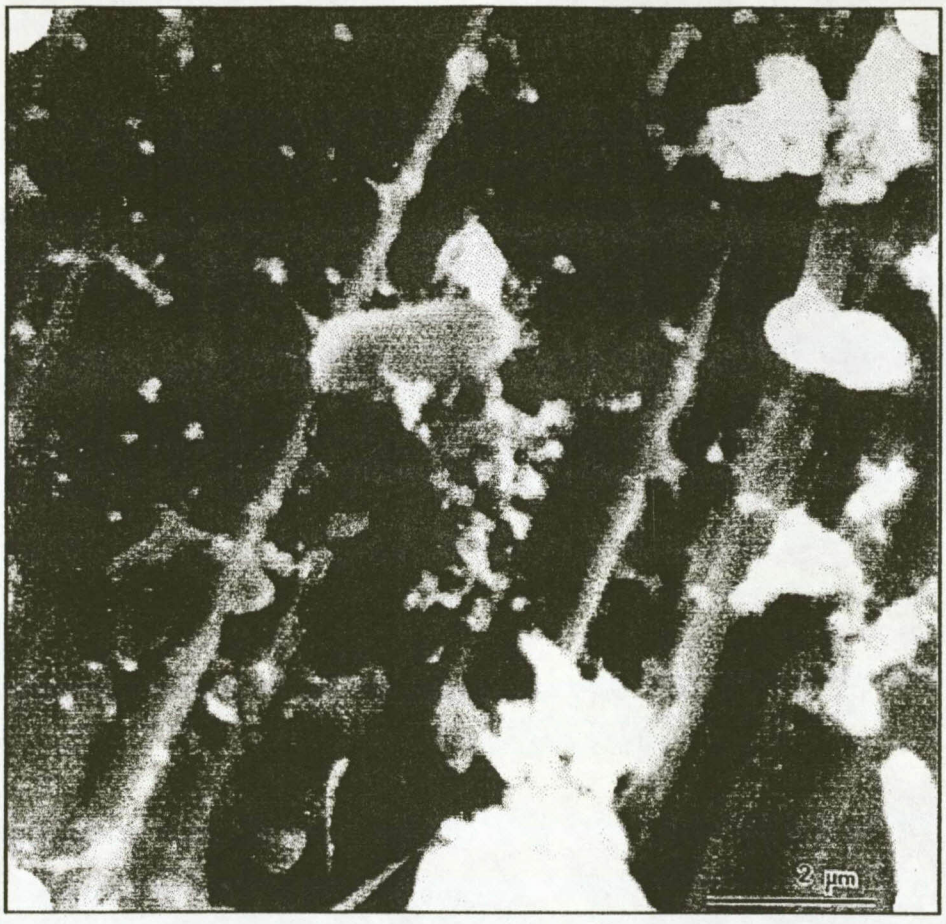


Figure 1. Adhered bacterial cell on a metal surface in a whitewater system of a paper mill (Donlan & Gibbon, http://www.MicrobeLibrary.org/images/biofilms).

The sessile microorganisms are responsible for most of the microbiologically associated problems experienced in the pulp and paper industry. These microorganisms are responsible for the production of biofilms or slimes that form on all surfaces exposed to water. Certain factors influence microbial metabolism and, therefore, microorganisms behave very differently when floating freely and when they are attached in a biofilm or even more from cells grown on laboratory media (Costerton & Lappin-Scott, 1989; Fletcher, 1984). McCoy (1987) stated that microbial communities in biofilms are up to seven times more metabolically active when compared to organisms in the planktonic state.

Biofilms consist of microbial cells, which include algae, nematodes, protozoa, fungi and bacteria (Martin, 1988; Robertson, 1994; Wiatr, 1994) as well as their

extracellular biopolymers (McFeters *et al.*, 1984; Johnsrud, 1997) and other material that have become entrapped in the extracellular matrix (Characklis, 1984). The interactions between fibres, water, microorganisms and the chemical additives present in a water system can give rise to a wide variety of deposits (Johnsrud, 1997). Biofilms generally consist of up to 95 % water (Characklis, 1984) while 5 to 25 % of the biofilm volume is made up by the various microorganisms (Caldwell *et al.*, 1992). Microorganisms are usually found in mixed cultures in nature and in pulp and paper mill water systems. Mixed microbial communities often have greater capabilities than those of the individual species (James *et al.*, 1995) and various types of interactions may exist between the different organisms (Appling, 1955; James *et al.*, 1995). James *et al.* (1995) stated that these interactions might have a significant effect on the species that are present in a biofilm and synergistic effects between the microorganisms are often more prevalent when fungi and bacteria grow together (Hughes, 1993). It can be speculated that more diverse microbial communities would be more difficult to control.

The encapsulated fast growing bacteria such as *Pseudomonas*, *Aerobacter*, *Arthrobacter*, *Alkaligenes*, *Proteus* and *Bacillus* species mostly dominate in industrial biofilms. Bacteria that are often found in paper and board machine biofilms include *Flavobacterium*, *Clavibacter*, *Sphaerotilus* and *Leptothrix* (Appling, 1955; Lutey, 1993; Vaisanen *et al.*, 1994), while fungal species include *Aspergillus*, *Penicillium* and *Cephalosporium* (Brewer, 1960). Robertson (1993) demonstrated that bacterial filaments dominate at neutral to alkaline pH, while more fungal filaments are present at an acidic pH. Bacteria grow between pH 4 and pH 9, with an optimum of 6 to 8, while fungi grow between pH 2 to pH 10, with an optimum of pH 3 to pH 7 (Hughes, 1993).

Generally, the outer layer of a biofilm consists of heterotrophic bacteria that deplete all available oxygen. These heterotrophic bacteria, therefore, establish an environment suitable for the growth of anaerobic sulphate reducing bacteria (Mueller, 1994) when sulphate and excess organic carbon are present (Robichaud, 1991). However, Johnsrud (1997) has reported that *Desulfotomaculum* and *Desulfovibrio* species could grow even when the bulk water contains oxygen concentrations close to

saturation. Anaerobic zones could also develop due to transfer limitations, which would stimulate the development of an anaerobic microbial population (Characklis, 1984; McFeters *et al.*, 1984).

The physical, chemical and biological properties of the biofilm are controlled by the environment in which the biofilm develops (Christensen & Characklis, 1989). Biofilm formation is influenced by the types of microorganisms present, nutrients supplied, moisture content, oxygen concentration, pH, temperature, ionic strength, substrate concentration and the presence of trace elements (Appling, 1955; Costerton & Lappin-Scott, 1989; Johnsrud, 1997; McFeters *et al.*, 1984; Mueller, 1994; Volk & LeChavellier, 1999). Biofilms are usually arranged for optimal absorption of nutrients and transfer of waste products (Stoner & King, 1994). As Vaatanen & Niemela (1983) reported, pH plays an important role in biofilm development. An increase in the pH value resulted in an increase in the total numbers of microbial colonies. Temperature was also observed to have a significant effect on bacterial growth in paper mills where the temperature is frequently maintained above the optimum temperature for mesophilic bacteria (Vaatanen & Niemela, 1983). It is clear that mills with a closed water system will experience more problems with microbial contamination than mills with a continuous flow of incoming water and water that leaves the system. Mills with a closed water system frequently experience an elevation in temperature.

Paper machine fluids provide an ideal environment for the growth of a wide variety of microorganisms (May, 1982; Martin, 1988; Robertson, 1993; Robertson & Taylor, 1993; Robichaud, 1991). Sugars released from the pulp, additives, the temperature and the recycling of water all contribute to the growth of microorganisms in the system (Vaatanen & Niemela, 1983). Volk & LeChavellier (1999) reported that biofilm thickness could directly be related to the biodegradable material that entered the system.

Additives do not only act as a nutrient source for the microorganisms, but could also be a source of microbial contamination (Hughes, 1993). Furthermore, the recyling of process water also contribute to an increase in the nutrient concentration, thus establishing a more favourable environment for the growth of microorganisms

(Barnes, 1984). Furthermore, if recycled fibre such as recycled paper is used, the microbial count can be approximately a 1000 times higher than in virgin pulp (Sorelle & Belgard, 1991), since recycled fibre can serve as an inoculum for both bacterial and fungal contamination into a paper mill water system. It is, therefore, evident that the prevention of biofilm formation on paper machine surfaces will not eliminate all microbial problems in paper mills. The microorganisms present in the planktonic phase, which adsorb to surfaces, still have to be controlled (Robertson, 1994).

Biofilms provide certain advantages for the sessile microorganisms. Microorganisms in biofilms are generally more resistant than planktonic microorganisms to the action of antimicrobial agents such as biocides and antibiotics (Costerton & Lappin-Scott, 1989, Robertson, 1994, Cloete *et al.*, 1998). Biofilm bacteria have, for example, been shown to be much more resistant to chlorine than free floating bacteria (Smith *et al.*, 2000). LeChavellier *et al.* (1988) reported that *Pseudomonas aeruginosa* was 500 to 3000 times more resistant to biocides and antibiotics in a biofilm than in the planktonic state. Thicker biofilms are also more resistant to biocides, since the extracellular material produced within the biofilm has been observed to react with the biocide and inactivate the chemicals present in the biocide (Mueller, 1994). Biocides would, therefore, preferably kill planktonic rather than sessile organisms (Vaisanen *et al.*, 1994). Biofilm thickness is influenced by the microbial species present in the biofilm, and biofilms comprised of mixed microbial species are often thicker and more stable than biofilms consisting of a single bacterial species (James *et al.*, 1995). Furthermore, biofilm development also protects the adsorbed organisms from the grazing of protozoa (Johnsrud, 1997; LeChavellier *et al.*, 1988). Biofilms are also advantageous to microorganisms because the organisms are exposed to a wider variety of nutrient and oxygen conditions (Fletcher, 1984; Martin, 1988; Nivens *et al.*, 1995; White *et al.*, 1999) within the biofilm and can survive in conditions below the required nutrient concentration (Characklis *et al.*, 1989). Biofilms also enable the formation of microniches such as anaerobic sites (Nivens *et al.*, 1995). The microorganisms are not washed away and thus have a better ecological advantage for reproduction (Characklis & Marshall, 1989). The disadvantages of microbial biofilms include competition for nutrients and terminal electron acceptors (White *et al.*, 1999)

and the availability of concentrated biomass for attack by predators (Nivens *et al.*, 1995).

MICROBIAL INDUCED CORROSION

Von Holy (1985) defined microbial induced corrosion (MIC) as the corrosion of a metal due to the microbial metabolism. During this process, atoms are exposed to an electron acceptor with a higher electron affinity than the potential donor. Oxidation occurs at the anodic site, and reduction occurs at the cathodic site (Brözel & Cloete, 1989). Microorganisms can either directly influence corrosion or establish conditions that result in corrosion. It has been estimated that up to 30 % of the maintenance cost in the pulp and paper industry is related to corrosion (Lutey, 1993). When water systems are closed, temperatures are generally increased. Consequently chemical reactions proceed at a faster rate than normally. Bennett (1985) stated that an increase of 7 °C in an acidic water system and a 20 °C increase in an alkaline water system would double the corrosion rate.

In closed water systems, the dissolved solids levels in the system increase, resulting in an increase in the corrosion cell currents. Suspended solid levels also increase in closed water systems. The increase in suspended solids results in an increase in the rate of biofilm formation that enhances the anaerobic conditions in the water system (Bennett, 1985). Although sulphate reducing bacteria are only active under anaerobic conditions, the interactions with other microorganisms may enhance their involvement in MIC (Wolfaardt & Cloete, 1992).

CONTROL OF MICROBIOLOGICALLY ASSOCIATED PROBLEMS

Microbiological control strategies should include the treatment of the incoming water, process material and the process water itself. Chemical treatments currently used to minimise biofilm development in water systems include the application of biocides, UV radiation, enzymes, synthetic dispersants and surfactants (Characklis, 1984, Cloete *et al.*, 1998). Only when contamination in all of these areas is under control, will biofilm formation decrease significantly (Stoner & King, 1994).

Biocides

Antimicrobial agents are added to paper machine water in order to kill or inhibit microorganisms. The addition of biocides significantly reduces the numbers of microorganisms available for attachment and subsequent biofilm formation. The spectrum of biocides can be broadened by combining active ingredients (Stoner & King, 1994). The efficacy of combined biocides is improved since no chemical compound is effective against all types of microorganisms (Appling, 1955).

Biocide efficacy is influenced by the specific types of organisms present, the active ingredients of the biocide used and the prevailing environmental conditions (Von Rege & Sand, 1998) including pH and temperature (Martin, 1988). System pH affects the efficiency of biocides since some products rapidly decompose at alkaline or acidic pH values. The system pH also determines which types of organisms will dominate within the system. Bacteria generally grow optimally between pH 6 and pH 8; while fungi would be dominant between pH 2 to pH 6 (Appling, 1955). Werker & Hall (1998) suggested that stringent pH control should improve the treatment reliability in pulp mill effluents. Knowledge of the different microorganisms in the water system and the interactions between the various organisms would also assist in the selection and dosage of the correct biocides.

Biofilm thickness has also been reported to influence biocide efficacy. Although biocide efficacy may be significant at the surface of biofilms exposed to the antimicrobial agent, the biocide efficacy may be substantially reduced inside the biofilm (Steward *et al.*, 1998). A study of biocide efficacy against *Pseudomonas aeruginosa* in biofilm showed that the biofilm development diminished biocide efficacy. It was reported that *Pseudomonas aeruginosa* was 150 to more than 3000 times more resistant to biocides and antibiotics when growing in biofilm than when growing in the planktonic state (Gorman, 1991; LeChavellier *et al.*, 1988). Steward *et al.* (1998) stated that the degree of biocide transport limitation depended on the biofilm thickness, bulk fluid biocide concentration, density of the neutralising sites in the biofilm and reaction rate between the biocide and the neutralising biomass.

Although biocides can reduce microbial activity, microorganisms cannot be removed totally from the water system (Von Rege & Sand, 1998). Sorelle & Belgard (1991) stated that the biocide cost for mills using recycled pulp could be almost 200 % higher than mills using virgin pulp, since contaminated recycled fibre can increase microbial fouling.

Biocides can be divided into the following major groups according to their action: oxidising biocides, non-oxidising biocides and biodispersants (Johnsrud, 1997). The major biocides can be grouped into the following categories (Tortora *et al.*, 1995; Pelczar *et al.*, 1993): phenol and phenolic compounds; alcohols; halogens; heavy metals and their compounds; organic acids; dyes; detergents; chlorohexidines; aldehydes; quaternary ammonium compounds; and gaseous agents.

EVALUATION OF BIOCIDE EFFICACY

Biocides are generally evaluated by measuring kill or inhibition (Robertson & Taylor, 1993). A reduction of more than 90 % of the microbial community when compared to an untreated control is used as an indication of biocide efficacy under predetermined conditions (Robertson, 1994). Since microorganisms are so diverse, a method used to enumerate one group of organisms might be inappropriate for enumeration of another group. Numerous techniques have been proposed for the evaluation of biocide efficacy. These techniques include plate counts, adenosine triphosphate (ATP) measurements and the most probable number (MPN) method.

Plate Counts

Various media and inoculation temperatures are employed for the various plate count procedures. Recent studies (Palojarvi *et al.*, 1997; Vestal & White, 1989) have, however, indicated that only between 0,01 and 1 % of all microbes are culturable on artificial media. Agar plate counts, therefore, generally underestimate the number of organisms present in a sample (White, 1984). Plate counts also favour the enumeration of nonfilamentous fungi and spores because plates are generally discarded when they show filamentous growth since it causes problems with enumeration. Enrichment cultures remove the microorganisms from their natural

habitat and only allow microbes with specific metabolic properties to grow under the specified cultural conditions (Vestal & White, 1989; Atlas & Bartha, 1993).

Plate counts, therefore, only supply limited information concerning the portion of the community that has the ability to grow on the selected media and under the specific conditions of incubation. Studies by Robertson (1993) confirmed that a large component of the organisms responsible for biofilm formation would be overlooked, when only plate counts were used as a means to assess biocide efficacy and the contribution of microorganisms to biofouling and biocorrosion would generally be underestimated. Furthermore, this approach is time-consuming and cumbersome and only reveals the presence of microbes that can be cultured on the chosen medium (Vestal & White, 1989). Plate counts also provide no information concerning the activity of the microorganisms or their physiological status (Von Rege & Sand, 1998).

Plate counts are, however, widely used in industry primarily due to their low cost and ease of application. The method is also used for continuous monitoring of the same environment since this method could provide trends in the variation of microbial numbers.

Adenosine Triphosphate (ATP) Measurements

Measurement of ATP is an indirect indicator of the metabolic status of the organisms within a sample, which is related to the energy charge of the cells (Von Rege & Sand, 1998). Adenosine triphosphate is present in all microorganisms and can, therefore, be measured, although the ATP concentration depends on the physiological state of the organism. Cellular ATP is detected when reduced luciferin reacts with oxygen to form oxidised luciferin in the presence of the luciferase enzyme, magnesium ions and ATP. Light is emitted and the amount of light is directly proportional to the concentration of ATP (Atlas & Bartha, 1993). Adenosine triphosphate measurements are generally not recommended for industrial and environmental studies since some microorganisms can alter their concentration of ATP with a change in nutritional or physiological conditions. Furthermore, ATP may also be adsorbed on particles in the environment giving a distorted microbial count (Atlas & Bartha, 1993).

Most Probable Number (MPN) Method

The MPN procedure gives a statistical estimate of the number of microorganisms present in the sample. Successive dilutions of the sample are made and replicate dilutions are scored as positive or negative. This pattern is used in conjunction with statistical tables to enumerate the microorganisms present in the sample (Atlas & Bartha, 1993). Since the MPN method is based on estimations, a high error frequency occurs (Melchiorri-Santolini, 1972).

ALTERNATIVE APPROACHES

Due to the inherent problems associated with the application of conventional microbiological methods for the enumeration and quantification of microorganisms in industrial and environmental samples, numerous alternative assays have been developed. These assays include molecular approaches as well as the analysis of both the functional (Buyer & Drinkwater, 1997) and the structural diversity (White *et al.*, 1996) of the microbial communities. These assays are independent of cell culturability and consequently circumvent many of the problems associated with conventional microbiological techniques.

Molecular Approaches

Van Damme *et al.* (1996) defined genotypic methods as those methods that make use of DNA or RNA molecules. Different molecular techniques may be used to obtain information about the microbial community structure. In some cases information on strain identification may be obtained without prior isolation and cultivation (Kohring *et al.*, 1994). Zhou *et al.* (1996) stated that the extraction of bacterial nucleic acids were useful to detect unculturable microorganisms. Ritz & Griffiths (1994) reported that nucleic acid hybridisation could be used to analyse shifts in the soil microbial community structure. Denaturing gradient gel electrophoresis (DGGE) is another method based on rRNA genes that can provide information about changes in the microbial composition (Muyzer *et al.*, 1993). Ribosomal RNA (rRNA) isolation and sequencing is also a useful tool for the determination of the community structure (Vestal & White, 1989). Although RNA can be used, DNA is a more stable target for

nucleic acid hybridisation (Sayler *et al.*, 1992). The amount of DNA extracted can be used to estimate the microbial biomass since the genetic composition of bacteria is relatively uniform (Sayler *et al.*, 1992). DNA fingerprinting and sequencing can also be used to discriminate bacterial isolates and clones (De Bruijn, 1992). Another method that can be used is protein fingerprinting for the determination of bacterial community structure. This method was applied to determine the community composition in an activated sludge system (Ehlers *et al.*, 1999). The advantage of polyacrylamide gel electrophoresis (PAGE) is that no culturing is necessary, therefore, the enrichment effect of conventional culturing methods is eliminated.

A major disadvantage of the application of molecular techniques is the quantitative recovery of microorganisms from the environment. White & 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 interpretation of data may also be problematic (Amann *et al.*, 1995). White (1994) suggested that nucleic acid studies should be supplemented by phenotypic information. The characterisation of microbial systems could also be enhanced by combing the signature lipid biomarker approach with 16S rDNA-based approaches (Liu *et al.*, 2000).

Analysis of Functional Diversity Based On Substrate Utilisation Profiles

Zak *et al.* (1994) defined the functional diversity of a microbial community as the numbers, types, activities and rates at which a suite of substrates are utilised. Although the exact numbers and taxonomic identities of the microbial species cannot be determined by analysis of microbial communities using the Biolog assay, the patterns of carbon source utilisation provide insight regarding the diversity within and among communities. Although this system was initially developed to identify pure bacterial strains, it is currently widely used to analyse the carbon source utilisation patterns of mixed microbial communities (Guckert *et al.*, 1996).

(Table 1). Each of the 95 wells contains a single compound that acts as a carbon, energy and electron source for the microorganisms (Lowit *et al.*, 2000). This method reflects the metabolic capabilities of a part of the community since it selects for organisms actively metabolising under the given conditions on the microtiter plate (Palojarvi *et al.*, 1997). The metabolic fingerprint obtained from the carbon source utilisation profile represents the physiological potential of the microbial community (Lowit *et al.*, 2000). The oxidation of various groups of organic compounds is thus assayed and not the growth (Garland & Mills, 1991). Biolog microtiter plates (Biolog Inc., Hayward, USA) have been widely used to describe the functional diversity of microbial communities in different environments or after certain treatments (Victorio *et al.*, 1996; Garland & Mills, 1991; Zak *et al.*, 1994). Garland & Mills (1991) were among the first to characterise the differences between habitats and between samples within the same habitat based on patterns of carbon source utilisation. The Biolog assay is a relative simple and rapid technique compared to other community level approaches such as DNA analysis (Buyer & Drinkwater, 1997).

Garland & Mills (1991) also demonstrated that differences in carbon source utilisation patterns were directly related to the separation of samples along axes of principle components. The Biolog approach yields a more sensitive and ecological relevant measure of the heterotrophic community structure and this technique has been shown to be useful in distinguishing microbial communities within various wastewater treatment systems (Schneider *et al.*, 1998). These treatment systems included municipal activated sludge, as well as bleached kraft mill effluents (Victorio *et al.*, 1996).

Table 1. Carbon sources present in Biolog GN microtiter plates (As adapted from Garland & Mills, 1991).

Carbohydrates	**Carboxylic acids**	**Amino acids**
N-Acetyl-D-galactosamine	Acetic acid	D-Alanine
N-Acetyl-D-glucosamine	cis-Aconitic acid	L-Alanine
Adonitol	Citric acid	L-Alanyl-glycine
L-Arabinose	Formic acid	L-Aspartic acid
D-Arabitol	D-Galactonic acid lactone	L-Glutamic acid
Cellobiose	D-Galacturonic acid	Glycyl-L-aspartic acid
i-Erythritol	D-Gluconic acid	Glycyl-L-glutamic acid
D-Fructose	D-Glucosaminic acid	L-Histidine
L-Fucose	D-Glucuronic acid	Hydroxy-L-proline
D-Galactose	α-Hydroxybutyric acid	L-Leucine
Gentiobiose	β--Hydroxybutyric acid	L-Ornithine
α-D-Glucose	γ-Hydroxybutyric acid	L-Phenylalanine
m-Inositol	ρ-Hydroxyphenylacetic acid	L-Proline
α-D-Lactose	Itaconic acid	L-Pyroglutamic acid
Lactulose	α-Ketobutyric acid	D-Serine
Maltose	α-Ketoglutaric acid	L-Serine
D-Mannitol	α-Ketovaleric acid	L-Threonine
D-Mannose	D,L-Lactic acid	D,L-Carnitine
D-Melibiose	Malonic acid	γ-Aminobutyric acid
β-Methyl-D-glucoside	Propionic acid	**Aromatic chemicals**
D-Psicose	Quinic acid	Inosine
D-Raffinose	D-Saccharic acid	Uronic acid
L-Rhamnose	Sebacic acid	Thymidine
D-Sorbitol	Succinic acid	Uridine
Sucrose	**Brominated chemicals**	**Polymers**
D-Trehalose	Bromosuccinic acid	Glycogen
Turanose	**Amides**	α-Cyclodextrin
Xylitol	Succinamic acid	Dextrin
Esters	Glucuronamide	Tween 80
Mono-methylsuccinate	Alaninamide	Tween 40
Methylpyruvate	**Amines**	**Phosphorylated chemicals**
Alcohols	Phenylethylamine	D,L-α-Glycerol phosphate
2,3-Butanediol	2-Aminoethanol	Glucose-1-phophate
Glycerol	Putrescine	Glucose-6-phophate

Victorio *et al.* (1996) reported that homogenisation of samples produced the most representative substrate utilisation profiles of the microbial community. Inoculum density had also been shown to influence colour development in Biolog plates (Garland & Mills, 1991; Zak *et al.*, 1994). The effect of variation in inoculum density could be eliminated by using various normalisation procedures. One proposed procedure is the normalisation of the inoculum size, but this is very time-consuming (Gamo & Shoji, 1999). Gamo & Shoji (1999) proposed the Biolog-MPN assay, but this method is very time-consuming and requires significant quantities of resources since it is based on successive dilutions of the same sample. An alternative approach is the application of the average well colour development (AWCD) technique, which normalises the data in order to compensate for any variation in inoculum density. The AWCD is the mean absorbance values for the 95 wells per reading time (Garland & Mills, 1991). In contrast, Kersters *et al.* (1997) reported that the dilution of samples to achieve equivalent densities was a more competent method. According to Guckert *et al.* (1996) additional information not available from any single time point analysis such as lag times, rates of colour development and maximum absorbance is, however, available during the integration of the absorbance versus time curves. Carbon source utilisation profiles are generally compared using multivariate analyses (O'Connell *et al.*, 2000).

Although it is acknowledged that changes in community structure could occur during the incubation of the Biolog plates, all the data are interpreted as a function of the microbial community structure from the original sample (Garland & Mills, 1991).

Analysis of The Structural Diversity Based On Signature Lipid Biomarker Analysis

Phospholipid fatty acid (PLFA) analysis has numerous advantages over other procedures for microbial estimation, since certain fatty acids (Table 2) are specific to bacteria or fungi and different groups of bacteria have different fatty acid compositions (Gillan & Hogg, 1984). Phospholipids can, therefore, be considered as a fingerprint of the microbial community (Peterson & Klug, 1994). Different groups of microorganisms synthesise a variety of PLFAs through various biochemical pathways and PLFAs can, therefore, be used as taxonomic markers (White & Ringelberg, 1996). When bacteria are grown under standardised conditions, they have a constant fatty acid composition, which is specific for a genus or even a species (Keweloh & Heipieper, 1996). This profile is often referred to as the organism's signature biomarker.

Signature lipid biomarker analysis is based on the liquid extraction and separation of microbial lipids from environmental samples, followed by quantitative analysis using gas chromatography and gas chromatography-mass spectrometry (White & Ringelberg, 1996). The signature lipid biomarker technique provides a good basis for a microbial identification system.

Factors such as the metabolic state of the organism, environmental changes and exposure to toxic substances influence the PLFA composition of the cell membranes (Frostegard *et al.*, 1993,1997). External stimuli such as temperature, pH, nitrogen source and salinity may also bring about a variation in the fatty acid profiles (Dowling *et al.*, 1986). Shifts in the microbial fatty acid profiles as a result of pH were also detected during this study (Chapter 3). The factor that most directly controls the composition of PLFA is temperature, since this directly influences the membrane function (Peterson & Klug, 1994).

Table 5. Examples of different types of fatty acids (As adapted from Raltedge & Wilkinson, 1988b).

FATTY ACIDS	Systematic name	Trivial name	Shorthand designation
Saturated straight chain fatty acids	Dodecanoic acid	Lauric acid	12:0
	Octadecanoic acid	Stearic acid	18:0
	Docosanoic acid	Behenic acid	22:0
Saturated branched chain fatty acids	13-Methyltetradecanoic acid	Isopentadecaanoic acid	13-Me-14:0
	10-Methyloctadecanoic acid	Tuberculostearic acid	10-Me-18:0
	2,4,6,8-Tetramethyloctacosanoic acid	Mycocerosic acid	2,4,6,8-Me-28:0
Monoenoic unsaturated fatty acids	*cis*-Hexadec-9-enoic acid	Palmitoleic acid	16:1ω7
	trans-Octadec-9-enoic acid	Elaidic acid	18:1ω9
	cis-Tetracos-15-enoic acid	Nervonic acid	24:1 ω9
Dienoic unsaturated fatty acids	*cis,cis*- Octadeca-9,12-dienoic acid	Linoleic acid	18:2ω6
	trans,trans-Octadeca-9,12-dienoic acid	Linelaidic acid	18:2ω6
Polyenoic unsaturated fatty acids	*cis,cis,cis*- Octadeca-9,12,15-trienoic acid	α-Linolenic acid	18:3 ω3
	cis,cis,cis,cis-Icosa-5,8,11,14-tetraenoic acid	Arachidonic acid	20:4 ω6
Hydroxy fatty acids	2-Hydroxyoctadecanoic acid	3-Hydroxystearic acid	3-OH-18:0
	15,16-Dihydroxyhexadecanoic acid	Ustilic acid	15,16-di-OH-16:0
Epoxy fatty acids	*cis*-12,13-Epoxy- *cis*-octadec-9-enoic acid	Vernolic acud	12,13-c (9*c*)
	9,10-Epoxyoctadecanoic acid	Epoxystearic acid	9,10 epoxy-18:0

Lipid Functions

Lipids are typical components of cellular membranes and act as a major storage form of carbon and energy. Lipids may also serve as insulation barriers to avoid thermal, electrical and physical shock water (Bohinski, 1987). Lipids also serve as precursors of many important substances (Bohinski, 1987). Furthermore, lipids are also frequently associated with photosynthetic processes in plants and microorganisms (Ratledge & Wilkinson, 1988).

Lipid Fractionation

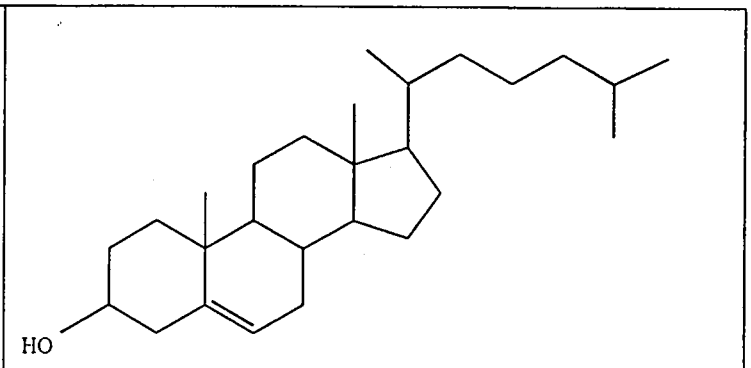
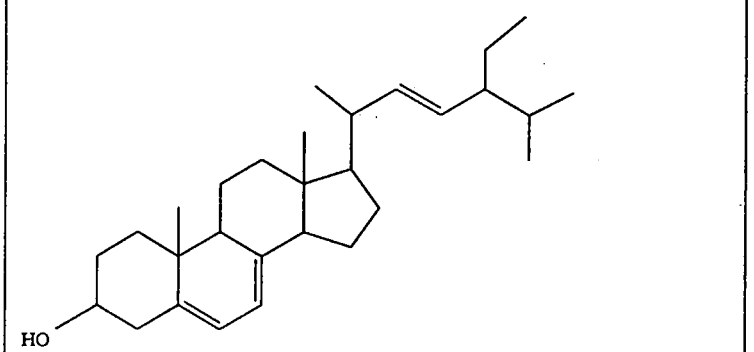
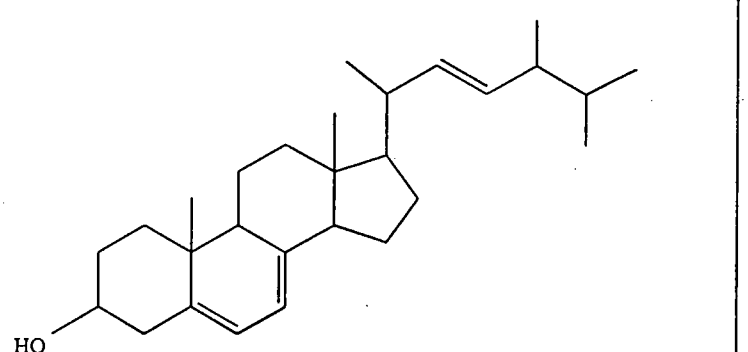
Lipids can be divided into the neutral and polar lipid fractions. Neutral lipids do not contain any charged atoms while polar lipids have a polar head group. The polar lipid fraction can be further divided into the glycolipid and phospholipid fractions. Fractionation into the neutral lipid, glycolipid and phospholipid fractions can be achieved with the use of silicic acid column chromatography and various solvents (Findlay & White, 1987; Kock & Ratledge, 1993).

Neutral lipids

This group of lipids are classically regarded as the waxes and the acylglycerols (Brennan, 1988). Acylglycerols are esters of trihydroxy alcohol, glycerol and fatty acids. A wax is an ester with constituent alcohol and acid components, both containing long hydrocarbon chains (Bohinski, 1987). These lipids do not contain any charged atoms (Gunstone & Herslof, 1992).

Sterols are classified as neutral lipids. Although sterols have a structural function, they can also act as precursors of hormones and bile acids. Sterols are present in animals (cholesterol), plants (stigmasterol) and fungi (ergosterol) (Table 3) (Gunstone & Herslof, 1992). Prokaryotes (including bacteria and cyanobacteria) contain no sterols, but in contrast they contain hopanes. Sterols are formed from squalene 2,3-oxide during an aerobic process while hopanoids are formed non-oxidatively (Coolbear & Threfall, 1989).

Table 3. Chemical structures of the most common sterols (As adapted from Gunstone & Herslof, 1992).

Cholesterol	
Stigmasterol	
Ergosterol	

Glycolipids

The term glycolipids is generally associated with all lipids linked to any type of carbohydrate component (Bohinski, 1987; Gunstone & Herslof, 1992). Glycolipids can be divided into acylated sugar derivatives that have a fatty acid esterified directly to the sugar moiety and lack glycerol, glycosyldiacylglycerols and complex glycolipids such as peptidoglycan glycolipids (Lynne, 1989). Glycolipids are responsible for protection against mechanical damage, aid in cell-to-cell recognition

and lubricate the cell surface against friction. Glycolipids also act as surfactants and emulsifiers (Ratledge & Wilkinson, 1988).

Poly-β-hydroxyalkanoic acids (PHA) are glycolipids. Polyhydroxyalkanoates act as intracellular storage polymers in microorganisms (Liu *et al.*, 2000). Poly-β-hydroxybutyrate (Figure 2) is an example of a PHA, which occurs as intracellular granules within bacteria (Pelczar *et al.*, 1993). Poly-β-hydroxyalkanoic acid is abundant in Gram positive and Gram negative bacteria. This polymer acts as an energy reserve in the bacteria. In fungi PHA form a minor compound, except in Basidiomycetes where it can form up to 22 % of the total fatty acids (LeChavellier & LeChavellier, 1988).

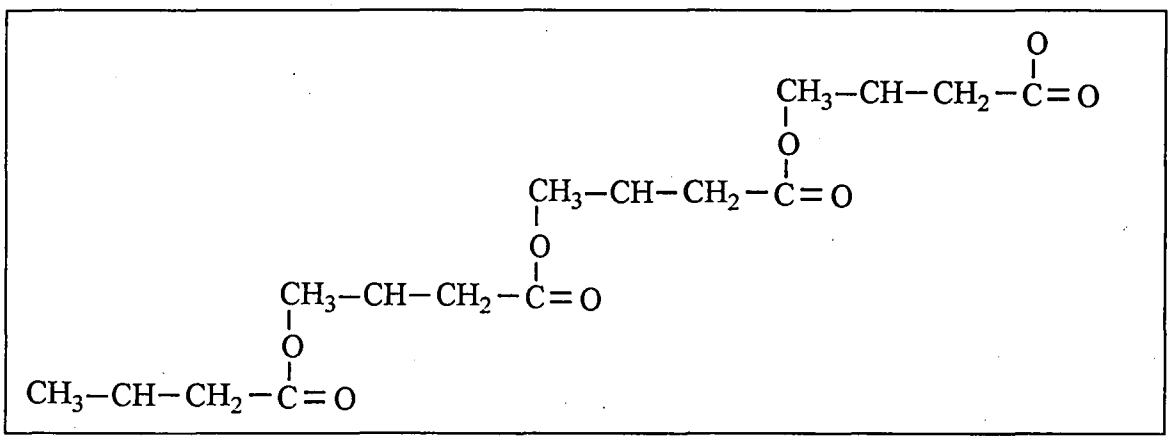
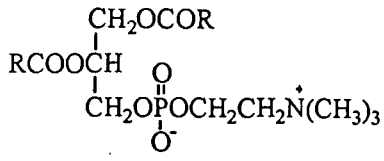
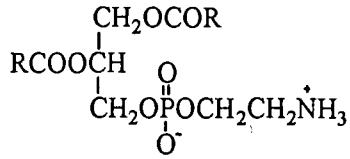
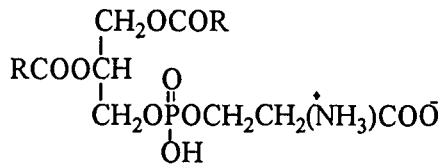
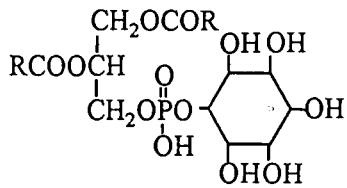
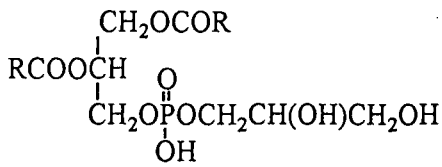
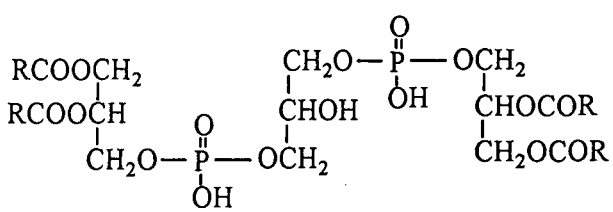
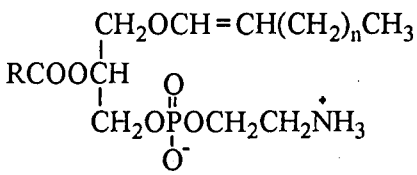


Figure 2. Chemical structure of Poly-β-hydroxybutyrate (As adapted from Pelczar *et al.*, 1993).

Phospholipids

Phospholipid fatty acids (PLFAs) usually have a saturated fatty acid on C1 and an unsaturated fatty acid on C2 of the glycerol backbone. Membrane phospholipids are a complex mixture of molecular species containing a variety of fatty acyl and head group compositions (Kaneda, 1991; Kim *et al.*, 1994). Phospholipid fatty acids can be divided into seven different classes each with its unique characteristics. These classes include: phosphatidylcholine; phosphatidylethanolamine; phosphatidylserine; phosphatidyl-inositol; phosphatidylglycerol; diphosphatidylglycerol; and plasmalogen (Table 4) (Gunstone & Herslof, 1992).

Table 4. Chemical structures of the various phospholipid classes (As adapted from Gunstone & Herslof, 1992).

Phosphatidylcholine	
Phosphatidylethanolamine	
Phosphatidylserine	
Phosphatidylinositol	
Phosphatidylglycerol	
Diphosphatidylglycerol	
Plasmalogen	

Phospholipids have no storage function, therefore they represent a constant portion of the cell mass (Noble *et al.*, 2000).

Fatty Acid Nomenclature

Fatty acids are designated as A:BωC, where A is the total number of carbon atoms, B is the number of double bonds, and C is the position of the double bond from the aliphatic (ω) end of the molecule. Double bond geometry is indicated as 'c' for *cis* and 't' for *trans*. The prefixes 'i' and 'a' denote iso- and anteiso – methyl branching, respectively. The prefix 'cy' designates a cyclopropyl component. The prefixes α and β show that the OH groups are situated on positions 2 and 3, respectively (Zelles, 1999).

Phospholipid Fatty Acids In Microbial Ecology

Microbial communities may be described by the quantification of the extractable cellular compounds that define the viable biomass and the community structure (Guezennec & Fialu-Medioni, 1996). Bacteria and eukaryotes produce different PLFAs, which facilitate the analysis of both groups of microorganisms in a single analysis (Noble *et al.*, 2000). Phospholipid fatty acids are also useful during the investigation of the nutritional status of organisms considering the loss of culturability of microorganisms that are dehydrated or injured but still viable (Macnaughton *et al.*, 1997). The analysis of PLFAs also provides a means to determine changes in the overall composition of the microbial community (Frostegard *et al.*, 1997).

Knowledge of the community structure allows the description of shifts within the community during development and succession as well as comparison between different biofilms (McFeters *et al.*, 1984). Werker & Hall (1998) demonstrated the use of fatty acid analyses in distinguishing between planktonic and sessile microbial populations. The authors looked at the correlations of different fatty acids to each other. The interference from non-microbial sources might cause problems (Gillan & Hogg, 1984), but in a controlled environment such as kraft mill effluent, non-microbial fatty acids from wood chip pulping would be indicated by the presence of linoleic acid (Werker & Hall, 1998). Vaisanen *et al.* (1994) stated that the proportion

of fatty acids in pulp and papermaking chemicals was insignificant. Signature lipid biomarker analysis can, therefore, be used successfully to characterise microbial communities in paper mill water systems. This was also the case in this study (Chapter 3).

Viable biomass

Assuming the fact that the viable culturable count of microorganisms from industrial and environmental samples only account for 0,1 to 10 % of the total microbial community (Macnaughton *et al.*, 1997), a more accurate and universal method for the quantification of microorganisms should be applied (White & Ringelberg, 1996). Viable biomass can be measured either as lipid phosphate (Guezennec & Fialu-Medioni, 1996; Hedrick & White, 1986) or as ester linked fatty acids (White *et al.*, 1996), since the presence of PLFA would signify the presence of cells with intact membranes (White & Ringelberg, 1996). A conversion factor of 5,9 x 10^4 cells per picomol PLFA (based on *E. coli*) was suggested by Kieft *et al.* (1994). A more applicable conversion factor for environmental samples is 2,5 x 10^4 cells per pmol PLFA, as suggested by A. Peacock (University of Tennessee, USA, personal communication). This conversion factor was derived from rapidly growing cells, 0,4 pg of dry weight per bacterial cell and 100 µmol PLFA per gram dry weight, which give the conversion factor of 2,5 x 10^4 cells per pmol PLFA.

Phospholipid fatty acid analyses provide a sensitive measure of the viable biomass present within a sample, since phospholipids are not used as reserve polymers and have a rapid turnover rate (Steward *et al.*, 1996). Lipid phosphate measurements have been shown to agree with other measures of biomass, including enzymatic activities (Hedrick & White, 1986), muramic acid levels (Dowling *et al.*, 1986), total adenosine triphosphate (Guezennec & Fialu-Medioni, 1996; Hedrick & White, 1986), respiratory activities (Hedrick & White, 1986) and total plate counts (Dowling *et al.*, 1986). However, many organisms can regulate their fatty acid and lipid composition in response to environmental conditions (Kieft *et al.*, 1994), in order to maintain the effective functioning of biological membranes (Guezennec & Fialu-Medioni, 1996) and samples should, therefore, be analysed under standardised conditions. Frostegard *et al.* (1997) quantified microbial biomass both as the total lipid phosphate and as

PLFA and concluded that PLFA analysis gave a better indication of the total viable biomass present within a sample. The ratio of diglyceride to PLFA has been reported to provide an estimate of the ratio of nonviable to viable biomass (Dowling *et al.*, 1986). Diglyceride fatty acids (DGFAs) are formed when cellular enzymes (phospholipases) hydrolyse the phosphate group of the phospholipid (Kieft *et al.*, 1994). The resulting diglyceride contains the same signature fatty acids as the phospholipids. The patterns of the DGFAs could, therefore, indicate the recently lysed components of the microbial community (White, 1995). Healthy biofilms generally have a DGFA:PLFA ratio of less than 0,5. An increase in DGFA:PLFA ratio from 0,4 to 0,7 was obtained in a *Mycobacterium smegmatis* biofilm as a result of biomass death due to chlorine exposure (White *et al.*, 1999).

Nutritional status

The lipid composition of microbes is the product of their metabolic pathways and is indicative of the phenotypic response of the organism to the environment (White *et al.*, 1996). White *et al.* (1996) suggested that specific patterns of PLFA could be used as indicators of physiological stress, for example toxicity, exposure to solvents, alcohols or acids (Mandelbaum *et al.*, 1997; Steward *et al.*, 1996).

The relative amounts of *trans* fatty acids are dependent on the growth rate, medium composition and environmental factors (Keweloh & Heipieper, 1996). It has been reported that exposure of microorganisms to toxic environments resulted in minicell formation (Mandelbaum *et al.*, 1997; White & Ringelberg, 1996) and a relative increase in specific *trans* monoenoic PLFA compared to the *cis* isomers (Gehron & White, 1982; Guckert *et al.*, 1991). Using *cis/trans* isomerisation, bacteria can adapt very quickly to toxic concentrations of organic substrates, thereby stabilising their membranes, which allows them to remain in physiologically acceptable conditions. The measurement of *cis* to *trans* isomerisation of unsaturated fatty acids can, therefore, be a relevant parameter to determine the physiological status of the microbial population in the presence of toxic pollutants (Keweloh & Heipieper, 1996). Halverson and Firestone (2000) found that the *trans* isomers decreased after an increase in water availability or as a result of a change from polyethylene glycol to sodium chloride as solute.

increase in water availability or as a result of a change from polyethylene glycol to sodium chloride as solute.

As suggested by Mandelbaum *et al.* (1997), the *trans:cis* ratio of 16:1 fatty acids and 18:1 fatty acids provides a general measure of stress or starvation. The concentration of *trans* monoenoic acids usually increase during starvation (Guckert *et al.*, 1986). *Trans:cis* ratios of higher than 0,1 are generally considered to be indicative of exposure to toxins or starvation (Guckert *et al.*, 1991; Keweloh & Heipieper, 1996; Steward *et al.*, 1996; White *et al.*, 1996) while ratios of 0,05 or less are generally considered to be indicative of non-stressed microbial communities (White *et al.*, 1996).

The presence of cyclopropyl fatty acids in microorganisms has also been identified as an indicator of physiological changes related to stress (Guckert *et al.*, 1991; Steward *et al.*, 1996) or anoxia (Kieft *et al.*, 1996). Starvation or a stationary growth phase results in the conversion of monoenoic fatty acids to cyclopropyl fatty acids. The presence of cyclopropyl fatty acids may also be stimulated by high temperatures, high magnesium ion concentrations and as a result of decreasing pH (Guckert *et al.*, 1986). A decrease in the water potential was also reported to result in an increase in cyclopropyl fatty acids, since most bacteria adapt to this situation by modifying the cell membrane by changing the phospholipids and thereby modifying their PLFAs (Cummings & Russell, 1996). White *et al.* (1999) stated that a cyclopropane PLFA:monoenoic PLFA ratio of greater than 0,1 could be regarded as being indicative of nutritional stress. This ratio could increase up to 2,5 as starvation or the stationary phase was prolonged. Cells that are growing exponentially have a cyclopropane PLFA:monoenoic PLFA ratio of less than 0,05 (Smith *et al.*, 2000).

Upon exposure to oxidising biocides (hypochlorite) (Smith *et al.*, 2000) oxirane (epoxide) PLFAs are formed at the expense of monoenoic PLFAs, which is a major component of the cell membrane in Gram negative bacteria (D.C. White, University of Tennessee, USA, personal communication). It could be speculated that *cis* to *trans* isomerisation is followed by the epoxidation of the alkene bond to yield the *trans* epoxidated fatty acid (D.C. White, personal communication). Smith *et al.* (2000)

reported that organisms containing epoxide fatty acids were rendered unculturable and could, therefore, be used as biomarkers for cell death. All the oxirane fatty acids detected were in the *trans* configuration.

Unbalanced growth conditions often occur when a suitable carbon source is present but one or more of the essential nutrients required for the formation of bacterial membrane lipids are lacking from the environment and the cells consequently cannot divide (Findlay & White, 1983; Ringelberg *et al.*, 1997; Zelles *et al.*, 1994). Under such conditions formation of PLFAs ceases and the carbon is stored as poly–hydroxyalkanoic acids (PHAs) (White & Ringelberg, 1996). It has been reported that endogenous storage lipids accumulate under conditions where cellular growth is repressed (Smith *et al.*, 1986). The formation of PHAs in bacteria or triglycerides in the microeukaryotes, which are endogenous storage lipids, relative to the PLFAs, could subsequently provide a measure of the nutritional status of the microbial community (Mandelbaum *et al.*, 1997; White *et al.*, 1996). White *et al.* (1999) reported that a PHA:PLFA ratio of more than 0,2 is indicative of unbalanced growth. It has also been reported that the PHA:PLFA ratio increased under conditions where heavy metal contamination was present. The PHA:PLFA ratio increased from 0,081 to 0,215 with an increase in copper concentration from 4,2 mg/kg to 150 mg/kg (Zelles *et al.*, 1994). White (1984) indicated that biofilms also accumulated PHAs relative to their total phospholipids under conditions of unbalanced growth. Valeur *et al.* (1988) reported that the relative amount of PHAs in sessile bacteria was one order of magnitude smaller, when compared to the PHA present in planktonic bacteria.

The loss of lipid phosphate would represent the loss of cellular membrane biomass. Subsequently, the ratio of lipid glycerol to lipid phosphate could also be used as a measure of the nutritional status of a microbial community (Gehron & White, 1982).

Respiratory quinones are also related to microbial physiology since quinone composition may serve as an indication of the degree of aerobic activity of the microbial community (White & Ringelberg, 1996). The common bacterial respiratory quinones are ubiquinones and naphtoquinones. Naphtoquinones consist of menaquinones and desmethylmenaquinones. Ubiquinones are found in eukaryotes.

and in some Gram negative bacteria. Menaquinones are present in Gram negative bacteria, Gram positive bacteria and Archaea, while desmethylmenaquinones have only been reported in some pathogenic enterobacteria and *Streptococcus faecalis* (Hedrick & White, 1986). Primary anaerobic cultures have much greater proportions of desmethylmenaquinones and menaquinones while aerobic cultures contain much more ubiquinones (Guckert *et al.*, 1985). Subsequently, the ratio of total naphtoquinones to total ubiquinone, could provide an indication of the extent of aerobic to anaerobic respiration (Hedrick & White, 1986).

Community structure

White *et al.* (1996) stated that analysis of lipids such as sterols (for the microeukaryotes - nematodes, algae and protozoa), glycolipids (for phototrophs and Gram positive bacteria) or the hydroxy fatty acids in the lipopolysaccharide of the lipid A (for Gram negative bacteria) could be used to provide a detailed community analysis. Another approach to determine the community structure is the analysis of signature lipid biomarkers (Vestal & White, 1989). Although PLFA analysis does not always allow detection of specific species present, it does provide an overview of the microbial community (White & Macnaughton, 1997). Menyawi *et al.* (2000) found that the analysis of fatty acid profiles was a rapid and accurate method for the identification of yeasts.

Signature lipid biomarker analysis does not list all the microorganisms present, unless a true biomarker for a specific species is present. The data are rather used to study changes in the major groups of organisms (Zelles, 1999). The more diverse the microbial community, the more diverse the PLFA profile is likely to be.

Polyunsaturated PLFAs are found almost exclusively in eukaryotes (White *et al.*, 1996) with C20:5ω3 and C20:4ω6 being the most abundant (Steward *et al.*, 1996). In exceptional cases polyunsaturated fatty acids are found in cyanobacteria (Zelles, 1999). Linoleic acid (C18:2) has been proposed as a fungal biomarker and the C18:2 content was observed to correlate well with the ergosterol content (Frostegard & Baath, 1996). Gram positive bacteria predominantly contain iso- and anteiso-branched saturated fatty acids formed by the branched-chain pathway (Frostegard *et*

al., 1993; Guezennec *et al.*, 1996; Ringelberg *et al.*, 1997; Tunlid *et al.*, 1989). Aerobic Gram negative bacteria contain monounsaturated lipids (Guezennec & Fialu-Medioni, 1996; White *et al.*, 1996) while anaerobic Gram negative bacteria contain branched saturated fatty acids (Guezennec *et al.*, 1996). Cyclopropyl fatty acid 17:0 is typical for Gram negative bacteria (Guezennec & Fialu-Medioni, 1996). Iso- and anteiso-branched chain fatty acids are predominant in Gram positive and sulphate reducing bacteria (Zelles, 1999). The contribution of monounsaturated fatty acids in Gram positive bacteria is very small (less than 20 %). Monounsaturated fatty acids can, therefore, be used as a general biomarker for Gram negative bacteria (Ratledge & Wilkinson, 1988). One drawback of SLB analysis is that Archaea cannot be detected by PLFA analysis since Archaea have ether rather that ester bonds in their membranes (Noble *et al.*, 2000).

Some microbial species are readily defined using the SLB methodology (Table 5) since they contain unique lipid components or lipid patterns (White *et al.*, 1996). *Desulfovibrio* spp. and *Desulfotomaculum* spp. have been reported to contain monoenoic 17-carbon fatty acids as major component (Dowling *et al.*, 1986). The membrane lipids of thio-oxidising bacteria are usually characterised by large amounts of monounsaturated fatty acids with either C16:1ω7 or C18:1ω7 predominating (Guezennec & Fialu-Medioni, 1996). Most *Methylomonas* and *Methylococcus* species have been reported to contain high levels of C16:1ω7t. The predominance of iso over anteiso (C15:0 and C17:0) is characteristic of sulphate reducing bacteria (Guezennec & Fialu-Medioni, 1996). Valeur *et al.* (1988) reported that a major difference existed between the PLFAs present in sessile and planktonic bacteria. Planktonic bacteria had a lower ratio of saturated to unsaturated C18 fatty acids, while sessile bacteria contained a larger proportion of C18 relative to C16 fatty acids (Valeur *et al.*, 1988).

Table 5. Specific signature lipid biomarkers assigned to different microorganisms.

Microorganisms	Biomarker	Reference
Diatoms	C16:1ω3t C16:4ω1 C22:4ω6 C20:5ω3 C20:5ω5	Findlay & Dobbs, 1993; Guckert *et al.*, 1991; Vestal & White, 1989
Actinomycetes	10Me-C17:0 10Me-18:0	Frostegard *et al.*, 1993; Peterson & Klug, 1994; Tunlid *et al.*, 1989
Protozoa	C20:3ω6 C20:4ω6	Vestal & White, 1989
Fungi	C18:3ω7c C18:3ω4c C18:2ω6c	Frostegard *et al.*, 1993; Peterson & Klug, 1994; Zelles *et al.*, 1994
Desulfobacter spp	cy 17:0 cy 19:0 10Me 16:0	Dowling *et al.*, 1986; Gillian & Hogg, 1984; Guezennec *et al.*, 1996; Kohring *et al.*, 1994; Steward *et al.*, 1996
Desulfovibrio spp	i/a C15:1ω7 i/a C17:1ω7	Guezennec *et al.*, 1996; Kohring *et al.*, 1994
Desulfotomaculum	C17:1ω7	Gillian & Hogg, 1984; Guezennec *et al.*, 1996
Desulfobulbus	C17:1ω6 C17:1ω8	Gillian & Hogg, 1984; Guezennec *et al.*, 1996
Methylosinus and *Methylocystis* spp.	C18:1ω8	Guezennec *et al.*, 1996
Clostridia	cy15:1	Vestal & White, 1989
Photosynthetic organisms	C16:1ω13t	Findlay & Dobbs, 1993
Green algae	C18:1ω9c C16:1ω3 C16:1ω13t	Guckert *et al.*, 1991
Microalgae	C16:3ω6	Vestal & White, 1989
Psychrophilic bacteria	C20:5 C22:6	Vestal & White, 1989
Bacteria (anaerobic desaturase pathway)	C18:1ω7c	Findlay & Dobbs, 1993
Type II methane oxidizing bacteria	C18:1ω8c	White *et al.*, 1996

CONCLUSIONS

Many problems are associated with the production of biofilms in the papermaking industry including the formation of holes and spotting and discolouration of the paper (Robertson & Taylor, 1993; Stoner & King, 1994). Microbial contamination, therefore, contribute significantly to losses in production and product quality. Antimicrobial agents are added to paper machine water in order to kill or inhibit microorganisms to reduce the numbers of microorganisms available for attachment and biofilm formation.

Biocide efficacy is currently evaluated using various culturing and biochemical assays. Due to the limitations of conventional microbial techniques and the fact that the viable count of microorganisms from environmental samples only account for 0,1 to 10 % of the total community (Palojarvi *et al.*, 1997; Vestal & White, 1989), numerous alternative approaches to study microbial communities *in situ* have been developed.

These approaches include the analysis of the functional diversity of the microbial population using the Biolog substrate utilisation assay as well as the evaluation of signature lipid biomarkers. Substrate utilisation may be used to characterise the functional differences among microbial communities (Buyer & Drinkwater, 1997), which reflect the metabolic capabilities of the part of the community that can actively metabolise the given substrates (Palojarvi *et al.*, 1997). Signature lipid biomarker detection provides a method that is quantitative, independent of cell culturability and allows the identification of bacteria that have distinctive PLFA patterns in a single analysis (Macnaughton *et al.*, 1997). Phospholipids can subsequently be considered as a fingerprint of the microbial community (Peterson & Klug, 1994), which provides a technique to determine the 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 (Mandelbaum *et al.*, 1997; Steward *et al.*, 1996).

Based on all the aforementioned literature it is evident that the evaluation of substrate utilisation profiles and signature lipid biomarkers could provide meaningful data concerning microbial communities without being subject to the disadvantages often associated with the conventional microbiological techniques. Furthermore, these techniques could also contribute significantly towards the understanding of microbial communities *in situ*. The analysis of carbon source utilisation profiles and signature lipid biomarkers could thus be of significant value in both environmental and industrial microbiology, and could contribute substantially toward understanding and controlling microbial communities in paper mill water systems.

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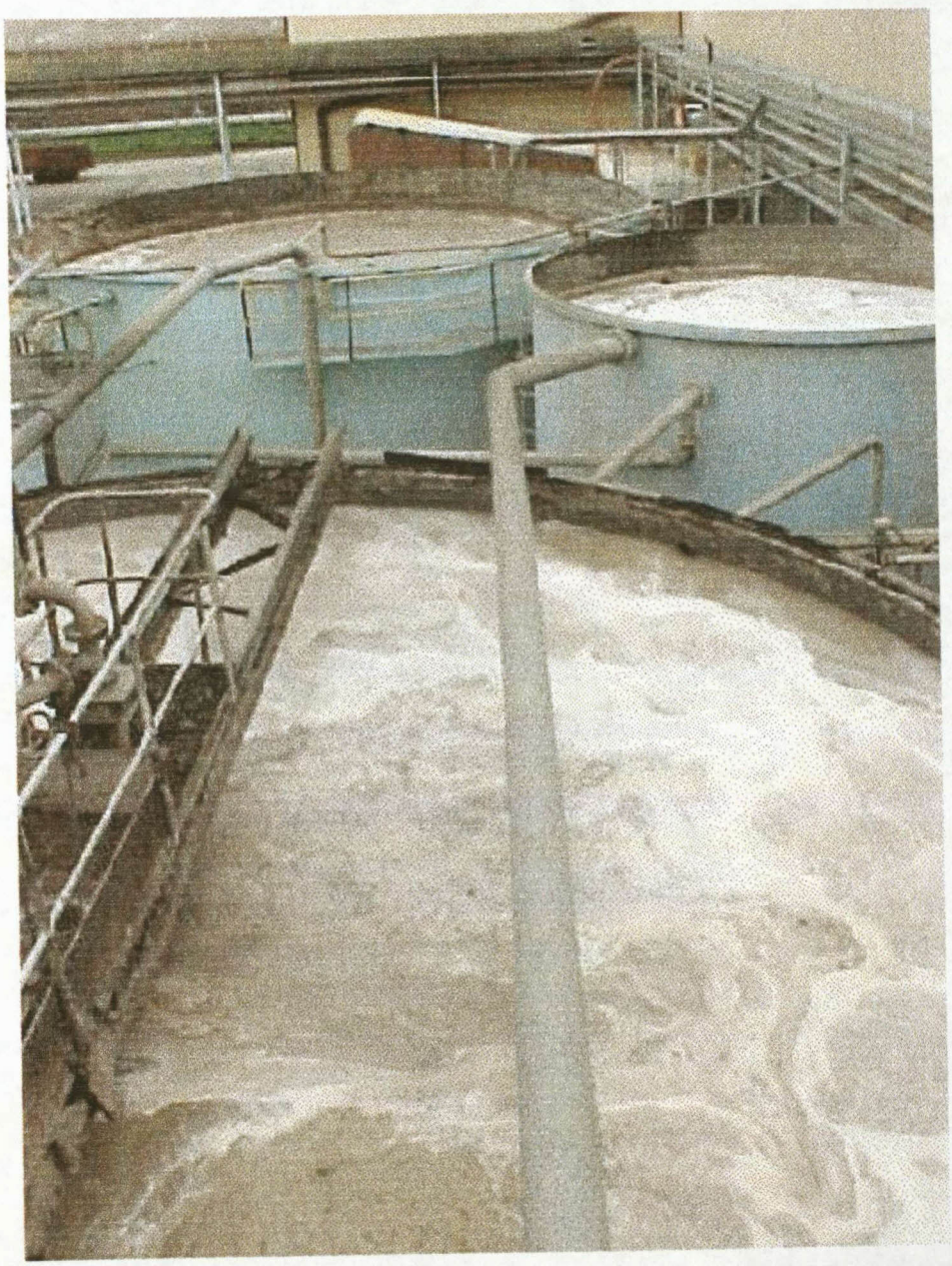
CHAPTER 2

FUNCTIONAL DIVERSITY OF THE MICROBIAL COMMUNITY IN A PAPER MILL WATER SYSTEM



ABSTRACT

Most microbiological studies of wastewater treatment systems rely on methods that are dependant on culturing. Due to the limitation of conventional methods, the numbers of microorganisms are largely underestimated and numerous alternative assays have been proposed. One possible alternative is the analysis of the functional diversity of the microbial community, which is based on carbon source utilisation profiles. The Biolog method was, therefore, evaluated in a paper mill water system. The influence of the production of various paper grades, biocide application and monthly shuts on the functional diversity of the microbial communities was determined. The communities in the planktonic as well as the sessile phases were analysed every two weeks for a period of one year. The average well colour development technique was used to transform the data obtained from the Biolog plates prior to multivariate statistical analysis (discriminant analysis). Results obtained during this study indicated that different microbial communities developed during the production of the different paper grades (liner versus fluting). A difference in the functionalities of the planktonic communities was evident after a single day of production of fluting or linerboard, while differences in the functional diversities of the sessile communities only became significant after five days of continuous production of a specific paper grade. Furthermore, the effect of different biocide dosage was more distinct in the planktonic than in the sessile phase. No clear trends concerning the period of time after a shutdown could be observed in the planktonic or sessile samples. Biodiversity indices showed that a high functional diversity existed in both the planktonic and sessile phases. On the basis of results obtained during this study, it could be concluded that the evaluation of substrate utilisation profiles was a sensitive measure, which enabled the detection of changes in the system and differentiation between microbial communities within the same water system.

INTRODUCTION

In paper mills, microbial biofilms play a considerable role in microbiologically induced corrosion, production of odours, paper breakages, spotting, holes and discolouration of paper. These aspects result in a loss of production and product quality that may have major economic implications for a paper mill (Stoner & King, 1994). It is, therefore, important to study biofilm formation and the factors that lead to biofilm formation.

The Sappi Cape Kraft paper mill, Milnerton, Cape Town, produces both fluting and linerboard from different sources of recycled fibre. The main distinction between these two grades of paper is the difference in composition and processing characteristics of the paper. The mill has a relatively closed water system with low effluent discharge. Microbial contamination and fouling are consequently enhanced primarily due both to the reuse of water and recycled fibre that serves as a continuous source of inoculum of microbial contamination (Sorelle & Belgard, 1991). Sorelle and Belgard (1991) reported microbial counts of up to 977 times higher when recycled furnish was used in comparison to virgin pulp. Increased microbial contamination may subsequently also result in an increase in biocide dosage and subsequent cost of production. Biofilm development within industrial systems can be controlled by the application of biocides, which are specific for certain organisms or environmental conditions. Biocide efficacy is generally monitored by using conventional microbiological techniques, including culturing, and biochemical assays.

Many problems are, however, experienced with the application and interpretation of results obtained using conventional microbiological techniques. Recent studies have indicated that less than 1 % of all microbes are culturable on artificial media (Vestal & White, 1989; Palojarvi *et al.*, 1997). Conventional culturing methods are restrictive due to the media used and the interruption of interactions that exists between microorganisms *in situ* (Kersters *et al.*, 1997). Sampling techniques that are applied in industrial microbiology generally result in microbial stress and the microorganisms may become dehydrated and damaged, which would prevent culturing (Macnaughton *et al.*, 1997). Most microbiological studies of wastewater treatment systems rely on cultivation, which quantifies a limited percentage of the microbial populations present

(Schneider *et al.*, 1998). Subsequently, incorrect conclusions may be made concerning the function and structure of the microbial community *in situ*. Due to the limitation of conventional methods, numerous alternative techniques to characterise microbial communities *in situ* have been developed.

One specific technique includes the characterisation of the functional diversity of the microbial community. The functional diversity of a microbial community can be defined as the numbers, types, activities and rates at which a suite of substrates are utilised (Zak *et al.*, 1994). Garland and Mills (1991) proposed that substrate utilisation using commercially available Biolog plates (Biolog Inc., Hayward, USA) could be used to characterise the functional differences among microbial communities. Although the Biolog system was initially developed to identify pure bacterial strains, it is currently widely used to analyse the carbon source utilisation patterns of mixed microbial communities (Guckert *et al.*, 1996). Biolog analysis can, therefore, reflect the metabolic capabilities of the part of the community that can actively metabolise the given substrates (Palojarvi *et al.*, 1997).

The application of the substrate utilisation assay using Biolog plates has been studied extensively in various environments. This approach has successfully been used to classify and compare heterotrophic microbial communities (Garland & Mills, 1991), follow microbial succession in developing compost (Carpenter-Boggs *et al.*, 1998) and characterise microbial communities in wastewater treatment systems (Victorio *et al.*, 1996). Schneider *et al.* (1998) stated that this approach yielded a more sensitive and ecologically relevant measure of heterotrophic community structure than conventional microbiological analyses.

The aim of this study was therefore to monitor the functional diversity of the microbial community in the water system of the Sappi Cape Kraft paper mill, Milnerton, over a period of one year. The influence of the production of various paper grades (linerboard / fluting), biocide usage and monthly shuts on the functional diversity of the microbial community in both the planktonic and sessile phases were also characterised.

MATERIALS AND METHODS

Mill Operations

The Sappi Cape Kraft paper mill, Milnerton, Cape Town, South Africa produces both fluting and linerboard from different sources of recycled fibre. A higher quality of recycled fibre, i.e. corrugated containers without any plastic, glue or staples is used during the production of linerboard, while the rest of the mixed office waste and magazines are used during the production of fluting. Fluting is produced at pH 6,5 to pH 7,5 while linerboard is produced at pH 4,5 to pH 5,5. Aluminium sulphate and rosin size are also added during the production of linerboard to increase the properties that enhance the printing quality, which makes storage under conditions of high humidity possible. No additional compounds are added to the fibre during the production of fluting.

During the period of evaluation, different biocides were added to the water system to control microbial fouling. Biocide BC4XL is an organosulphur (sulphone) and quaternary ammonium compound combination with optimum effectivity at a pH of 4 to pH 8. It is a broad spectrum biocide that is effective against bacteria and fungi. Biocide BC6 is an organosulphur (carbamate) compound that functions optimally at pH 4 to pH 10. Although this is also a broad-spectrum biocide, it is more effective against sulphate reducing bacteria. Biocide SNP3002 is a quaternary ammonium compound and organotin combination that functions optimally at a pH 4 to pH 10. This broad spectrum biocide is more effective against fungi than bacteria. All the biocides are applied in the clarifier.

Furthermore, during the period of evaluation, routine shutdowns of the papermachine were performed approximately every four weeks for maintenance and cleaning procedures. These shutdowns did not involve any boilout procedures.

Sample Collection

Duplicate planktonic and sessile samples (approximately 50 ml) were collected from the Cape Kraft mill every two weeks for a period of one year. Planktonic samples

(planktonic phase) were collected from the Former #5 overflow, while biofilm (sessile phase) samples were collected from the splash plate at Former #2.

Sample Preparation

The samples were cooled during transport and were processed within 24 hours of sampling. A stock solution of phosphate buffer was prepared by dissolving 12,36 g Na_2HPO_4, 1,80 g NaH_2PO_4 and 85,0 g NaCl in 1 L deionised water (Guckert *et al.*, 1996). This solution was filter sterilised (0,22 µm) and stored at 4 °C. The buffer was prepared by mixing 100 ml of the stock solution in 1 L deionised water. Approximately 5 ml of biofilm or 10 ml of water from the various samples was added to 30 ml of phosphate buffer and homogenised (Heidolph Diax 600, Germany) as described by Guckert *et al.* (1996) and centrifuged (500 x g) to remove any particulate matter. The supernatant was standardised to a turbidity of 0,25 to 0,35 absorbance units (420 nm) using the phosphate buffer (Guckert *et al.*, 1996).

Inoculation

The standardised microbial suspensions were poured into a reagent reservoir and aliquots (150 µl) were added to each well of the GN Biolog microtiter plates (Biolog Inc., Hayward, USA) using a multi-channel pipettor. Duplicate Biolog plates were evaluated for absorbance changes at 590 nm using a Labsystems iEMS microtiter plate analyser (Labsystems, Helsinki, Finland) since Zak *et al.* (1994) concluded that 590 nm is the peak absorbance of tetrazolium violet. Following an initial (time 0) reading, the Biolog plates were incubated at 25 °C in the dark. The reduction of tetrazolium violet in each well was measured after 12, 24, 36, 48 and 64 hours of incubation.

Data Processing

The optical density (OD) values (Appendices A & B) in each Biolog microplate were corrected for the background colour in the control well (A1) without carbon source. Negative values were converted to zero and the data obtained from the Biolog microplates were analysed using the average well colour development (AWCD) technique as described by Garland (1996). According to O'Connell *et al.* (2000) multiple readings and standardization of each sample are the most commonly used

approach. In the AWCD technique, any variance in the inoculum density was accounted for by dividing the absorbance of each well by the average absorbance for the whole plate, giving the standardised OD. Standardised patterns rather than the absolute values were consequently compared.

During this study, patterns were compared from the intermediate phase of the Biolog incubation and an AWCD of 0,3 to 0,5 absorbance units was used as the reference point for multivariate statistical analysis of the data (Fritze *et al.*, 1997; Heuer & Smalla, 1997). Biolog results were analysed using the NCSS 97 software (Statistical Solutions, Ireland). During the discriminant analysis, stepwise variable selection was employed. Multivariate analysis (discriminant analysis) was performed on the AWCD transformed data. Multivariate analyses are frequently employed to compare substrate utilisation profiles from various samples (O'Connell *et al.*, 2000). A carbon source was assumed to be important for the discrimination of compared microbial communities when the standardised OD was significantly different ($p \leq 0,005$) between samples and its weighted sum of factor-loadings was higher than the median, suggesting a significant contribution to the multivariate statistical difference (Heuer & Smalla, 1997).

Since the group membership during this study was known *a priori*, multivariate statistical analysis using discriminant analysis was very applicable (Fritze *et al.*, 1997). Discriminant analysis selects those variables that yield the best separation according to the given groups. Hypothesis testing and a more rigorous analysis of catabolic profiles can be achieved using discriminant analysis (Buyer & Drinkwater, 1997).

The Wilks' lambda was used to test the significance of the discriminant function. A value near zero indicates an accurate model while a value near one indicates a poor model. The F value tested the significance of the Wilks' lambda. The significance level of the F-test is represented by p where a value less than 0,05 is considered significant (Hintze, 1997).

The functional diversity of the analysed microbial communities was quantified using a substrate diversity index (Magurran, 1988; Zak *et al.*, 1994):

$H' = - \Sigma [p_i \ln (p_i)]$ .....(1)

Where p_i is the ratio of the corrected OD_{590} value for a carbon source to the sum of the corrected OD_{590} values for all substrates.

The substrate equitability of the microbial populations was also determined. The substrate equitability (J) is a rescaling of the substrate diversity index (Magurran, 1988; Zak *et al.*, 1994):

$J = H' / H'_{max}$ .....(2)

Where H'_{max} is the maximal substrate diversity index for the plates.

The similarity between samples was calculated using Sorenson's measure for quantitative data (Equation 3) (Magurran, 1988). The matrix obtained (Appendix C) was used to construct a dendogram with the Group Average (Unweighted pair group) method (NCSS 97, Statistical Solutions, Ireland).

$C_N = 2jN / (aN + bN)$ .....(3)

Where C_N = Sorenson's measure of similarity;

aN = the sum of the turbidities for sample a;

bN = the sum of the turbidities for sample b and

jN = the sum of the lower of the two turbidities for each carbon source in the two samples compared.

RESULTS AND DISCUSSION

Influence of Production of Different Paper Grades on the Functional Diversity of the Microbial Community

Analysis of Planktonic Samples

The functional diversity of the planktonic microbial communities differed significantly (Wilks' Lambda: 0,52; F = 12,4; $p \leq 0,05$) (Figure 1). The first canonical score, obtained from the discriminant analysis accounted for 100% of the total variability observed within the data set.

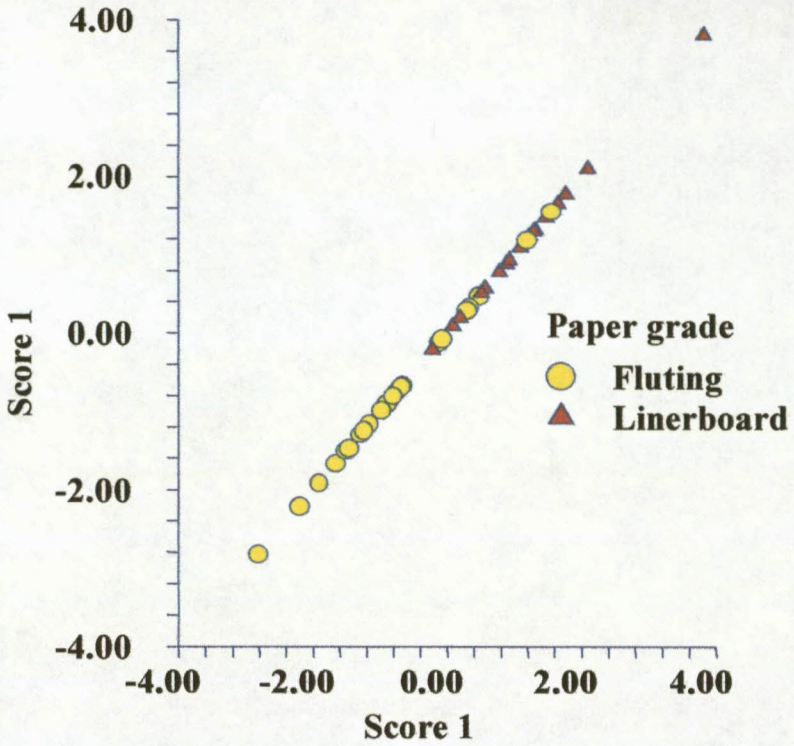


Figure 1. Ordination plot of the canonical-variate scores obtained for the planktonic microbial community after one day of production of a specific paper grade.

Significant differences were observed between the microbial communities in the planktonic phase when fluting and linerboard were produced. These differences occurred within a single day after changing the production process (Figure 1), although some overlapping between the functional diversity of the microbial communities during the production of linerboard and fluting was apparent.

Based on the results of the discriminant analysis performed on the planktonic samples obtained after six days of production of a specific paper grade, it was evident that the functional diversity of the planktonic microbial communities differed significantly (Wilks' Lambda: 0,25; $F = 32,1$; $p \leq 0,05$) (Figure 2). The first canonical score, obtained from the discriminant analysis accounted for 100% of the total variability observed within the data set.

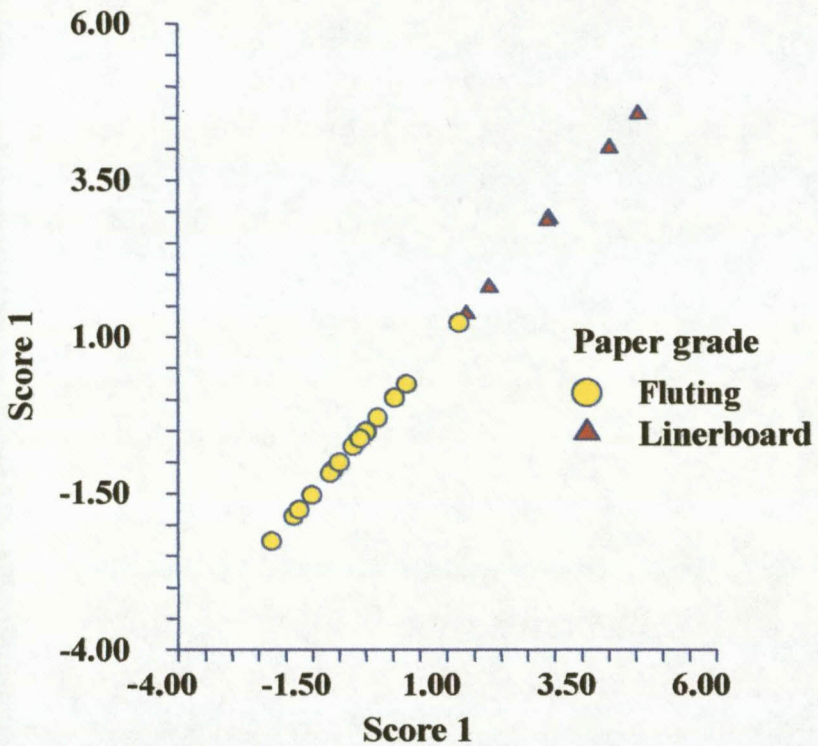


Figure 2. Ordination plot of the canonical-variate scores obtained for the planktonic microbial community after six days of production of a specific paper grade.

On the basis of the results presented in Figure 2, it is evident that when the specific production process continued for an extended period (six days), the differences in the functional diversity of the planktonic microbial communities became more pronounced and less overlapping in the overall functionality of the microbial communities was evident, when compared to that observed after one day of production. The decrease in the Wilks' Lambda value compared to that after a single day of production of a specific paper grade shows a significant improvement in the predictability of the model since values close to zero are indicative of a very accurate model (Hintze, 1997).

The shifts in the functional diversity of the microbial community might be caused by the differences in pH during the production of fluting and linerboard, as well as the

various chemical additions made to the fibre during the production of linerboard. The acidic conditions during the production of linerboard would favour the growth of fungi and acidophilic bacteria (Hughes, 1993). In contrast, the production of fluting would primarily select for bacterial growth, since fluting was produced at higher pH values (Hughes, 1993).

The catabolic capabilities (AWCD) of the microbial communities, differed significantly ($p < 0,05$) in their ability to utilise three of the 95 different carbon sources after one day (Table 1) and two of the 95 different carbon sources supplied on the Biolog microtiter plate after six days of production of a specific paper grade (Table 2).

Table 1. The mean AWCD and standard deviation of the independent variables found to differ significantly between the various planktonic samples as obtained after one day of production of a specific paper grade.

	Production grade	
Variables	**Fluting**	**Linerboard**
Tween 80	0,178 ± 0,339	0,449 ± 0,475
α-Keto valeric acid	0,250 ± 0,422	0,050 ± 0,160
L-histidine	0,551 ± 0,670	1,215 ± 0,474

Table 2. The mean AWCD and standard deviation of the independent variables found to differ significantly between the various planktonic samples as obtained after six days of production of a specific paper grade.

	Production grade	
Variables	**Fluting**	**Linerboard**
α-Keto glutaric acid	0,566 ± 0,478	1,331 ± 0,688
Urocanic acid	8,732E-02 ± 0,670	0,624 ± 0,272

Differential catabolism of an amino acid (L-histidine), a polymer (Tween 80) and a carboxylic acid (α-Keto valeric acid) was responsible for differentiation of the microbial communities present in the various planktonic samples after one day of production of a specific paper grade (Table 1). Differential utilisation of an aromatic

chemical (Urocanic acid) and a carboxylic acid (α-Keto valeric acid) was responsible for differentiation of the microbial communities present in the various planktonic samples after six days of production of a specific paper grade (Table 2). These results indicated that different microbial populations, each with a unique capacity to utilise specific substrates, had developed in the planktonic samples during the production of fluting and linerboard. Victorio *et al.* (1996) also reported that microbial communities from pulp mill effluent treatment systems could be differentiated by the utilisation of urocanic acid and L-histidine.

Analysis of Sessile Samples

In contrast to the results as obtained for the planktonic microbial community, the results of the discriminant analysis of the substrate utilisation profiles of the sessile samples indicated that the functional diversity of the microbial communities only differed significantly (Wilks' Lambda: 0,46; $F = 15,2$; $p \leq 0,05$) after five days of production of a specific paper grade (Figure 3). The first canonical function accounted for 100% of the total observed variability in the data set.

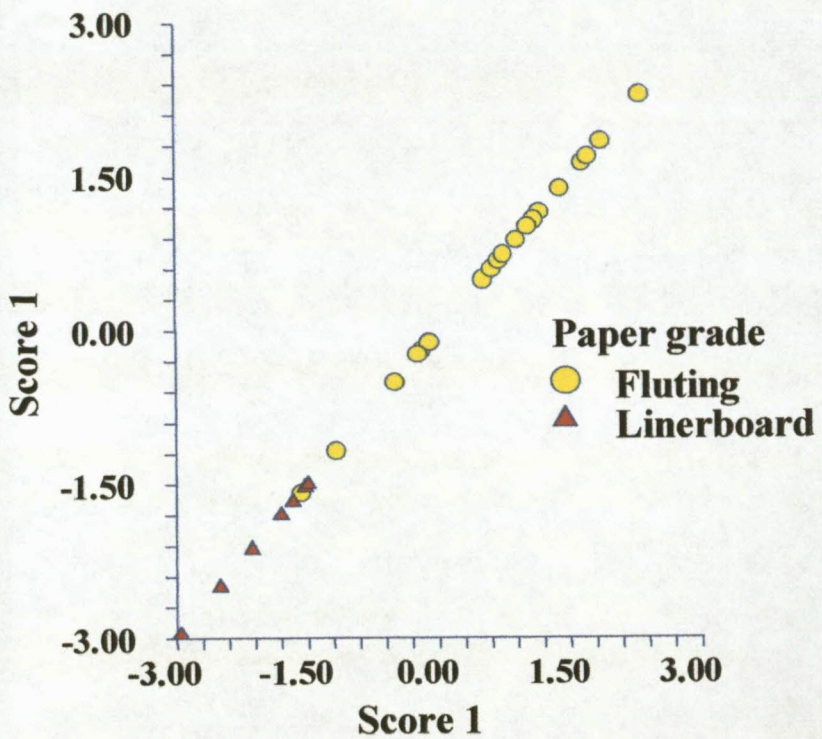


Figure 3. Ordination plot of the canonical-variate scores for the sessile microbial community after five days of production of a specific paper grade.

The results obtained for the sessile samples indicated that no immediate shift in the functional diversity of the microbial communities present in the sessile phase could be observed. The results obtained indicated that the functional diversity of the microbial communities in the biofilm remained relatively constant and did not respond immediately to the change in the production of different grades. This is in contrast to the results obtained for the microbial community present within the planktonic phase of the paper mill water system. These results illustrate and confirm the protective effect of biofilm development where the microorganisms are not as exposed to a change in environmental conditions.

The functional diversity of the microbial communities in the various sessile samples differed significantly ($p < 0,05$) in their ability to utilise two of the 95 different carbon sources supplied on the Biolog microtiter plate after five days of production of a specific paper grade (Table 3). Differential catabolism of a carboxylic acid (D-saccharic acid) and a phosphorylated chemical (D,L-α-glycerol phosphate) was responsible for differentiation of the microbial communities present in the various sessile samples after five days of producing a specific paper grade. These results indicate that different microbial populations, each with a unique function, had developed in the sessile samples due to the production of fluting and linerboard.

Table 3. The mean AWCD and standard deviation of the independent variables found to differ significantly between the various sessile samples after five days of production of a specific paper grade.

	Production grade	
Variables	**Fluting**	**Linerboard**
D-saccharic acid	1,168 ± 0,653	1,599 ± 0,319
D,L-α-glycerol phosphate	0,560 ± 0,363	0,158 ± 0,282

Influence of Different Biocides on the Functional Diversity of the Microbial Community within the Water System

Based on the results of the discriminant analysis (multivariate analysis) it is evident that the functional diversity of the microbial communities in the planktonic samples differed significantly (Wilks' Lambda: 0,40; $F = 21,2$; $p \leq 0,05$) based on the use of different biocides within the water system (Figure 4). The first canonical function accounted for 100% of the total observed variability in the data set.

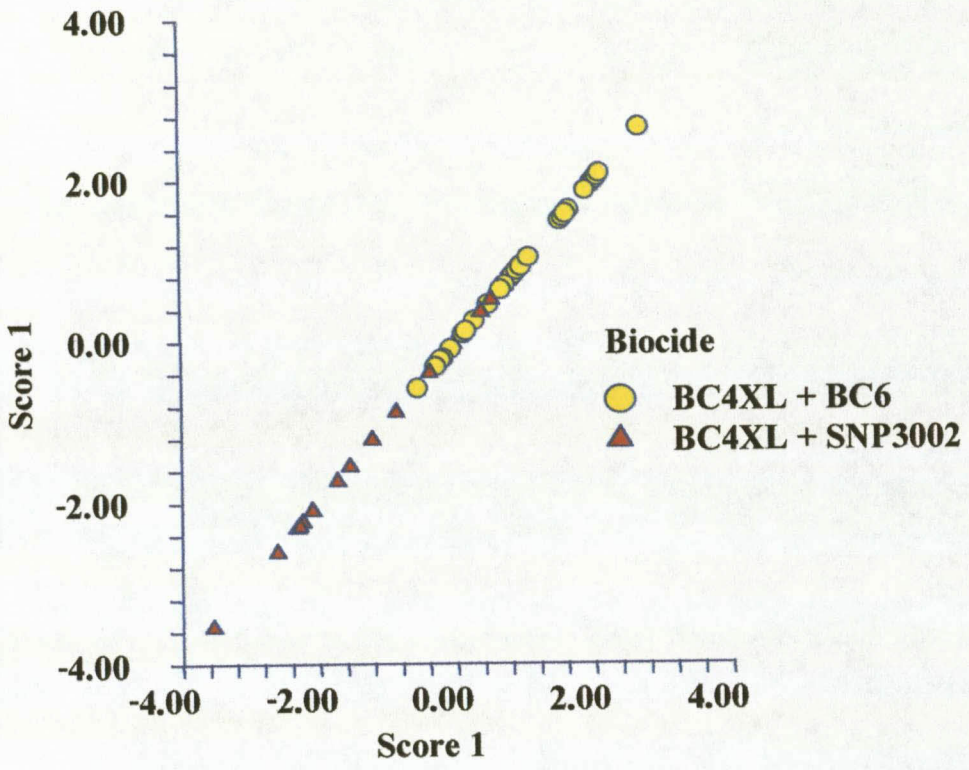


Figure 4. Ordination plot of the canonical-variate scores for the planktonic microbial community based on different biocide usage.

Based on the results obtained from the discriminant analysis it is evident that the functional diversity of the microbial communities in the sessile samples also differed significantly (Wilks' Lambda: 0,61; $F = 28,0$; $p \leq 0,05$) when different biocides were used within the water system (Figure 5).

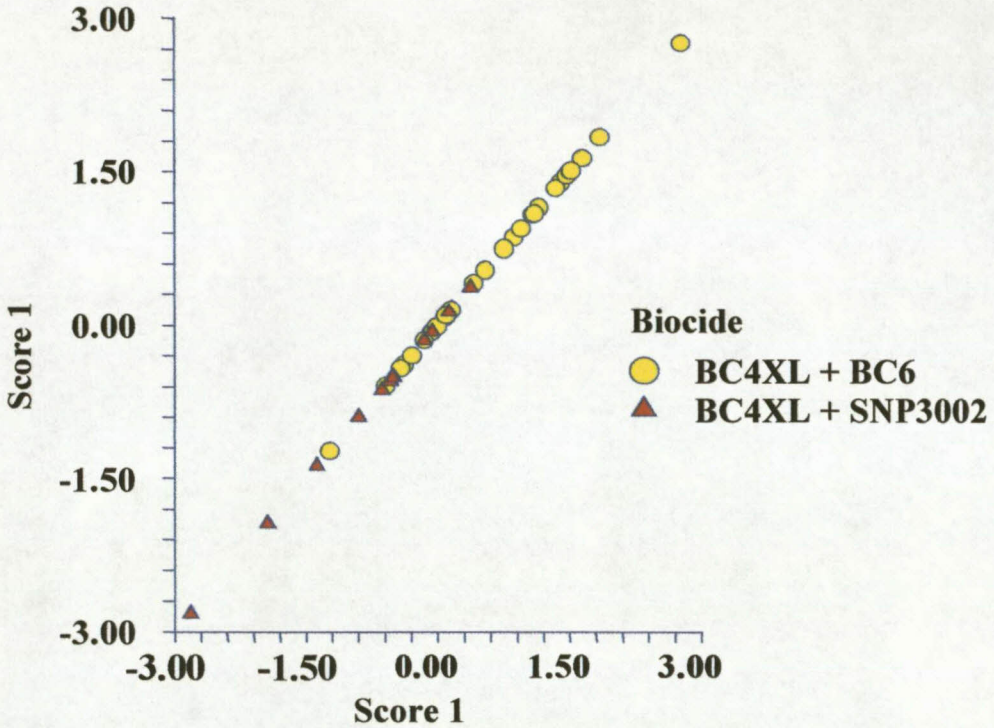


Figure 5. Ordination plot of the canonical-variate scores for the sessile microbial community based on different biocide usage.

Robertson (1994) reported that microorganisms in biofilms are more resistant to biocides than free-floating microorganisms. In our study the effect of different biocide dosages was more distinct in the planktonic than in the sessile phase (Figure 4 & 5), illustrating the protective effect of biofilms. A better separation between the various planktonic samples was observed since less overlapping between the various samples was evident. As was evident from the results, the biocides used, selected for microbial communities with differences in their functional diversities.

The catabolic capabilities of the microbial communities in the planktonic phase differed significantly ($p < 0,05$) in their ability to utilise three of the 95 different carbon sources (Table 4). However, the sessile communities differed significantly in their ability to utilise only one of the 95 different carbon sources based on different biocides used (Table 5). Differential catabolism of a carbohydrate (α-D-lactose) and the carboxylic acids D-gluconic acid and propionic acid was responsible for differentiation of the microbial communities present in the various planktonic samples as a result of the different biocides applied. Differential catabolism of a carbohydrate

(maltose) was responsible for differentiation of the microbial communities present in the various sessile samples as a result of the different biocides applied. These results indicated that different microbial populations had developed in the planktonic and sessile samples as a result of the different biocides applied. Slight overlapping between the functional diversity of the microbial communities was, however, observed. This may be the result of overlapping functionalities caused by the application of different biocides. The shifts in the functional diversity of the microbial communities could possibly be caused by the differences in biocide composition.

Table 4. The mean AWCD and standard deviation of the independent variables observed to differ significantly between the various planktonic samples based on the use of different biocides.

	Biocides	
Variables	**BC4XL + BC6**	**BC4XL + SNP3002**
α-D-lactose	1,435 ± 0,648	1,512 ± 1,536
D-gluconic acid	2,193 ± 0,627	1,094 ± 0,757
Propionic acid	0,256 ± 0,297	1,465 ± 1,705

Table 5. The mean AWCD and standard deviation of the independent variables observed to differ significantly between the various sessile samples based on the use of different biocides.

	Biocides	
Variable	**BC4XL + BC6**	**BC4XL + SNP3002**
Maltose	2,278 ± 0,593	1,223 ± 0,768

Influence of Paper machine Shutdown on the Functional Diversity of the Microbial Community within the Water System

On the basis of multivariate statistical analysis of substrate utilisation profiles no clear trends concerning the age (weeks after a shutdown) for the sessile phase could be observed. This could possibly be due to inefficient cleaning during the shuts. Based on the results of the discriminant analysis it was evident that the functional diversity of the microbial communities in the planktonic phase differed based on the time that has elapsed after cleaning during a shut.

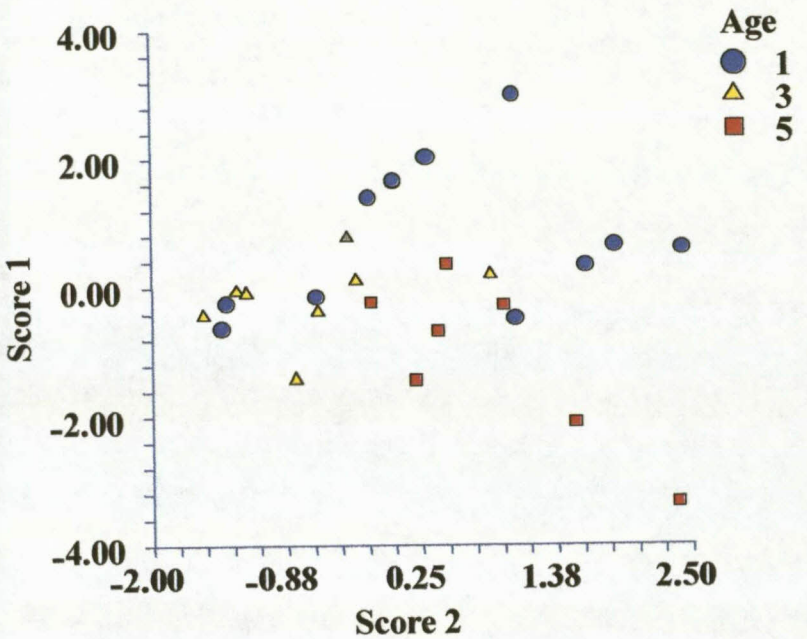


Figure 6. Ordination plot of the canonical-variate scores for the planktonic microbial community based on the age (weeks that had elapsed) after a shutdown.

Biodiversity

On the basis of the substrate diversity values obtained using the substrate diversity (H) indices (Equation 1), it is evident that a high substrate diversity existed in all the planktonic and sessile samples (Table 6). According to Magguran (1998) values of the Shannon index fluctuate between 1,5 and 3,5 and rarely increase above 4,5. Values obtained during the present study generally were within the higher range of

diversity, indicating that very high substrate diversity values were obtained. The high substrate diversity values obtained during this study was confirmed by the fact that during the present study most of the carbon sources were utilised, contributing to the very high Shannon indices.

The substrate equitability (*J*) indices (Equation 2) obtained for the various planktonic and sessile samples did not show any trends or significant differences (Table 7). Magguran (1988) stated that a high evenness (equitability) would imply a high diversity. It can therefore be concluded that a high functional diversity existed in all the planktonic and sessile samples. Zak *et al.* (1994) concluded that two sites with identical substrate richness, evenness and diversity could result from the catabolism of different substrates. This is in correspondence with results obtained during this study where high functional diversity existed in the planktonic and sessile samples, although the phases could be differentiated based on the utilisation of different substrates. The fluctuations in the substrate diversity values are possibly the result of the continuous change in the various production parameters (paper grade and biocide dosage). No correlation between the parameters and the different indexes could, however, be found, since the combination of the different parameters might influence the communities that develop.

Table 6. Substrate diversity values (H) obtained for the various samples.

	Planktonic phase		Sessile phase	
Sample #	H Value	Standard Deviation	H Value	Standard Deviation
1	4,341	0,038	4,259	0,054
2	4,258	0,041	4,310	0,014
3	4,193	0,079	4,332	0,086
4	4,133	0,198	3,876	0,026
5	3,875	0,055	4,137	0,009
6	3,941	0,065	4,004	0,160
7	4,394	0,064	3,772	0,243
8	4,129	0,052	3,957	0,133
9	4,381	0,028	4,028	0,145
10	4,153	0,150	4,232	0,044
11	4,035	0,149	3,861	0,034
12	4,268	ND	3,830	ND
13	3,878	ND	4,332	ND
14	3,992	ND	4,112	ND
15	4,167	0,041	4,324	0,074
16	4,039	0,037	3,852	0,292
17	3,862	0,203	4,251	0,066
18	4,138	0,030	4,411	0,103
19	4,402	0,016	4,253	0,016
20	4,159	0,086	4,157	0,254
21	4,101	0,147	4,432	0,030
22	4,251	0,070	3,583	0,183
23	3,361	1,468	3,895	0,355
24	3,619	0,439	3,699	0,218
25	2,878	0,402	4,046	0,052

ND = Analysis not performed in duplicate

Table 7. Substrate equitability indices (*J*) obtained for the various samples.

	Planktonic phase		Sessile phase	
Sample #	*J* Value	Standard Deviation	*J* Value	Standard Deviation
1	0,953	0,008	0,935	0,012
2	0,935	0,009	0,946	0,003
3	0,921	0,017	0,951	0,019
4	0,908	0,043	0,851	0,006
5	0,851	0,012	0,908	0,002
6	0,865	0,014	0,879	0,035
7	0,965	0,014	0,828	0,053
8	0,907	0,011	0,869	0,029
9	0,962	0,006	0,884	0,032
10	0,912	0,033	0,929	0,010
11	0,886	0,033	0,848	0,007
12	0,937	ND	0,841	ND
13	0,852	ND	0,951	ND
14	0,877	ND	0,903	ND
15	0,915	0,009	0,950	0,016
16	0,887	0,008	0,846	0,064
17	0,848	0,044	0,934	0,014
18	0,909	0,007	0,96	0,023
19	0,967	0,003	0,934	0,003
20	0,913	0,019	0,913	0,056
21	0,901	0,032	0,973	0,007
22	0,934	0,015	0,787	0,040
23	0,738	0,322	0,85	0,078
24	0,795	0,096	0,812	0,048
25	0,632	0,088	0,889	0,011

ND = Analysis not performed in duplicate

In contrast to the Shannon diversity index, Sorenson's index emphasises the degree of substrate utilisation. In the dendogram of the similarity in carbon source utilisation of the samples within the sessile phase (Figure 7), the clusters obtained (Appendix C) could be distinguished based on their Shannon indices. The Shannon index emphasises the amount of carbon sources that are utilised. Cluster A represents a high degree of substrate utilisation (average value of 6,422), while cluster B represents a cluster that only utilises a small variety of carbon sources (average value of 2,900).

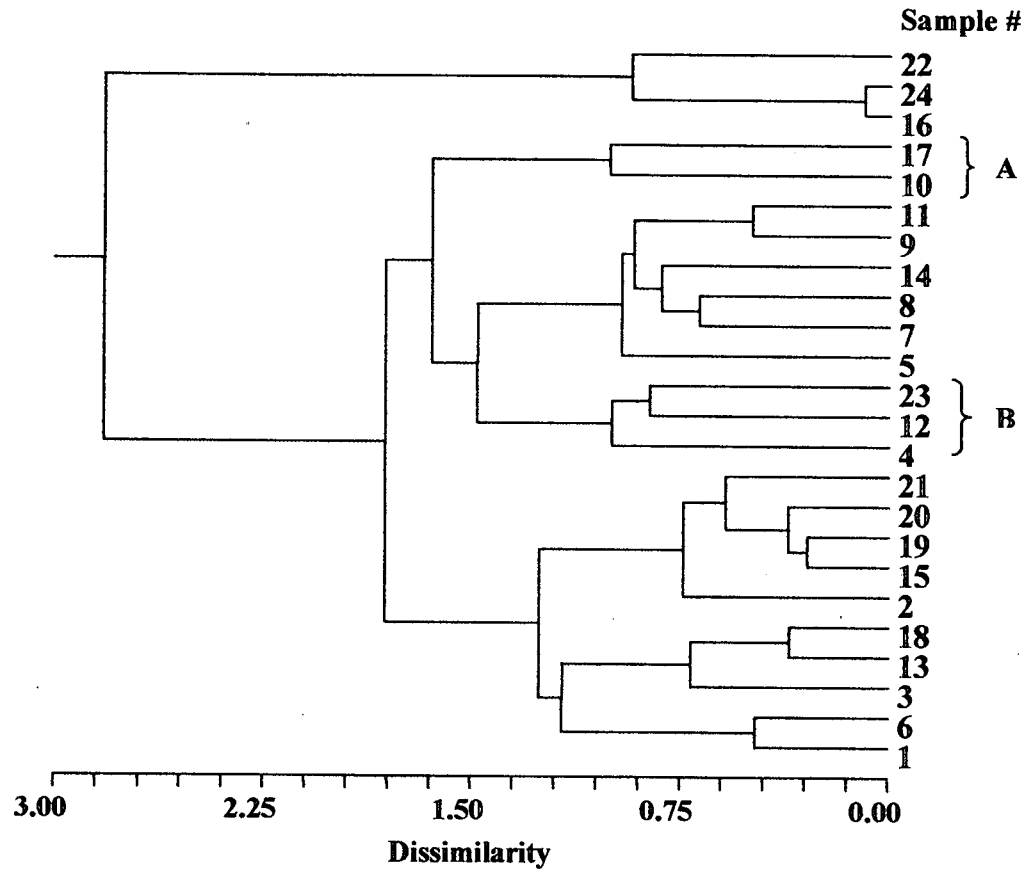


Figure 7. Similarity in carbon source utilisation patterns between the various samples as obtained from the sessile phase.

In contrast to the sessile phase, no clustering could be performed based on the similarity in carbon source utilisation of the samples within the planktonic phase (Figure 8). This could possibly be attributed to the rapid shifts in the microbial communities within the planktonic phase. It could be speculated that the community within the sessile phase was more stable than the community within the planktonic phase.

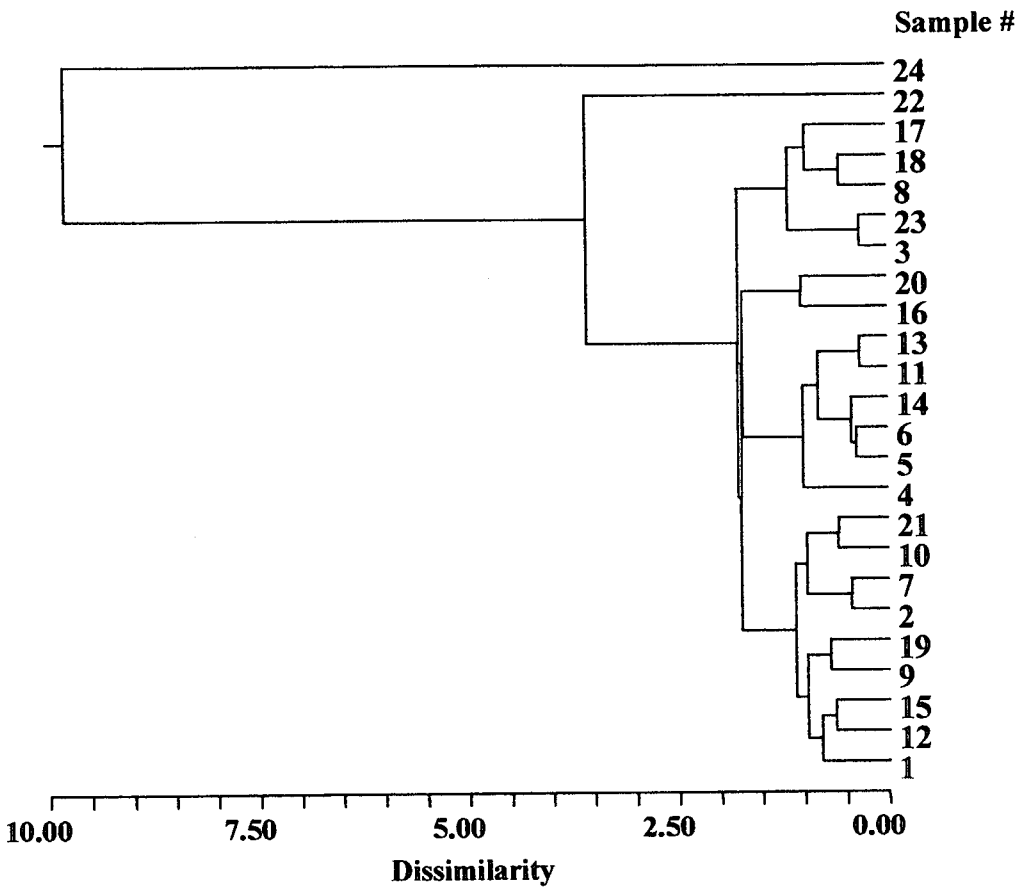


Figure 8. Dissimilarity in carbon source utilisation patterns between samples between the various samples as obtained from the planktonic phase.

CONCLUSIONS

On the basis of the results obtained during this study it was evident that unique microbial communities had developed in the water system when linerboard and fluting were produced. The shifts in the functional diversity were probably caused by the difference in pH and chemical composition of the planktonic phase during the various production processes. Although the planktonic and sessile microbial communities could utilise a wide range of substrates, the communities could only be differentiated by the utilisation of a few substrates, illustrating that microbial communities with different functionalities had developed within each phase. These results were in correspondence with results as obtained by Kaiser *et al.* (1998), who reported that substrate utilisation was a sensitive method to detect differences in the metabolic responses of mixed microbial communities. The planktonic microbial communities in the water system were much more sensitive to changes in the external environment with the shifts in the functional diversity of the microbial communities being much more pronounced and rapid when compared to the sessile microbial communities. This could possibly be due to the protective effect of biofilms and the fact that attached microorganisms may be better adapted than those occurring in the planktonic phase (Robertson, 1994).

Furthermore, the results of this study suggest that the various biocides applied, selected for communities with differences in their functional diversities, although slight overlapping was evident. The overlapping of the microbial communities may be the result of overlapping functionalities caused by the selection of specific microbial populations by the biocides. These results suggest that different biocides should, therefore, be used when different products are manufactured. The implementation of a microbial control strategy or programme at the plant will have to take the variation in the production process into consideration and that biocides will have to be selected for a specific production process. Biocide dosage into the water system would have a dramatic and immediate effect on the microorganisms present in the planktonic phase, whilst the response in the sessile phase would be delayed.

No clear trends concerning the age of populations (time that had elapsed after a shutdown) on the functional diversity of the microbial community in the sessile phase

could be observed. This observation could possibly be due to inefficient cleaning during the shutdown period. In contrast, differences in the functional diversity of the microbial communities within the planktonic phase were observed.

The high substrate equitability values (values close to 1) indicated that the microbial communities in the planktonic and sessile samples were growing in the same proportion on all of the carbon sources. The high substrate diversity values obtained (values close to 4), also implied a high functional diversity. Based on the clustering of the results obtained using Sorensen's index, it was evident that the community within the sessile phase was more stable than the community within the planktonic phase.

On the basis of the results obtained during this study, it was evident that the statistical evaluation of carbon source utilisation profiles provides an alternative technique to conventional culturing which is sensitive enough to detect shifts in the microbial communities due to changes in the operational procedure of the mill (mode of paper production and biocide application). The detection of shifts in the microbial community is in correlation to data obtained by Schneider *et al.* (1998). The authors found that phenotypic fingerprinting was a more sensitive method than conventional microbiological methods to detect changes in the microbial community. It may thus be concluded that the evaluation of substrate utilisation using the Biolog assay was a sensitive measure to detect changes in the system and to differentiate between microbial communities within the same system. This approach could be of significant value during the selection and implementation of a microbial control programme within the water system and paper plant.

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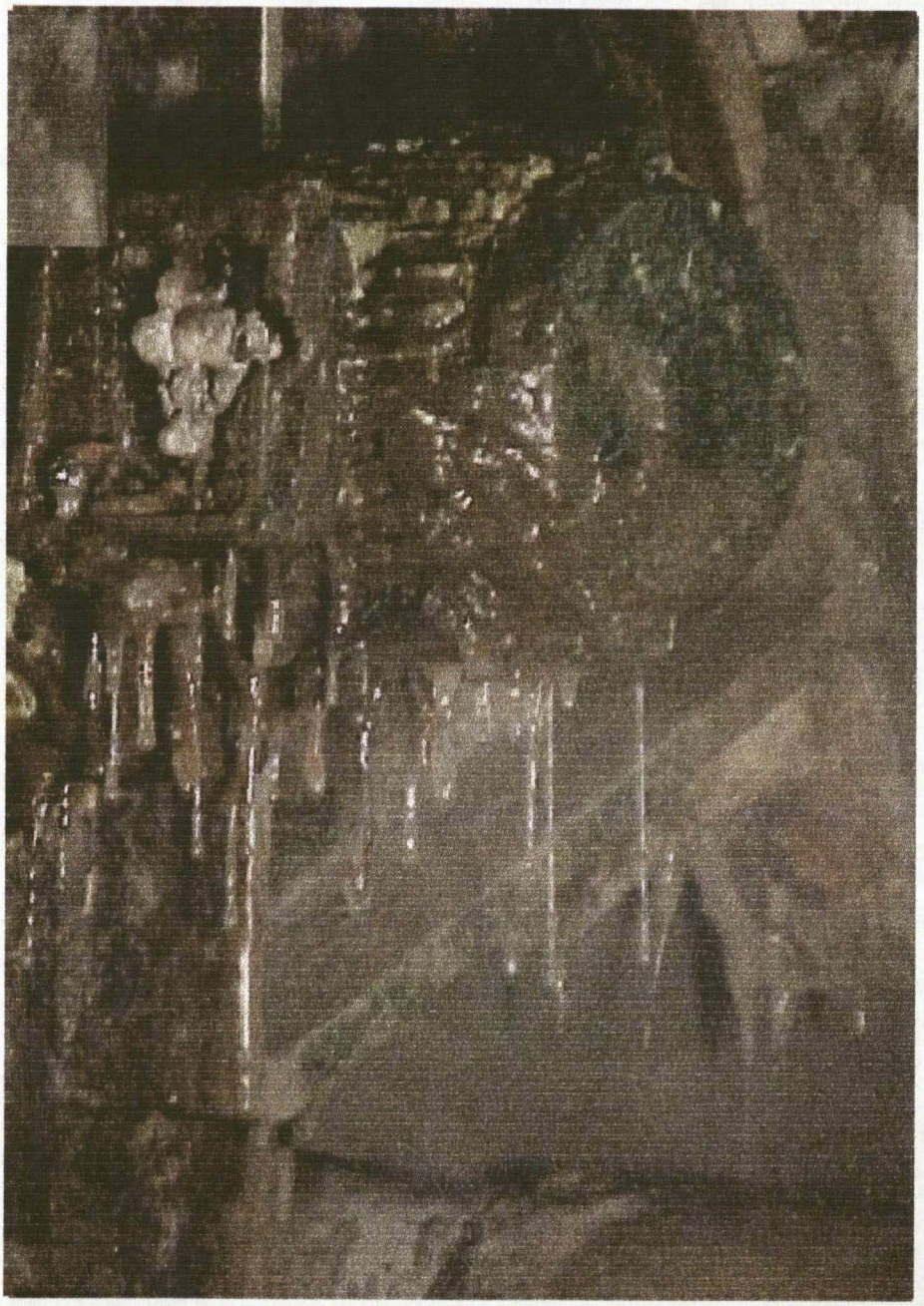
CHAPTER 3

STRUCTURAL DIVERSITY OF THE MICROBIAL COMMUNITY IN A PAPER MILL WATER SYSTEM



ABSTRACT

Microbial populations are generally enumerated using conventional microbiological techniques. Due to the limitations of these techniques, the microbial numbers are generally underestimated since conventional methods can only quantify a limited percentage of the microbial populations. Numerous alternative techniques have subsequently been developed. One possible alternative technique involves the analysis of signature lipid biomarkers. This method provides the means to determine changes in the microbial community composition. The aim of this study was, therefore, to evaluate the applicability of SLB analysis in a paper mill water system. The changes in the microbial community at a paper mill were monitored for one year. Samples from both the sessile and planktonic phases were subjected to signature lipid biomarker analysis as well as conventional culturing techniques. The ratio of diglyceride fatty acids to phospholipid fatty acids (PLFAs) was determined in order to provide an estimate of the ratio of the non-viable to viable microorganisms within the biomass. High biomass mortality was generally observed. The *trans* to *cis* values obtained were also indicative of stressed microbial communities. Analysis of the PLFAs present in the sessile and planktonic phases revealed the presence of a large diversity of microorganisms. The same trends in the number of cultured cells and the counts obtained with PLFA analysis were observed, although the cell counts obtained with PLFA analysis were approximately a 1000 times higher. Differences in the abundance and groups of various fatty acids occurred when different board grades were produced. On the basis of the results obtained, it was evident that the analysis of signature lipid biomarkers provided significantly more information concerning microbial communities than conventional culturing and this method was found to be suitable for application in a paper mill water system.

INTRODUCTION

Many problems in the papermaking process are frequently associated with the production of microbial biofilms. Microbial problems can lead to poor runnability and lower production rates that have severe economic implications for a paper mill. Microbial control at the Sappi Cape Kraft paper mill, Milnerton, South Africa, is very important due to the degree of closure and the recycled fibre that are used at the mill. The closure of the water system generally results in an increase in the degree of biofilm formation and subsequent corrosion (Bennett, 1985). It has been reported that the use of recycled fibre increases the microbial contamination of the water system. Microbial counts can be up to 977 times higher in recycled fibre than in virgin pulp (Sorelle & Belgard, 1991).

Most microbiological studies of wastewater treatment systems rely on culture dependent methods. Vestal & White (1989) reported that less than 1 % of all microorganisms are culturable on artificial media. This leads to an underestimation of microbial numbers since conventional methods only quantify a very limited percentage of the microbial populations actually present (Schneider *et al.*, 1998). Numerous alternative techniques to characterise microbial communities *in situ* have consequently been developed. One possible alternative is the analysis of signature lipid biomarkers.

Signature lipid biomarker analysis provides a method that is quantitative, independent of cell culturability and allows the identification of microorganisms that have distinctive PLFA patterns in a single analysis (Macnaughton *et al.*, 1997). Peterson & Klug (1994) proposed that phospholipids could be considered as a fingerprint of the microbial community. This method 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 PLFA are indicative of physiological stress, nutritional status as well as the viable biomass of the microbial population (Mandelbaum *et al.*, 1997; Steward *et al.*, 1996).

The proportion of fatty acids in pulp and papermaking chemicals is insignificant (Vaisanen *et al.*, 1994). These advantages make signature lipid biomarker analysis an

attractive technique for the estimation of microbial biomass in paper mill water systems.

The application of the signature lipid biomarker approach has been studied extensively in various environments. This method has been used to track microbial succession in developing compost (Carpenter-Boggs *et al.*, 1998), estimate bacterial and fungal biomass (Frostegard & Baath, 1996), study changes of microbial communities during starvation and desiccation (Kieft *et al.*, 1994), compare the phylogenetic relationship between dissimilatory sulphate-reducing bacteria (Kohring *et al.*, 1994) and compare biofilm and suspended microorganisms in wastewater treatment systems (Werker & Hall, 1998).

The aim of this study was, therefore, to apply signature lipid biomarker analysis to study the microbial populations in the water system at the SAPPI Cape Kraft paper mill, Milnerton, South Africa. The study was conducted over a period of one year to evaluate the technique as a possible indicator of shifts in the microbial community as a result of changes in the process parameters within the plant.

MATERIALS AND METHODS

Mill operations

The Sappi Cape Kraft paper mill, Milnerton, produces both fluting and linerboard from different sources of recycled fibre. The main difference between the production of fluting and linerboard is the difference in pH and the chemical composition of the water. During the period of evaluation, different biocides were added to the water system to control microbial fouling (BC4XL, BC6 and SNP3002). Routine shutdowns were performed approximately every four weeks as maintenance cleaning procedures. The mill operations were discussed in more detail in Chapter 2.

Sample Collection

Duplicate water (planktonic) and biofilm (sessile) samples were obtained from the Cape Kraft paper mill, Milnerton, Cape Town, South Africa every two weeks over a period of one year. The samples for the structural analysis were immediately frozen

in liquid nitrogen while the samples for the conventional analyses were cooled to 4 °C and transported on dry ice. Samples were processed within 24 hours of sampling.

Lipid Extraction and Fractionation

The planktonic samples (approximately 10 ml) were filtered using Whatman 4A filter paper and the biomass was freeze dried using a Dura-Dry MP II freeze-dryer (FTS Systems, Stone Ridge, USA). Biomass from the sessile samples (approximately 10 g) was freeze dried without any further treatment. Total lipids were extracted overnight from the lyophilised biomass using a mixture of chloroform and methanol (150 ml, 2:1 v/v) and separated by washing the extract twice with distilled water (25 ml). The solvents were evaporated using a Bibby RE 100 rotary evaporator (Sterilan, Staffordshire, England). The lipids were transferred to preweighed vials using diethylether (2 ml) and dried overnight in a vacuum oven (50 °C) in the presence of P_2O_5 to determine the mass of the total lipid fraction of each sample. The total lipid fractions were fractionated on activated (105 °C for 12 h) silicic acid columns (21 g) using various solvents as described by Kock and Ratledge (1993). The silicic acid was allowed to reach room temperature before it was mixed with 90 ml of chloroform and poured into glass columns. The neutral, glyco, and phospholipid fractions were eluted using 1,1,1,-trichloroethane (150 ml), acetone (100 ml) and methanol (100 ml), respectively. The different lipid fractions were dried overnight in a vacuum oven (50 °C) in the presence of P_2O_5. The amount of lipids in each fraction was determined gravimetrically. The vials were stored under nitrogen gas (-20 °C) until further processing.

Determination of Non-Viable / Viable Biomass Using the Neutral Lipid Fraction

The neutral lipid fraction was dissolved in chloroform (600 µl) and spotted onto thin-layer chromatography (TLC) plates (60A, Whatman). Lipid standards containing mono, di, and triglycerides as well as an arachidonic acid standard (free fatty acid) were spotted onto the side lanes of the plates. Authentic mono, di, and triglyceride standards were purchased from Sigma Aldrich (SA). The TLC plates were developed in petroleum ether (60 to 80 °C bp), diethylether and acetic acid (85:15:1) at 4 °C. The side lanes were stained in an iodine chamber and the corresponding diglyceride

lane on the TLC plates were scraped off. The diglyceride fraction was then filtered through a column stopped with glass wool using chloroform (2 ml) as eluent. The diglycerides were dried under a constant stream of nitrogen and dried in a vacuum oven (50°C) in the presence of P_2O_5. The diglycerides were methylated using trimethyl sulphonium hydroxide (TMSH) (200 µl) and subjected to GC analysis (Jeffrey, 1996).

Determination of the Microbial Community Structure Using the Phospholipid Fraction

The phospholipid fraction was dissolved in chloroform (200 µl), methylated by the addition of TMSH (200 µl) (Jeffrey, 1996) and analysed with gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) for the determination of the community structure. Dodecanoic acid (C12:0) (4 µl) was used as internal standard to enable the quantification of the other fatty acids present in the sample. Authentic lipid standards to allow verification of the various PLFAs via GC and GC/MS analysis (methyl palmitelaidate, methyl-palmitoleate, methyl-eicosapentadecanoate, methyl-14-methylhexadecanoate, methyl-12-methyltridecanaote, methyl-13-methylpentadecanoate, 37 component FAME mix, grain fatty acid methyl ester mix and bacterial acid methyl ester mix) were purchased from Sigma Aldrich (SA), Supelco Inc. (USA) and Matreya Inc. (USA). *Trans* and *cis* isomers of C18:1 were determined with GC/MS analysis. A conversion factor of 2,5 x 10^4 cells per pmol PLFA (A. Peacock, University of Tennessee, USA, personal communication) was used for microbial enumeration (Appendices D & E).

Gas Chromatography Conditions

The lipid fractions were analysed using a Hewlett Packard 5890 series II gas chromatograph 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 six minutes. Thereafter the temperature increased by 3 °C/min to a maximum of 245 °C. Nitrogen was used as a carrier gas at a flow rate of 5 ml/min.

Gas Chromatography-Mass Spectrometry Conditions

Verification of the identity of the various compounds was obtained by using a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard 5972 GC Mass Selective 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 to a maximum of 300 °C. Helium was used as a carrier gas at a flow rate 1 ml/min.

Determination of Viable Cell Counts Using the Standard Plate Count Procedure

Serial dilution series (10^{-2} to 10^{-8}) using distilled water, were prepared for every sample obtained (1 ml of water or 1 g biofilm) and viable cell counts were obtained using the nutrient agar pour plate technique (Walter, 1967). The plates were incubated at 30 °C for five days and the colonies were enumerated.

Data Processing

Signature lipid biomarker results were analysed using the NCSS 97 software (Statistical Solutions, Ireland). During discriminant analysis, stepwise variable selection was employed. Differences between groups of fatty acids (terminally branched saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids) were considered to be significant where $p \leq 0{,}005$. Since the group membership during this study was known *a priori*, multivariate statistical analysis using discriminant analysis was applicable (Fritze *et al.*, 1997) where the discriminant analysis selects for those variables that yield the best separation according to the specified groups.

RESULTS AND DISCUSSION

Determination of Non-Viable / Viable Biomass

Diglyceride fatty acids (DGFAs) are formed when cellular enzymes (phospholipases) hydrolyse the phosphate group of the phospholipids (Kieft *et al.*, 1994). The resulting diglycerides contained the same signature fatty acids as the phospholipids. Therefore,

the ratio of DGFAs to phospholipid fatty acids (PLFAs) provide an estimate of the ratio of non-viable to viable microorganisms in the biomass. It has been reported that healthy biofilms generally have a DGFA to PLFA ratio of less than 0,5:1 (White *et al.*, 1999). The DGFA to PLFA ratios obtained during this study generally indicate a high degree of biomass death (Table 1). It can thus be concluded that the microbial communities present in the sessile and planktonic phase experience a high degree of mortality. The mortality can possibly be ascribed to the effect of biocide dosage. During 10 of the 25 weeks, the ratio of DGFA to PLFA in the sessile phase was less than 0,5:1 indicating that the biofilms were healthy. In comparison, the planktonic samples showed a high degree of biomass death over the whole study. This indicated that the microorganisms in the planktonic phase were more susceptible to the biocide application and shifts in the production mode.

Table 1. Ratios of diglyceride to phospholipid fatty acids (DGFA / PLFA) as obtained in the sessile and planktonic phases over the one year period.

	Ratio of DGFA : PLFA	
Sample #	**Sessile**	**Planktonic**
1	0,247*	1,549
2	0,418*	1,228
3	4,302	2,756
4	6,498	1,751
5	2,936	0,903
6	6,121	2,548
7	1,226	6,044
8	0,653	10,354
9	1,000	10,048
10	1,308	2,869
11	4,150	4,321
12	0,893	3,354
13	0,201*	2,723
14	1,000	1,708
15	1,000	1,807
16	0,088*	1,726
17	1,000	2,136
18	0,791	1,000
19	0,290*	2,463
20	1,652	0,918
21	0,378*	4,346
22	0,095*	1,077
23	0,149*	ND
24	0,123*	0,762
25	0,097*	2,287

ND = Not detemined

* DGFA : PLFA ratio of less than 0,5:1

Determination of the microbial community structure

It has been reported by Mandelbaum *et al.* (1997) that the *trans* to *cis* ratio of the fatty acids 16:1 and 18:1 can be used as a general measure of stress or starvation since these *trans* monoenoic acids usually increases during starvation. *Trans* to *cis* ratios of higher than 0,1:1 are generally indicative of exposure to toxins or starvation while ratios of 0,05:1 or less are generally indicative of non-stressed communities (White *et al.*, 1996). Seventeen of the 18 *trans* to *cis* values obtained for the sessile samples and all 18 of the *trans* to *cis* values obtained for the planktonic samples had a ratio of higher than 0,1:1, which may be considered to be indicative of stressed microbial communities (Table 2). The stress might be ascribed to the application of biocides as well as the continuous variation in the external parameters due to changes in the production mode (linerboard vs fluting). The change in the pH and the additives added during the production of linerboard may cause environmental stress for the microbial communities present in the planktonic and sessile phases. Using *cis/trans* isomerisation, bacteria can adapt very quickly to toxic concentrations of organic substrates, thereby stabilising their membranes, which allow them to retain an intracellular physiological balance (Guckert *et al.*, 1991).

Analysis of the PLFAs present in the sessile and planktonic phases revealed the presence of a large diversity of microorganisms within the water system. It was reported that polyunsaturated PLFAs are found almost exclusively in eukaryotes (White *et al.*, 1996) and Frostegard & Baath (1996) proposed that C18:2 should be used as a fungal biomarker. The fatty acid, C18:2 could, therefore, be used as an indicator of the number of the eukaryotic organisms present in the water and biofilm. On the basis of the results obtained during this study, it is evident that fungi were more abundant when linerboard was produced than when fluting was produced (Figure 1). An 8,81 % increase in the concentration of C18:2 was obtained in the sessile phase while a 144,90 % increase was obtained in the planktonic phase for C18:2. These results were expected, since fungi grow optimally at a pH of 2 to 6 (Hughes, 1993), which was the pH range of the water when linerboard was produced.

Table 2. *Trans / cis* ratios of C18:1 obtained in the sessile and planktonic phases over the one year period.

	Ratio of *trans / cis* C18:1	
Sample #	**Sessile**	**Planktonic**
1	2,356	2,663
2	1,385	2,490
3	2,745	1,235
4	5,570	2,129
5	1,078	0,615
6	0,549	1,984
7	0,302	1,183
8	0,680	1,315
9	2,299	0,414
10	2,233	0,300
11	2,665	0,385
12	0,073*	0,866
13	0,527	0,859
14	1,531	1,328
15	1,124	0,447
16	0,539	1,521
17	0,395	ND
18	0,341	1,051

ND = Not determined (*Trans / cis* ratios for samples 19 to 25 ND)
* *Trans / cis* ratios of 0,05:1 or less

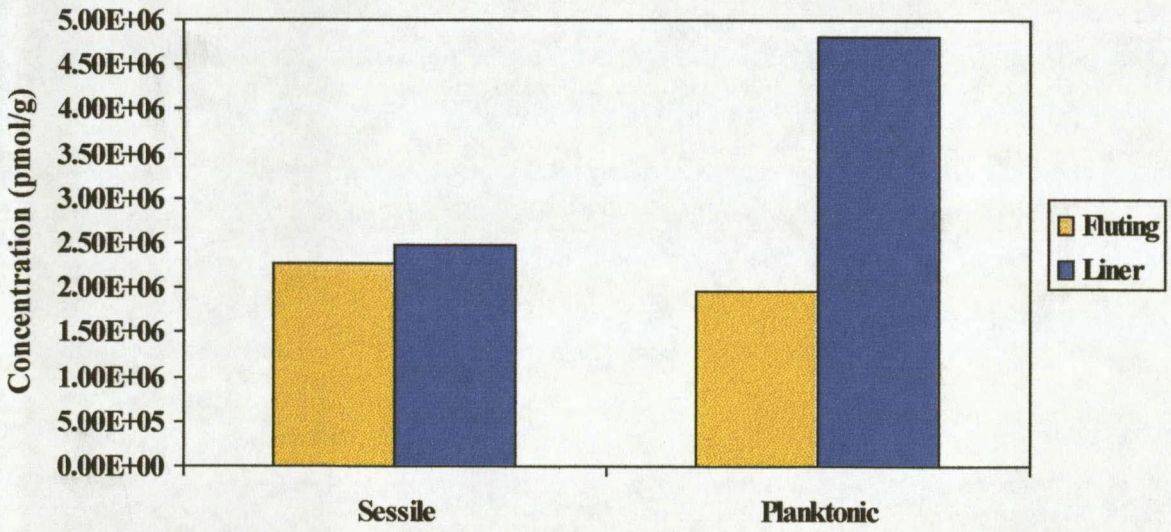


Figure 1. Concentration (pmol/g) of C18:2 present in the sessile and planktonic phases during the production of different paper grades.

Terminally branched saturated fatty acids are generally considered to be indicative of the presence of Gram positive organisms (Zelles, 1999). On the basis of the results obtained for the concentration of terminally branched saturated fatty acids, it is evident that the distribution of Gram positive organisms varied significantly between the various phases (Figure 2). A 34,23 % increase in the concentration of terminally branched saturated fatty acids was evident in the biofilm when linerboard was produced whilst a 49,54 % increase was observed in the planktonic samples when fluting was produced. These results could have significant implications for the biocide dosage programme. It is, therefore suggested that the biocide application programme be changed during the production of fluting and linerboard, which confirms the results as obtained during the analysis of substrate utilisation (Chapter 2).

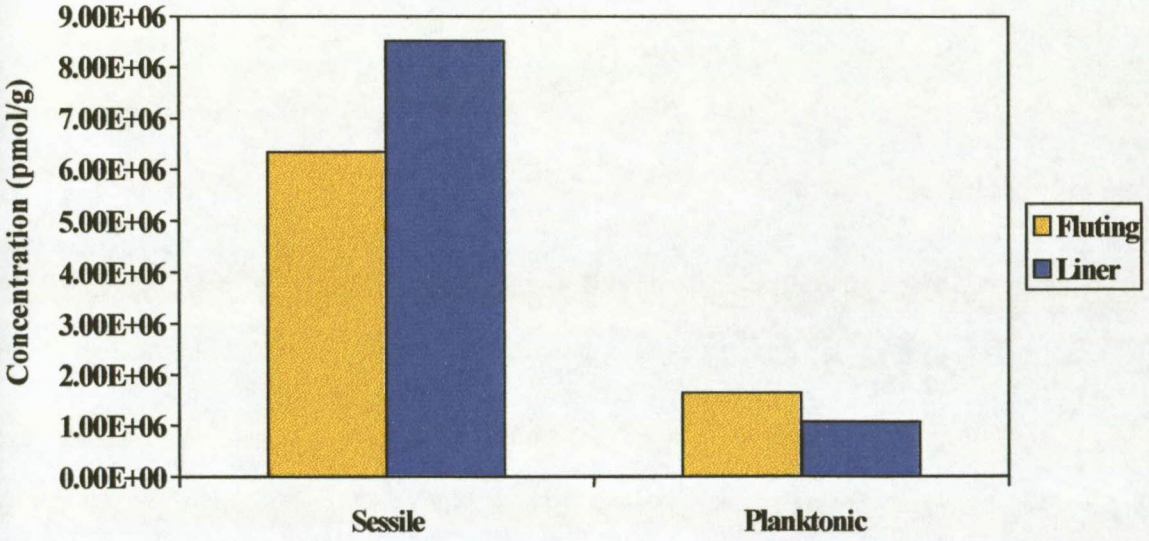


Figure 2. Concentration (pmol/g) of terminally branched saturated fatty acids present in the sessile and planktonic phases during the production of different paper grades.

Monounsaturated fatty acids (e.g. C16:1, C17:1) are generally considered to be indicative of Gram negative organisms (Ratledge & Wilkinson, 1988). On the basis of the results obtained during this study, it is evident that the Gram negative organisms were more abundant in both the sessile and planktonic samples when linerboard was produced (Figure 3). A 66 % increase in the concentration of monounsaturated fatty acids was obtained in the sessile phase when linerboard was produced, while a 117 % increase was obtained in the planktonic phase. The higher

concentrations of monounsaturated fatty acids in both the sessile and planktonic samples during the production of linerboard were in contrast to the terminally branched saturated fatty acids. This is possibly indicative of the fact that Gram negative bacteria were distributed throughout the water system (planktonic and sessile phases), while the Gram positive bacteria were more prevalent in the sessile phase. The signature lipid biomarker approach was, therefore, effective in detecting shifts within the microbial community.

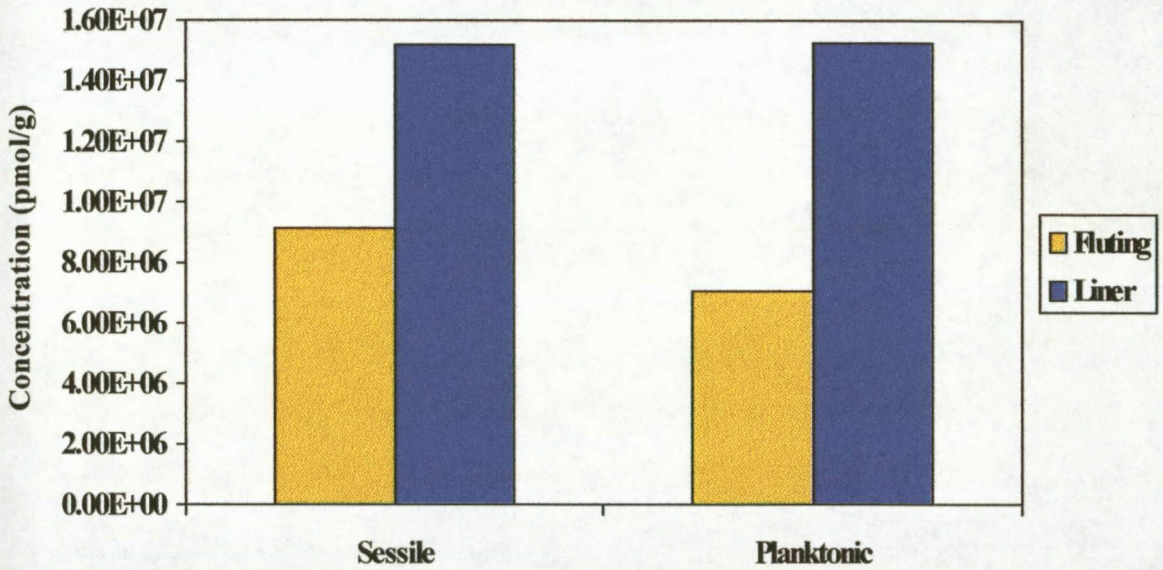


Figure 3. Concentration (pmol/g) of monounsaturated fatty acids present in the sessile and planktonic phases during the production of different paper grades.

Valeur *et al.* (1988) reported that planktonic bacteria had a lower ratio of saturated to unsaturated C18 fatty acids, while sessile bacteria contained a larger proportion of C18 relative to C16 fatty acids. These results were confirmed during this study where a lower ratio of saturated to unsaturated C18 fatty acids was observed in the planktonic phase (Figure 4) and a larger proportion of C18 to C16 fatty acids (Figure 5) was observed in the sessile phase.

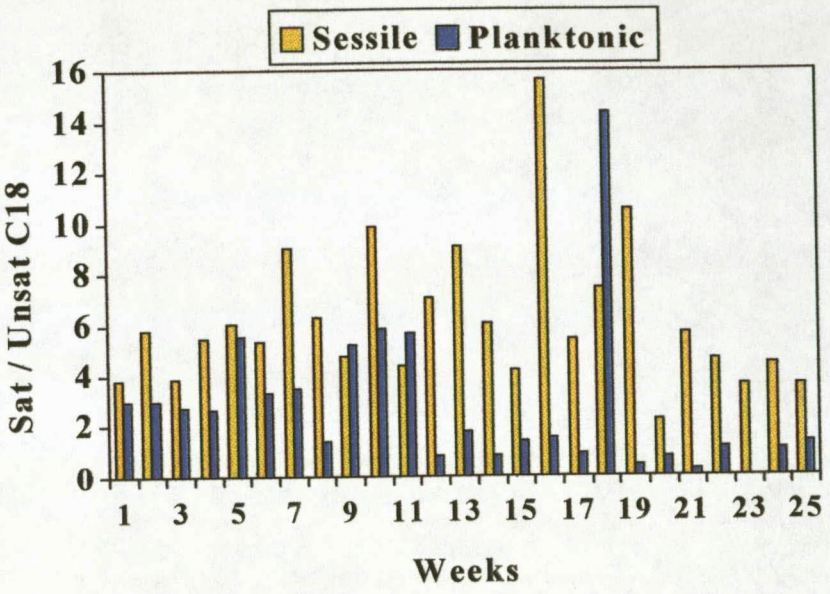


Figure 4. Comparison of saturated (Sat) to unsaturated (Unsat) C18 in the sessile and planktonic phases over the one year period.

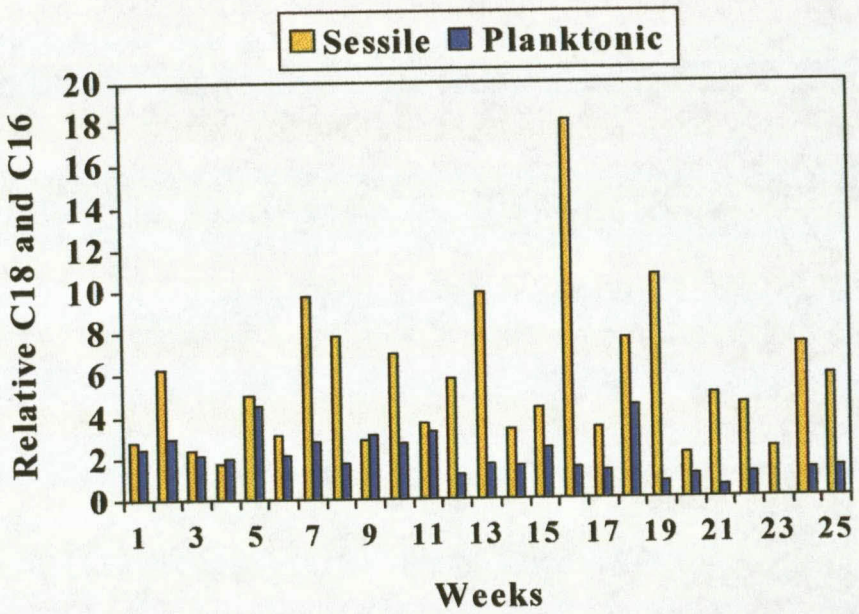


Figure 5. Comparison of relative C18 and C16 composition in the sessile and planktonic phases over the one year period.

Frostegard *et al.* (1993) reported that Gram positive bacteria predominantly contain branched saturated fatty acids while monounsaturated fatty acids are considered to be

a general biomarker for Gram negative bacteria (Ratledge & Wilkinson, 1988). The eukaryotic community can, therefore, be correlated to the polyunsaturated fatty acids (White *et al.*, 1996). Normal saturated fatty acids occur in most microorganisms and are, therefore, considered to be ambiguous.

The compositions of the communities in the planktonic and sessile phases based on different groups of fatty acids are illustrated in Figures 6 and 7. It is evident that the microbial community composition in the planktonic samples (Figure 4) changed dramatically between weeks 9 to 12. During this period the relative abundance of the Gram positive community (as indicated by terminally branched saturated fatty acids) was reduced while the eukaryotic community (as indicated by polyunsaturated fatty acids) increased substantially. During the same time the relative abundance of the eukaryotic community increased and gradually decreased in the sessile samples (Figure 5). Based on the results obtained, it is evident that the Gram positive microorganisms constituted a larger part of the microbial community in the sessile than in the planktonic phase. The changes in the microbial community structure during the year could possibly be ascribed to the continuous changes in the process parameters (paper grade produced, biocide application and shutdown).

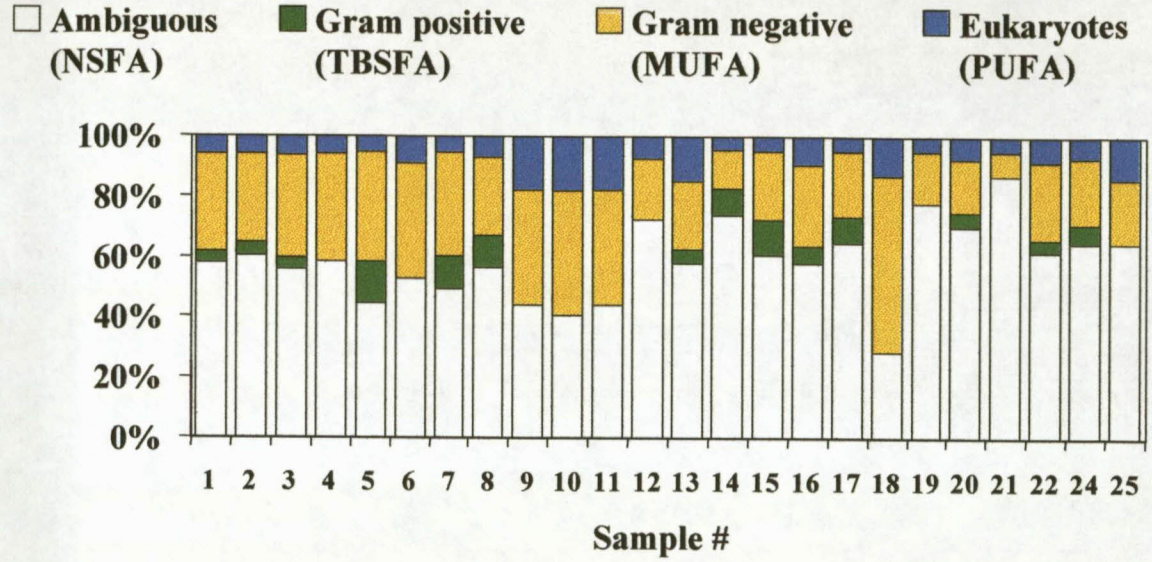


Figure 6. Microbial community composition in the planktonic phase based on different groups of fatty acids. NSFA = Normal saturated fatty acids; TBSFA = Terminally branched saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.

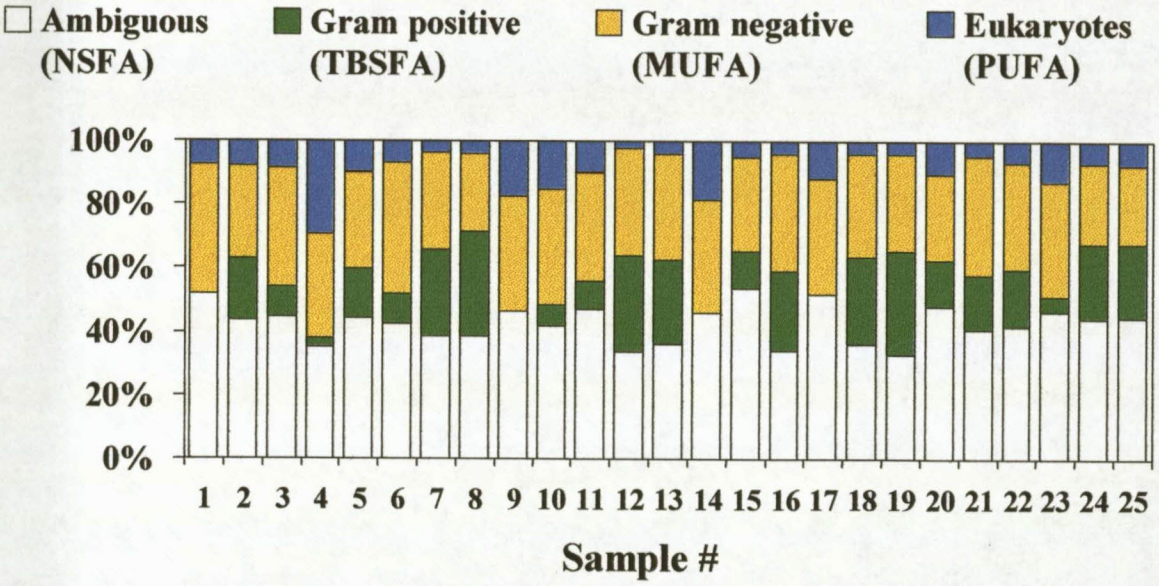


Figure 7. Microbial community composition in the sessile phase based on different groups of fatty acids. NSFA = Normal saturated fatty acids; TBSFA = Terminally branched saturated fatty acids; MUFA = Monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.

The same trends in the number of cultured cells and the theoretically calculated number of cells on the basis of PLFA analysis were observed for both the sessile and planktonic samples (Figure 8 & 9). The cell counts for PLFA analysis were obtained using a conversion factor of 2,5 x 10^4 cells per pmol of PLFA (A. Peacock, University of Tennessee, USA, personal communication) derived from the analysis of rapidly growing cells and assuming 0,4 pg of dry weight per bacterial cell and 100 µmol PLFA per gram dry weight. On the basis of these results, it is evident that the cell counts obtained for the planktonic phase were more stable and consistent than the cell counts obtained for the sessile phase. This could possibly be ascribed to particulate matter such as starch and fibre that adhered to the microorganisms present in the sessile phase, which would influence the weight of the sample being analysed.

Generally the cell counts obtained with PLFA analysis were much higher than the counts obtained with the conventional culturing technique. This could possibly be attributed to the fact that less than 1 % of all microbes are culturable on artificial media (Vestal & White, 1989; Palojarvi *et al.*, 1997). Culturing methods are selective due to the media used and the interactions that exist between the various microorganisms (Kerster *et al.*, 1997). Culturing techniques generally remove the microorganisms from their natural habitat and only allow microbes with specific metabolic properties to grow under the specified cultural conditions (Vestal & White, 1989; Atlas & Bartha, 1993). In contrast, PLFA analysis enabled the detection of all the microorganisms that are present in the environment.

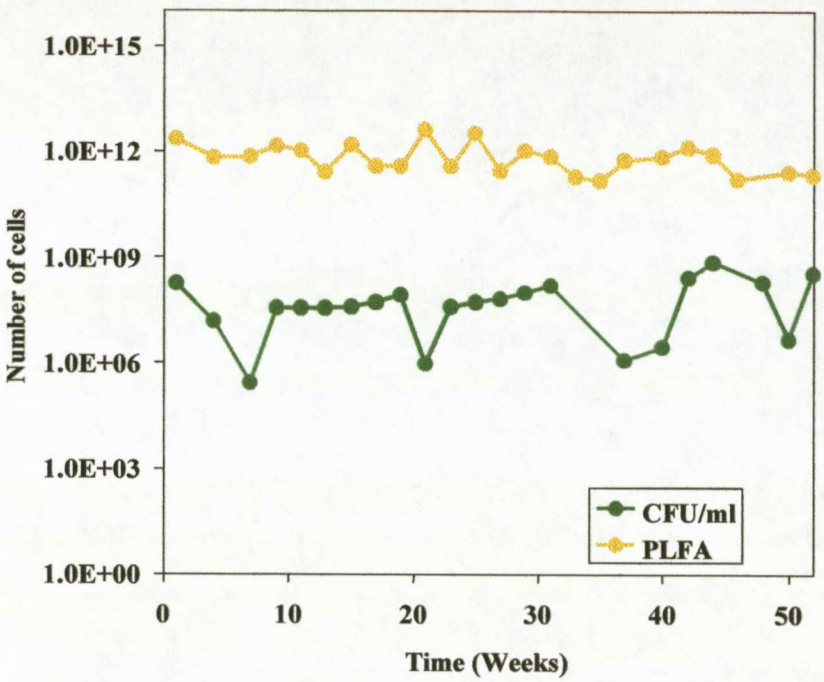


Figure 8. Comparison of numbers of cells obtained with phospholipid fatty acid analysis (PLFA) and plate counts (CFU/ml) for the planktonic samples as obtained over a period of one year.

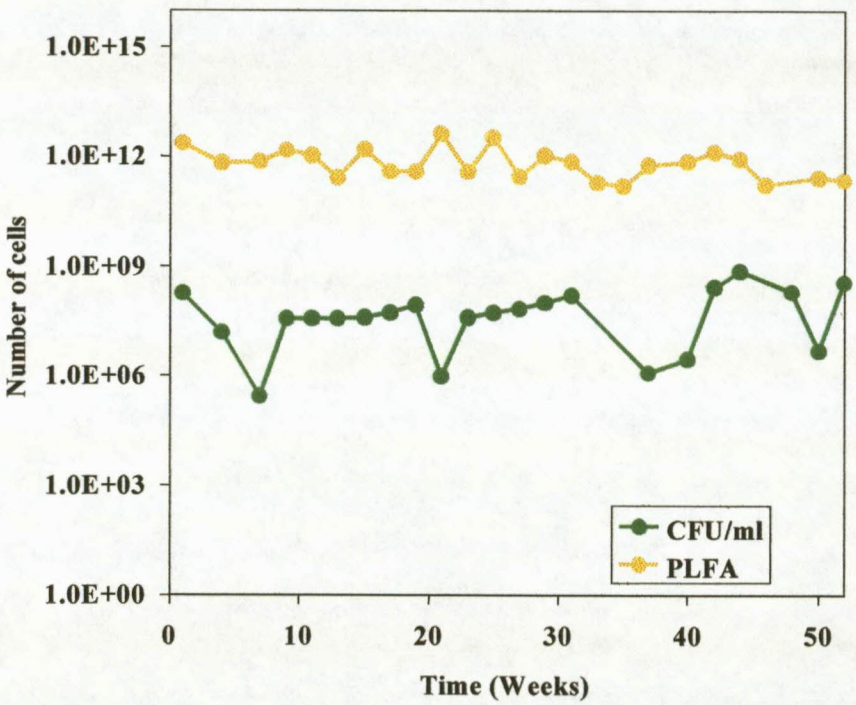


Figure 9. Comparison of numbers of cells obtained with phospholipid fatty acid analysis (PLFA) and plate counts (CFU/ml) for the sessile samples as obtained over a period of one year.

The structural diversity was based on differences between fatty acids. Differences in the abundance and groups of fatty acids (terminally branched saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids) in the samples occurred when fluting and linerboard were produced. These differences were apparent in the planktonic and sessile phases on the day when production of a specific grade started (Figures 10 & 11). Based on the results of the discriminant analysis it was evident that the diversity of the planktonic microbial communities differed significantly (Wilks' Lambda: 0,16; $F = 4,1$; $p \leq 0,05$) on the day of a change in production grade. The diversity of the sessile microbial communities did not differ significantly (Wilks' Lambda: 0,35; $F = 1,9$; $p > 0,05$) on the day when there was a change in the paper grade. The differences in the fatty acids might be ascribed to the microbial adaptation to the change in the environment due to the change in chemical composition of the water during the change in production grade. 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).

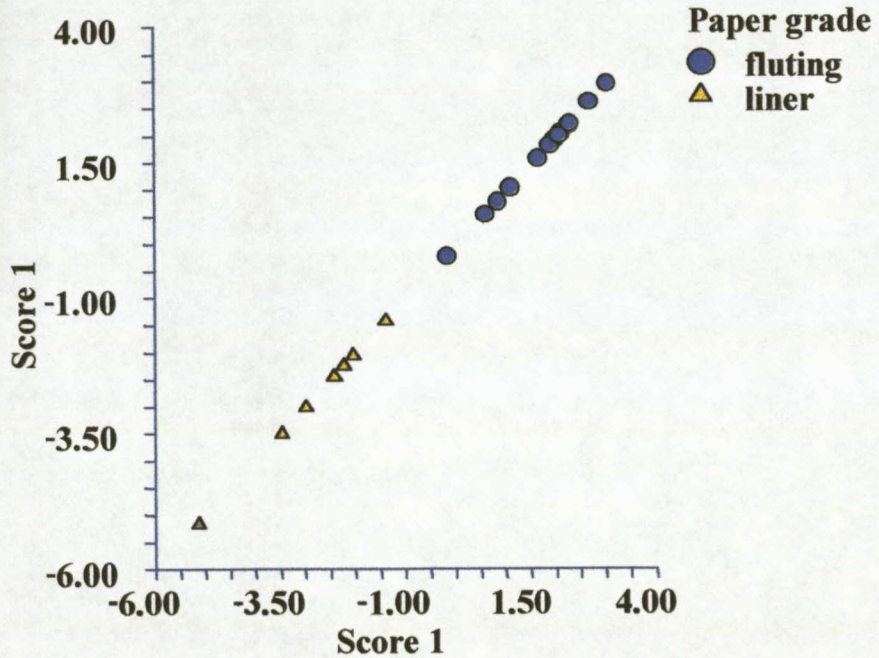


Figure 10. Ordination plot of the canonical-variate scores based on the combined effect of the different groups of fatty acids as obtained for the planktonic microbial community after one day of production of a specific paper grade.

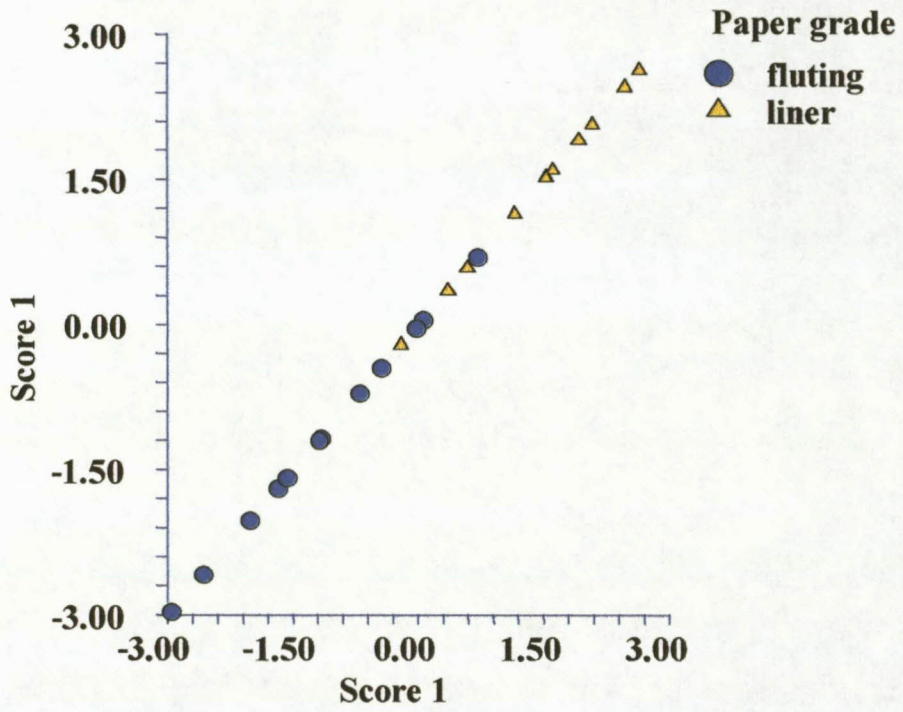


Figure 11. Ordination plot of the canonical-variate scores based on the combined effect of the different groups of fatty acids as obtained for the sessile microbial community after one day of production of a specific paper grade.

After five days of continuous production of fluting or linerboard difference in the groups of lipids (terminally branched saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids) indicated that two distinct microbial communities developed in the planktonic and sessile samples (Figure 12 & 13). Based on the results of the discriminant analysis it was evident that the diversity of the planktonic microbial communities differed significantly (Wilks' Lambda: 0,06; $F = 1,6$; $p \leq 0,05$) after an extended period of production of a specific paper grade, although the diversity of the sessile microbial communities did not differ significantly (Wilks' Lambda: 0,021; $F = 8,0$; $p > 0,05$). This indicated that the differences in the microbial lipids become more pronounced after an extended period of time.

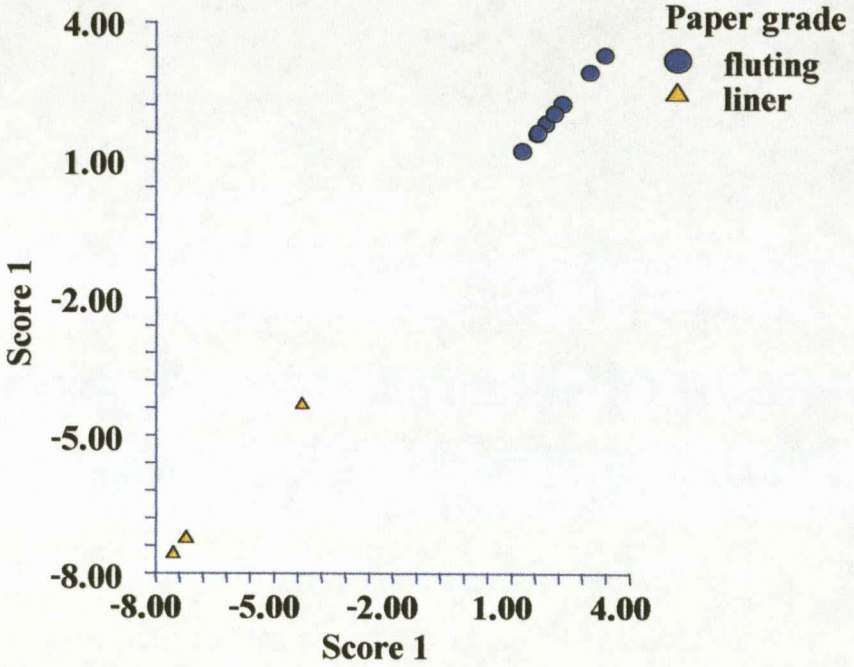


Figure 12. Ordination plot of the canonical-variate scores based on the combined effect of the different groups of fatty acids as obtained for the planktonic microbial community after five days of production of a specific paper grade.

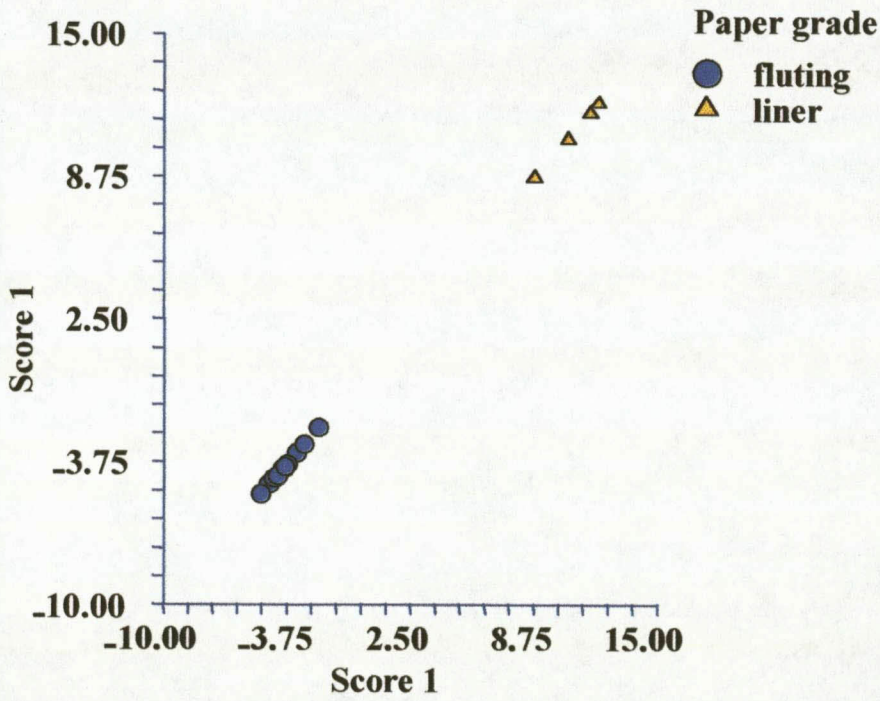


Figure 13. Ordination plot of the canonical-variate scores based on the combined effect of the different groups of fatty acids as obtained for the sessile microbial community after five days of production of a specific paper grade.

CONCLUSIONS

On the basis of the results obtained during this study, it may be concluded that a high degree of cell mortality was evident in both the planktonic and sessile phases, as indicated by the high DGFA to PLFA ratios. This could be ascribed to the biocides applied in the water system. The high *trans* to *cis* ratios obtained in the planktonic and sessile phases were indicative of stressed microbial communities. It may be concluded that the stress was a result of the biocide application or the change in the production grade. During a change from fluting to linerboard, the pH changed and additives were added to the fibre.

A large diversity of microorganisms was present in the planktonic and the sessile phases. A difference in the abundances of different groups of fatty acids was observed during the production of fluting and liner. It may be concluded that the production of the different paper grades selected for different microbial communities in both the sessile and planktonic phases, which was in correlation with results obtained by Werker & Hall (1998) who demonstrated the use of fatty acid analyses to distinguish between planktonic and sessile microbial populations. The signature lipid biomarker approach was effective in detecting shifts within the microbial community.

A lower ratio of saturated to unsaturated C18 fatty acids was observed in the planktonic community and a larger proportion of C18 to C16 fatty acids was observed in the sessile community. These results confirmed the report of Valeur *et al.* (1988) who studied the differences in the lipid composition between planktonic and sessile cells of a Gram negative bacterium.

Similar trends were observed in the cell counts as 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 stress the underestimation of microorganisms as obtained by conventional microbiological methods. This study demonstrated that signature lipid biomarker analysis was a valid approach to study changes within a paper mill water system and corresponded with results obtained by Werker & Hall (1998) who concluded that the

analysis of microbial lipids could be used to compare microbial communities within and between experiments.

On the basis of the results obtained during this study, it may be concluded that differences in the structural diversity occurred within the microbial community when fluting and linerboard were produced. These deductions were made based on the combined effect of the different groups of fatty acids. The abundance of terminally branched saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids varied with the change in production mode. The results obtained from the structural diversity were in accordance with results found in this study (Chapter 2) where the functional diversity also varied with a change in production mode.

Knowledge of the different microorganisms in the water system and the interactions between the various organisms will also assist in the selection and dosage of the correct biocides. It can be concluded that a significant amount of information concerning the structure of the microbial community could be obtained during this study using the analysis of signature lipid biomarkers. Although this technique could be considered as too expensive for routine application, it could aid in the management of the water system and especially in the evaluation of biocide efficacy.

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CHAPTER 4

MICROBIOLOGICAL AUDITS OF THE MICROBIAL COMMUNITY IN THE CAPE KRAFT PAPER MILL WATER SYSTEM

ABSTRACT

Due to the positive results obtained in the two previous studies, it was decided to apply carbon source utilisation and signature lipid biomarker analyses at the Sappi Cape Kraft paper mill during two separate microbiological audits. The first audit was performed to assess the efficacy of the current biocide dosage programme at the mill. These studies included a survey of the whole system from furnish through to the wet-end of the paper machine and water treatment plant. Chemical, microbiological and physical aspects were included in the survey. Indications of possible microbial resistance to the biocides being applied were observed. Alternative biocides were, therefore, recommended. Phospholipid fatty acid analyses revealed the presence of a large diversity of microorganisms in the various unit operations confirming the results found in the previous study. Cluster analysis of the substrate utilisation data indicated that unit operations could be grouped according to similarities in their functional diversities. These groups could be dosed with separate biocide programmes. The second microbiological audit was performed to evaluate the effect of the recommended changes made to the microbial control programme. The results of the second microbiological audit indicated a considerable improvement in the microbial control programme.

INTRODUCTION

Many problems in the papermaking process are frequently associated with the production of biofilms. Deposits that break loose may result in paper breakages, spotting, holes and discolouration (Stoner & King, 1994). Paper breakages and other defects may affect the efficiency and the profits of the mill. Microbiologically induced corrosion and biofilm formation could lead to reduced machine speed and lower production rates that have economic implications for a paper mill. Biofilm development could be controlled with biocide applications, and an effective microbial control programme should include the selection of efficient biocides, the selection for optimal application points of biocides and the monitoring of the efficacy of the control programme (Moran, 1992).

Previous research indicated that the analysis of carbon source utilisation patterns (substrate utilisation patterns) and signature lipid biomarkers could be successfully applied to evaluate the functional and structural diversity of microbial populations in paper machine water systems (Chapters 2 and 3). Many of the problems frequently associated with the culturability of microorganisms were circumvented by the application of these techniques. Based on the previous results, it was evident that these techniques could be used to correlate process parameters to the functional and structural diversity of the microbial community, which could assist in better management of the water systems.

The aim of this investigation was, therefore, to study the microbial population in the water system of the Sappi Cape Kraft paper machine, to identify potential problems areas and to develop a successful microbial control program. This study involved a survey of the water system from furnish through to the wet-end of the paper machine and water treatment plant in order to improve the microbial control programme at the paper mill. The first microbial audit was conducted in July 1999 to evaluate the prevailing control measures at Cape Kraft and the second audit was performed during April 2000 to evaluate the effect of the recommended changes made to the control measures.

MATERIALS AND METHODS

During the two separate audits, fluting was produced at the mill, which is the predominant paper grade produced by Cape Kraft.

Sample Collection

Water samples (planktonic phase) were collected from various unit operations in the Cape Kraft water system (Table 1, Figure 1). Samples for the analysis of the structural diversity (signature lipid biomarkers) were immediately frozen in liquid nitrogen and transported on dry ice, while the samples for the analysis of the functional diversity (carbon source utilisation patterns) were only cooled (4 °C). The conventional microbiological analyses were performed on site.

Table 1. Collection points for planktonic samples.

Sample #	Collection point
1	Dump chest
2	Broke chest
3	Cloudy water chest
4	Filler line machine chest
5	Top line machine chest
6	Filler line head box
7	Top line head box
8	Silo 1
9	Silo 2
10	Silo 3
11	Silo 4
12	Silo 5
13	White water chest
14	Effluent buffer
15	Clarified water
16	Unclarified water
17	Clarifier sludge
18	Clarifier water
19	Industrial water

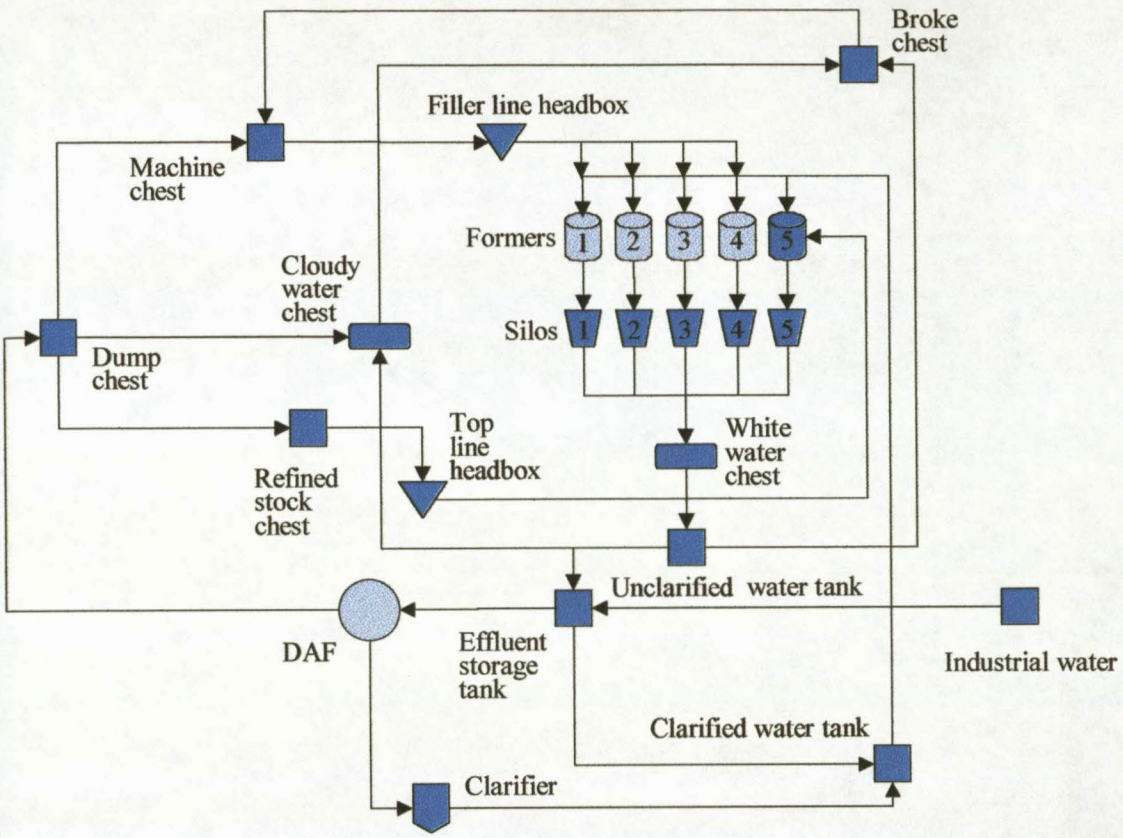

Figure 1. A simplified diagram of the water system at Cape Kraft. Unit operations where samples were collected are shaded in darker blue.

Physical Parameters

The physical parameters were determined in the different unit operations. These included the determination of the pH, temperature and oxidation-reduction potential (ORP) of the various samples.

Conventional Culturing

Conventional microbiological cultivation included enumeration of total aerobic bacteria (TAB), fungi and sulphate reducing bacteria (SRB) (Tappi Test Methods). Serial dilution series (10^{-1} to 10^{-6}) using distilled water were prepared for all the samples using 1 ml of water. Total aerobic bacteria were enumerated using plate count petrifilms (Merckoplate) that were incubated at 37 °C for 48 hours in order to determine the general contamination of the planktonic community. Fungal counts were performed on Sabouraud dextrose petrifilms (Merckoplate) and incubated at

37 °C for 48 hours. Sulphate reducing bacteria were enumerated on iron-sulphate agar (C.A. Milsch) and incubated anaerobically at 37 °C for seven days.

Toxicity of Biocides

The Minitox test, which is based on oxidation-reduction colour changes, was used to simultaneously determine the minimum inhibitory concentration (MIC) of different biocides (Chemserve-Nalco). Samples for the Minitox test were taken from the No. 5 former overflow. The MIC was determined during the first audit using a colourimetric reaction. The following products (SAPC, Chloorkop) were tested for their biocidal efficacy: SNP 3058 (glutaradehyde); BC 4XL (organosulphur, sulphone); SNP 3002 (QAC and organotin combination); SNP 103 (MBT); BC6 (organosulphur, carbamate); SNP 105 (isothiazoline); and SNP 301 (TCMTB). The different biocides were tested at concentration ranges from 1ppm to 500 ppm.

Sensitivity of the Microbial Community to the Various Biocides

The sensitivity of the microbial community to the various biocides was determined using the Tra-Cide analysis (Nalco-Sapco, Chloorkop, South Africa).). The Tra-Cide analysis is based on the measurement of total adenosine triphosphate (ATP) as an indicator of the total microbial load in the water as well as the measurement of toxicity towards luminescent bacteria, which provides information concerning the concentration of the biocides in the water system. Samples from the No. 5 former overflow were taken every 30 minutes for analysis, using Nalco Tra-Cide equipment.

Signature Lipid Biomarker Analysis

The planktonic samples (approximately 10 ml) were filtered using Whatman 4A filter paper and the biomass was freeze dried using a Dura-Dry MP II freeze-dryer (FTS Systems, Stone Ridge, USA). Total lipids were extracted overnight from the lyophilised biomass using a mixture of chloroform and methanol (150 ml, 2:1 v/v) and separated by washing the extract twice with distilled water (25 ml). The solvents were evaporated using a Bibby RE 100 rotary evaporator (Sterilan, Staffordshire, England). The lipids were transferred to preweighed vials using diethylether (2 ml) and dried overnight in a vacuum oven (50 °C) in the presence of P_2O_5 to determine the mass of the total lipid fraction of each sample. The total lipid fractions were

fractionated on activated (105 °C for 12 h) silicic acid columns (21 g) using various solvents as described by Kock and Ratledge (1993). The silicic acid was allowed to reach room temperature before it was mixed with 90 ml of chloroform and poured into glass columns. The neutral, glyco-, and phospholipid fractions were eluted using 1,1,1,-trichloroethane (150 ml), acetone (100 ml) and methanol (100 ml), respectively. The different lipid fractions were dried overnight in a vacuum oven (50 °C) in the presence of P_2O_5. The amount of lipids in each fraction was determined gravimetrically. The vials were stored under nitrogen gas (-20 °C) until further processing.

The phospholipid fraction was dissolved in chloroform (200 µl), methylated by the addition of TMSH (200 µl) (Jeffrey, 1996) and analysed with gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS) for the determination of the community structure. Dodecanoic acid (C12:0) (4 µl) was used as internal standard as to enable the quantification of the other fatty acids present in the sample (Appendices F & G). Authentic lipid standards to allow verification of the various PLFAs via GC and GC/MS analysis (methyl palmitelaidate, methyl-palmitoleate, methyl-eicosapentadecanoate, methyl-14-methylhexadecanoate, methyl-12-methyltridecanaote, methyl-13-methylpentadecanoate, 37 component FAME mix, grain fatty acid methyl ester mix and bacterial acid methyl ester mix) were purchased from Sigma Aldrich (SA), Supelco Inc. (USA) and Matreya Inc. (USA). A conversion factor of 2,5 x 10^4 cells per pmol PLFA were used for microbial enumeration (A. Peacock, University of Tennessee, USA, personal communication).

Gas Chromatography Conditions

The lipid fractions were analysed using a Hewlett Packard 5890 series II gas chromatograph 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 six minutes. Thereafter, the temperature increased by 3 °C per minute until a maximum of 245 °C was achieved. Nitrogen was used as a carrier gas at a flow rate of 5 ml/min.

Gas Chromatography-Mass Spectrometry Conditions

Verification of the identity of the various compounds was obtained with a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard 5972 GC Mass Selective 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 per minute to a maximum of 300 °C. Helium was used as a carrier gas at a flow rate of 1 ml/min.

Analysis of Substrate Utilisation Profiles

A stock solution of phosphate buffer was prepared by dissolving 12,36 g Na_2HPO_4, 1,80 g NaH_2PO_4 and 85,0 g NaCl in 1 L deionised water (Guckert *et al.*, 1996). This solution was filter sterilised (0,22 µm) and stored at 4 °C. The buffer was prepared by mixing 100 ml of the stock solution in 1 L deionised water. Approximately 10 ml of water from the various samples were added to 30 ml of phosphate buffer and homogenised (Heidolph Diax 600, Germany). The resulting suspensions were centrifuged (500 x g) to remove any particulate matter and the supernatant was standardised to a turbidity of 0,25 to 0,35 absorbance units (420 nm) using a phosphate buffer (Guckert *et al.*, 1996).

The standardised microbial suspensions were poured into a reagent reservoir and 150 µl aliquots were added to each well of the Biolog microtiter plates. Duplicate GN Biolog plates (Biolog Inc., Hayward, USA) were evaluated for absorbance changes at 590 nm using a Labsystems iEMS plate analyser (Labsystems, Helsinki, Finland). Following an initial (time 0) reading, the Biolog plates were incubated at 25 °C in the dark. The reduction of tetrazolium violet in each well was measured after 12, 24, 36 and 48 hours of incubation.

The data obtained from the Biolog microplates (Appendices H & I) were analysed using the average well colour development (AWCD) technique (Garland, 1996). An average well colour development of between 0,3 and 0,5 Absorbance units was used as the reference point for statistical analysis of the data (Heuer and Smalla, 1997). Results obtained from the substrate utilisation assay were analysed using the NCSS 97 software (Statistical Solutions, Ireland). Ward's minimum variance clustering

algorithm was used to construct a dendogram of the substrate utilisation data since the lowest delta values that indicated the goodness of fit (Hintze, 1997), were obtained using this algorithm ($\Delta = 0,14$).

Biodiversity

The species diversity indices were calculated with the following equations (Magurran, 1988):

$H' = -\sum p_i \ln p_i$ (1)

Where H' = Shannon index or degree of substrates utilised and p_i = the proportional turbidity observed in the ith well.

$p_i = a_i / \sum a$ (1.1)

Where a_i = the turbidity of the ith well and $\sum a$ = the total turbidity observed in all wells of the sample.

$d = a_{max} / \sum a$ (2)

Where d = the Berger-Parker index of dominance, a_{max} = the highest turbidity observed for all wells and $\sum a_i$ = the total turbidity observed in all the wells of the sample.

RESULTS AND DISCUSSION

Physical Parameters

The extreme variation in pH and temperature values in the various unit operations suggested the possibility of various and noticeably different habitats, which may exist in the different unit operations. During the second audit, the pH values were similar to the values as obtained during the first audit. However, temperature values showed changes of up to 15 °C when compared to the first audit (Table 2). Vataanen and Niemela (1983) stated that temperature has a significant effect on bacterial numbers illustrating that more stringent control of the system temperature might reduce microbial contamination and related problems such as corrosion.

The ORP values obtained in the first audit indicated that reductive environments were present in most of the unit operations, especially in the clarifier sludge (Table 2). During the second audit, it was found that the conditions in the larger part of the water

system had become more oxidative. The change from a reductive to an oxidative environment would minimise the problems frequently associated with anaerobic zones. The development of anaerobic zones and resistance to biocides are indicative of biofilm formation (Johnsrud, 1997). Visually, biofilm formation was minimised by the addition of the new biocides.

Table 2. Levels of pH, oxidation-reduction potential (ORP) and temperature in the planktonic phase of the different unit operations during the two separate audits.

	pH		ORP (mV)		Temperature (°C)	
Sample Point	**1st Audit**	**2nd Audit**	**1st Audit**	**2nd Audit**	**1st Audit**	**2nd Audit**
Dump Chest	5,7	5,7	14	17	45	60
Machine Chest	5,8	5,8	-98	22	35	35
Refined Stock Chest	5,7	5,7	-19	-21	37	37
Broke Chest	6,4	6,4	69	48	33	34
Filler Headbox	5,8	5,8	-49	-89	35	37
Top Line Headbox	5,7	5,7	-6	-7	39	36
Silo 1	6,0	6,0	-145	25	33	37
Silo 2	5,8	5,8	-123	107	33	35
Silo 3	6,0	6,0	-121	54	33	35
Silo 4	5,8	5,8	-19	-35	33	34
Silo 5	6,1	6,1	-11	45	33	35
White Water Chest	6,1	6,1	-60	-97	29	33
Effluent Storage Tank	5,7	ND	-162	ND	33	ND
Clarified Water Tank	5,2	5,2	-228	-89	32	23
Unclarified Water Tank	5,0	5,0	-260	-90	12	23
Clarifier Water	5,1	5,1	-315	-126	34	32
Clarifier Sludge	4,3	4,3	-404	-223	31	33
Cloudy Water Chest	6,3	6,3	94	-9	34	35

ND = Not determined

Conventional Culturing

Relatively high total aerobic bacterial (TAB) levels (>5,5x10^7) were observed throughout the system during the first audit (Figure 2). The high TAB counts could increase the tendency for slime formation. The TAB levels in most of the unit operations had decreased significantly after a change in the biocide programme, with the exception of the effluent buffer and unclarified water unit operations. These

results suggest a significant improvement in the programme to control microbial contamination.

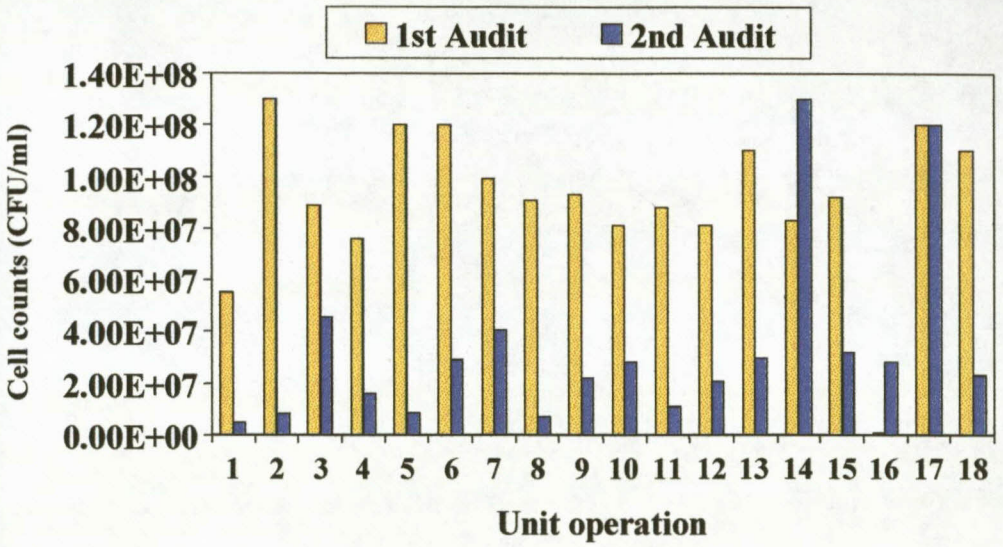

Figure 2. Total aerobic bacteria (TAB) (CFU/ml) as detected in the different unit operations during the two separate audits.

Relatively high levels of fungal contamination were also detected in the whole system during the first audit (Figure 3). This could possibly be ascribed to the slightly acidic conditions of the system. With the exception of effluent buffer unit operation, the fungal levels had decreased substantially after the change in the microbial control programme.

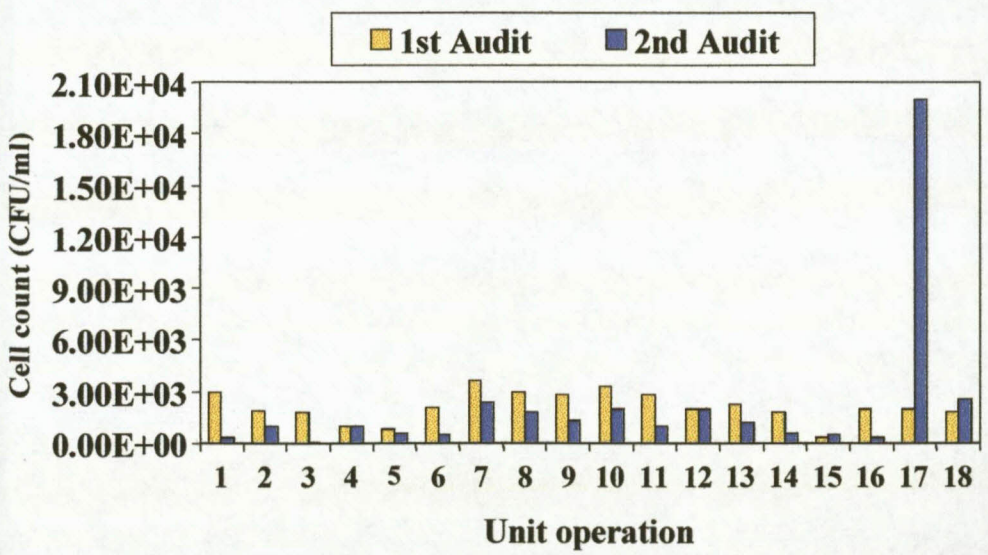

Figure 3. Fungi (CFU/ml) as detected in the different unit operations during the two separate audits.

High SRB levels (>1,8x10^3 CFU/ml) were detected in all the unit operations during the first audit (Figure 4), which confirmed the high anaerobic activity and low ORP values (Table 2). According to Vataanen and Niemela (1983) low redox potential values inhibit the growth of aerobic bacteria, and the growth of anaerobes is consequently promoted. During the second audit the SRB levels were lower in the larger part of the water system (Figure 4). This could possibly also be ascribed to the higher ORP values in the various unit operations (Table 2).

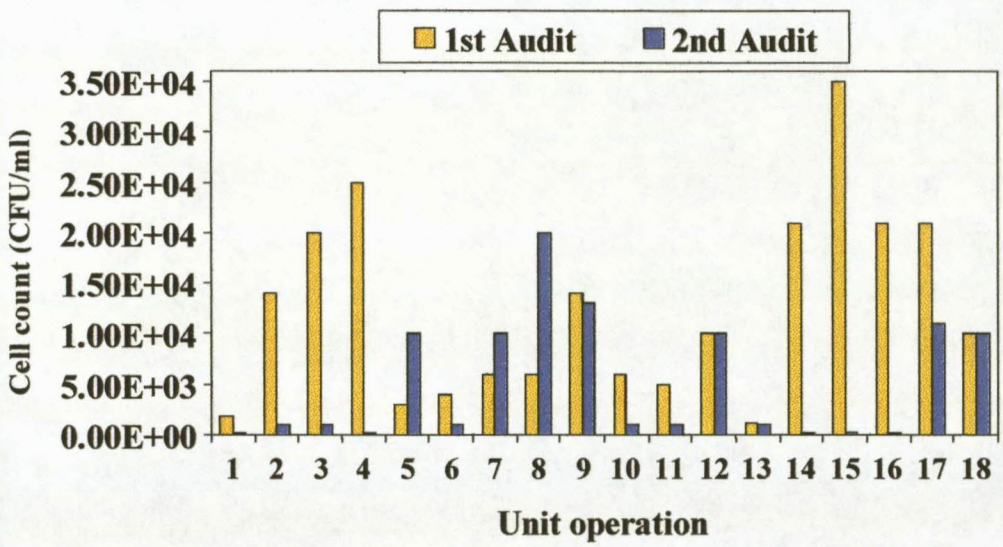

Figure 4. Sulphate reducing bacteria (SRB) (CFU/ml) as detected in the different unit operations during the two separate audits.

Toxicity of Biocides

The Minitox test was only performed during the first audit to determine the inhibitory effect of the different biocides and in order to assist the recommendation of more effective biocides. The testing of toxicity was performed at various biocide concentrations in order to determine the most effective level of biocide required to inhibit or kill the microorganisms in the water system (Moran, 1992). The Minitox test indicated that the biocides SNP 3058, SNP 301 and BC6+ were the most effective non-oxidizing biocides against the microbial population in the No. 5 former overflow (Table 3). These biocides were, therefore, selected for application in the water system. The biocides that had previously been applied at the mill (BC 4XL and SNP 3002) had very high MIC values, possibly indicating that microbial resistance to these

biocides might have been acquired or that the biocides were ineffective against the specific microbial populations present in the water system. The other biocide that had been applied to the water system, BC6, had a very low MIC value, indicating that the biocide was still active against the microbial communities in the water system.

Table 3. The minimum inhibitory concentration (MIC) values of the different biocides tested.

Biocide	MIC (ppm)
SNP 3058	16
BC 4XL	250
SNP 3002	500
SNP 103	250
BC6	8
SNP 105	500
SNP 301	32

Sensitivity of the Microbial Community to the Various Biocides

During the first audit the results of the Tra-Cide analysis indicated that the levels of biocide as reflected by relative toxicity units (RTU) fluctuated over time (Figure 5). On the other hand, the levels of microorganisms as reflected by relative light readings (RLR) remained relatively constant (Figure 5). Very low toxicity was, therefore, observed and with no notable effect on the microbial population. These results confirmed the assumption that microbial resistance to the specific biocides had possibly developed.

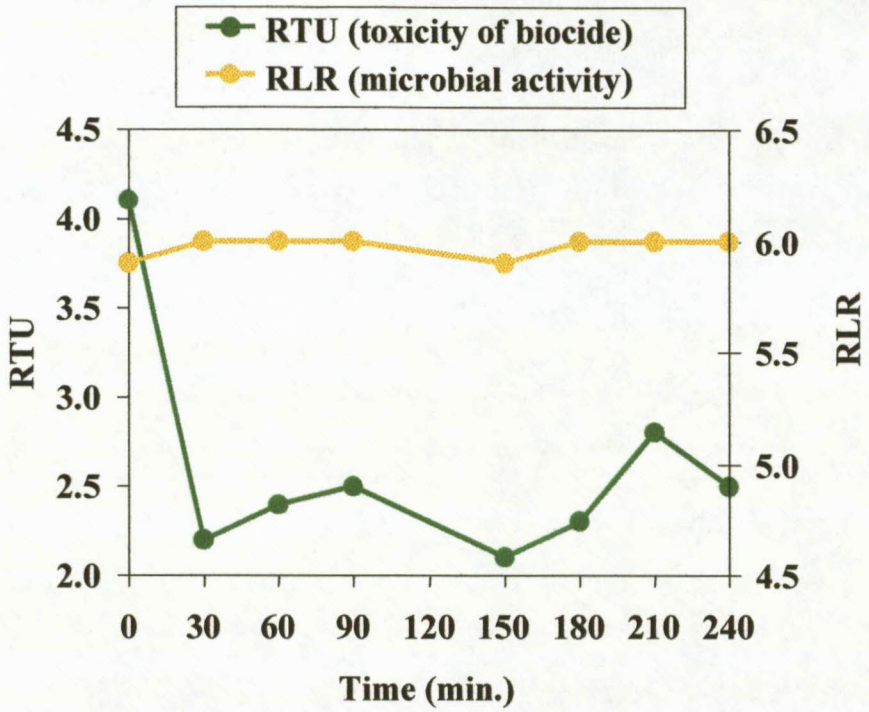

Figure 5. Comparison of the toxicity of the biocides in the water system as reflected by relative toxicity units (RTU) with the microbial activity as reflected by relative light readings (RLR) during the first audit.

During the second audit the biocides were more effective in controlling microbial contamination. The effectiveness of the biocides being applied was demonstrated by the decreased levels of microorganisms as the levels of biocide increased (Figure 6). It may, therefore, be concluded that the application of the new biocides at the mill was effective in reducing the microbial activity within the water system.

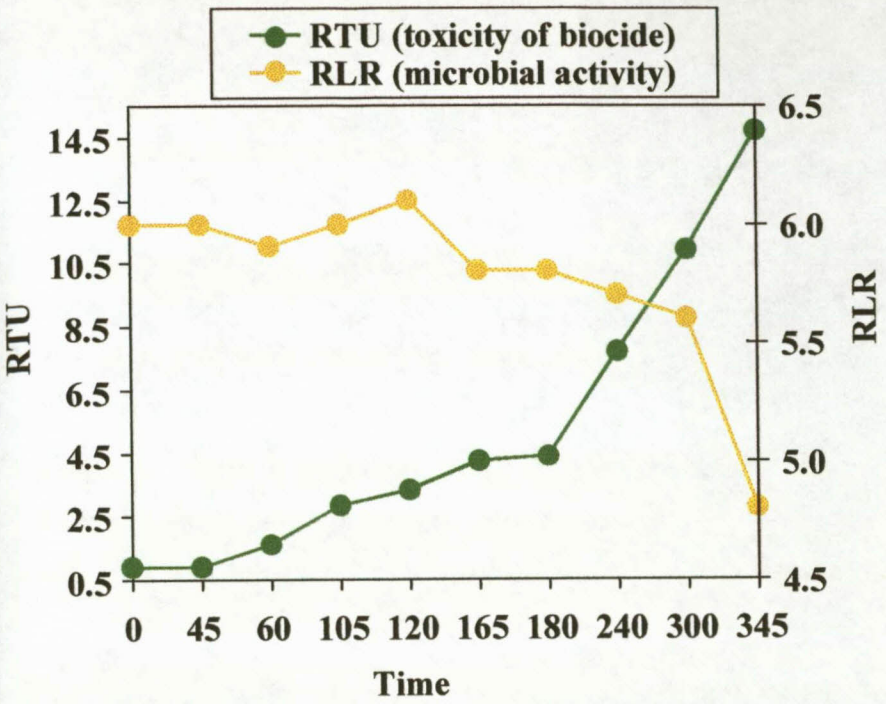

Figure 6. Comparison of the toxicity of the biocides in the water system as reflected by relative toxicity units (RTU) with the microbial activity as reflected by relative light readings (RLR) during the second audit.

Structural Diversity

Analysis of the PLFAs revealed the presence of a large diversity of microorganisms in the different unit operations during the first and second microbial audits. The levels of microbial contamination detected in most of the unit operations based on the analysis of signature lipid biomarkers, had decreased noticeably since the first audit (Figures 7, 8 & 9). Based on the relative abundance of the concentration of monounsaturated fatty acids, the Gram negative fraction of the community had decreased substantially after a change in the biocide programme (Figure 7). These organisms are of specific interest since the sulphate reducing bacteria (SRB) are included in this group of microorganisms (Pelczar *et al.*, 1993). The SRB play a significant role in microbial fouling and corrosion (Wolfaardt & Cloete, 1992). A decrease in the SRB population may, thus, be beneficial to reduce maintenance costs at the plant.

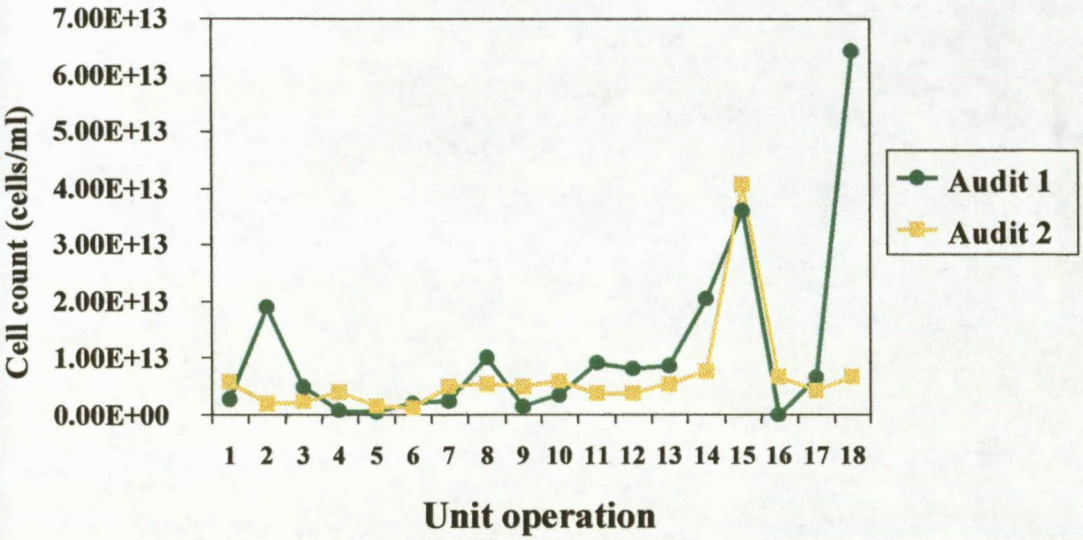

Figure 7. Comparison of the relative levels of Gram negative microorganisms (cells/ml) as detected with PLFA analysis in the different unit operations during the first audit and second audit. The relative abundance are calculated based on the assumption that monounsaturated fatty acids are considered to be indicative of the presence of Gram negative organisms.

After a change in the microbial control programme, the Gram positive microorganisms detected with PLFA anlaysis were almost completely under control (Figure 8). In some unit operations (broke chest (2), cloudy water chest (3), top line machine chest (5) and filler line headbox (6)) no Gram positive microorganisms were detected. Terminally branched saturated fatty acids are generally considered to be indicative of the presence of Gram positive organisms (Zelles, 1999).

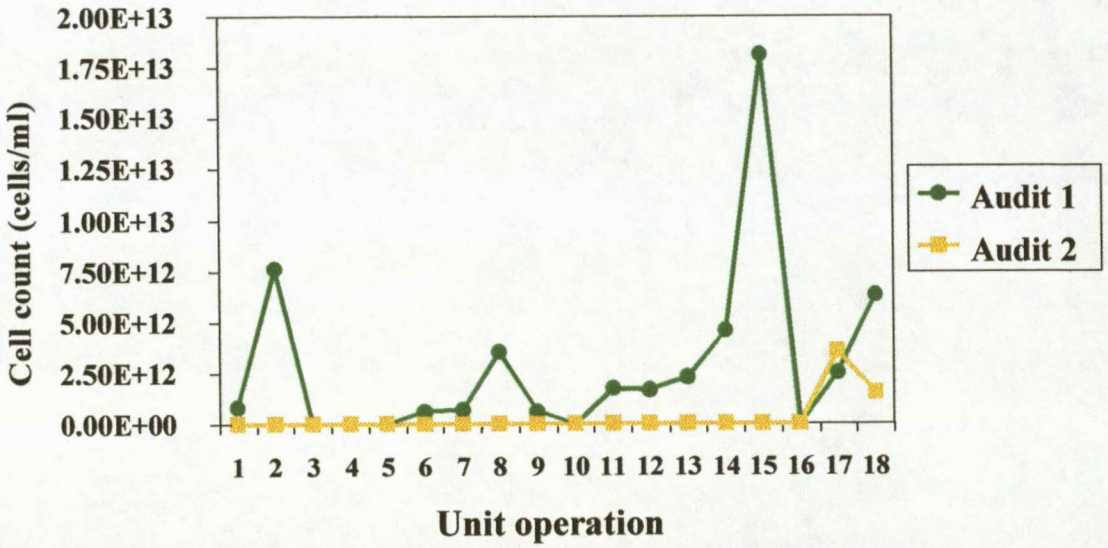

Figure 8. Comparison of the relative levels of Gram positive microorganisms (cells/ml) as detected with PLFA analysis in the different unit operations during the first audit and second audit. The relative abundance are calculated based on the assumption that terminally branched saturated fatty acids are considered to be indicative of the presence of Gram positive organisms.

Assuming that C18:2 can be considered a biomarker of fungal biomass, it was evident that the levels of fungal contamination in the water system had decreased substantially after the biocide dosage programme had been changed at the mill (Figure 9). Fungi contribute substantially to the microbial fouling at a mill and it is, therefore, of extreme importance to control the numbers of the fungal community in order to reduce biofilm build-up.

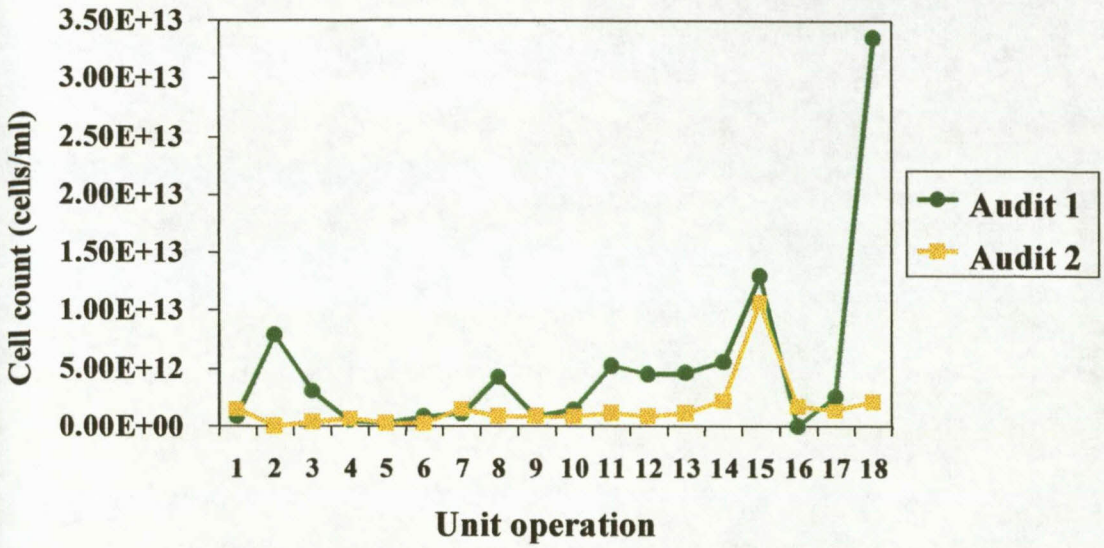

Figure 9. Comparison of the fungal levels (cells/ml) as detected with PLFA analysis in the different unit operations during the first audit and second audit. The relative abundance are calculated based on the assumption that C18:2 are considered to be a biomarker for fungi.

Culturing of viable cells indicated that the microbial numbers were similar in all the unit operations during the first audit (Figure 10). However, phospholipid fatty acid analysis indicated that a large variation in the number of organisms was present in the various unit operations. The higher counts and the fluctuations in the counts between the different unit operations could possibly be ascribed to the unculturable fraction of the community, which could only be detected with the PLFA analysis (Macnaughton *et al.,* 1997). Phospholipid fatty acid analysis, therefore, provided a better reflection of the variation within the system.

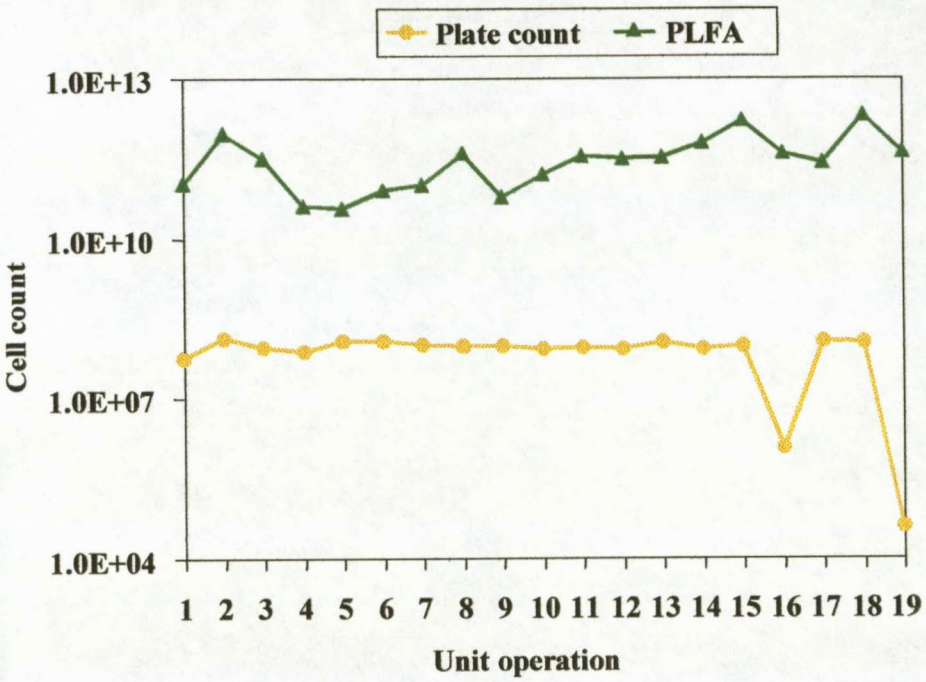

Figure 10. Comparison of cell counts as obtained with PLFA analysis (cells/ml) and plate counts (CFU/ml) at the different sampling points during the first audit.

Based on the results as obtained during this study, it was evident that the number of cultured cells and cell numbers based on PLFA analysis followed the same trend between the different unit operations during the second audit (Figure 11). After comparison of the cell numbers obtained from PLFA analysis during the first and second audit, it was clear that the cell counts had increased substantially after the change in the biocide dosage programme (Figure 12). This increase was in contrast to the results as obtained with conventional microbiological cultivation techniques, possibly indicating the presence of a larger unculturable fraction. It must, however, be kept in mind that it was not cell counts that were important, but the composition of the community. The higher counts obtained from the water samples did not contribute directly to biofilm formation and other microbiologically associated problems. Previous studies indicated that biofilm formation was not necessarily a problem when the cell counts were very high (Appling, 1955).

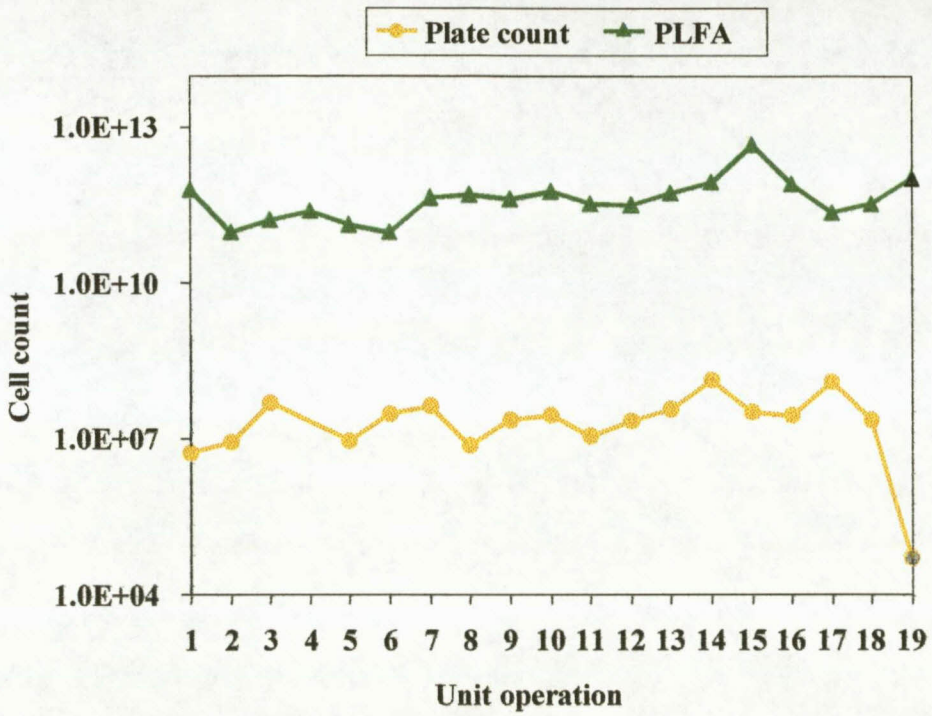

Figure 11. Comparison of cell counts as obtained with PLFA analysis (cells/ml) and plate counts (CFU/ml) at the different sampling points during the second audit.

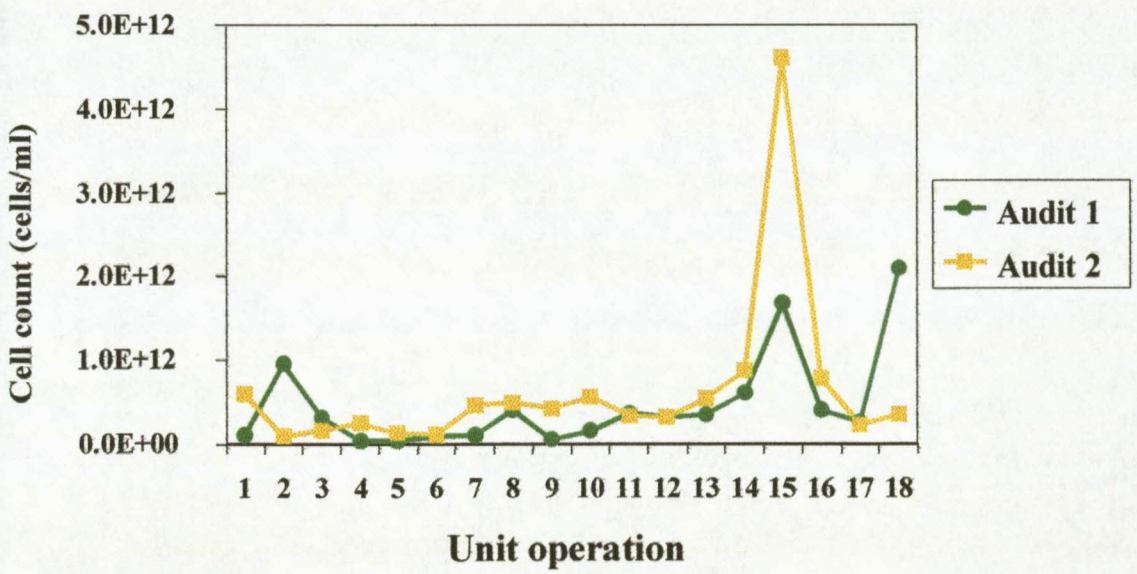

Figure 12. Comparison of cell counts as obtained with PLFA analysis (cells/ml) during the first and second audit.

Functional Diversity

The functional diversity of microbial populations in the water system of Cape Kraft during the first audit is best illustrated by a dendogram that was derived from hierarchical cluster analysis using Ward's minimum variance clustering algorithm (Figure 13). Two major clusters could be identified based on the similarity of the substrate utilisation profiles within the various unit operations.

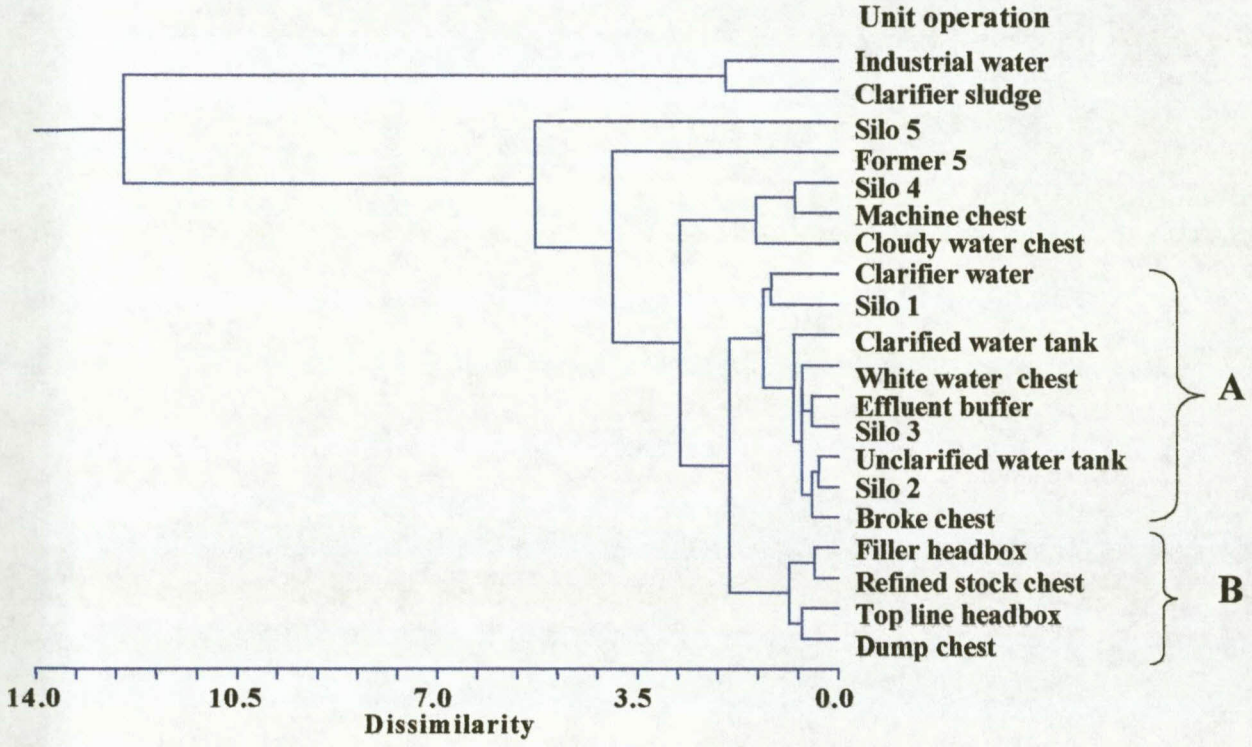

Figure 13. Dendogram obtained from the results of the substrate utilisation as obtained during the first audit using Ward's minimum variance clustering algorithm.

Two major clusters (A & B) could be identified with populations utilising similar substrates and also of unit operations that are linked in the water system (Figure 14). These results suggest that the functional diversity of the microbial communities within these unit operations is similar and that these unit operations could be dosed with similar biocides. The new biocide application points were, therefore, on the showers, the clarifier, the machine chest and the refined stock chest. The new application points for the biocide dosage fell into the clusters as obtained with the carbon source utilisation approach.

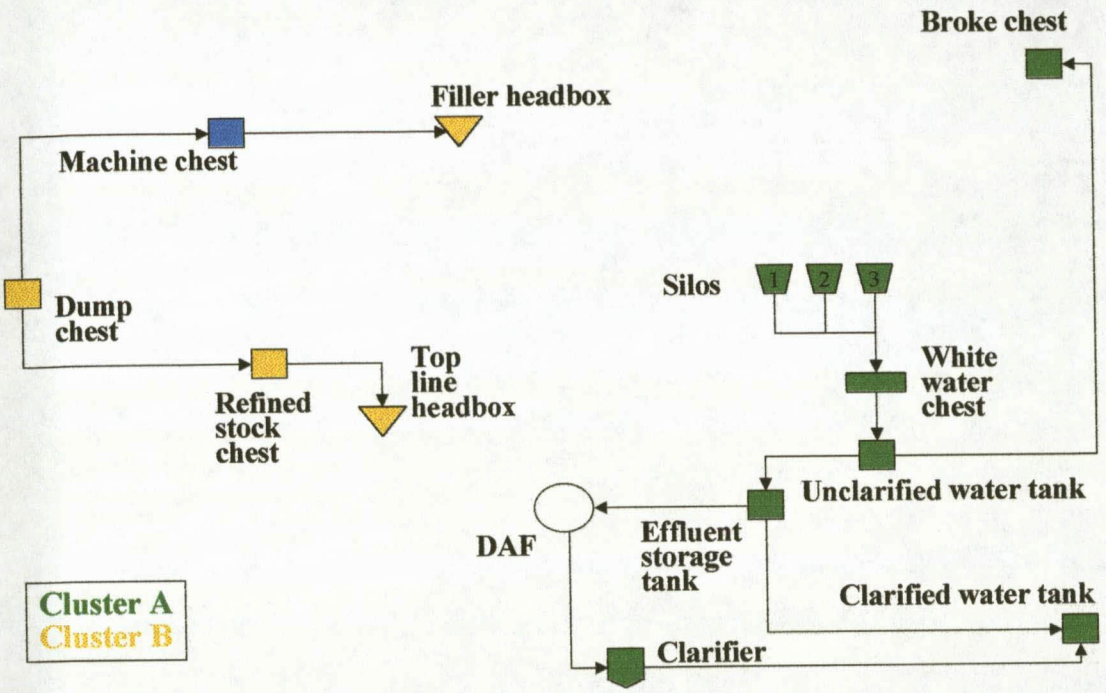

Figure 14. A summary of the water flow between the various unit operations from clusters obtained from Ward's minimum variance clustering algorithm. White areas were not sampled and blue areas were outside clusters.

The dendogram obtained from the hierarchical clustering of the substrate utilisation data from the second audit showed clusters with high dissimilarity (Figure 15). The degree of dissimilarity of the clusters obtained during this audit was much higher than obtained in the first audit and grouping of unit operations for similar biocide treatment was, therefore, not possible. The results obtained possibly reflected the ability of the new biocides being applied in the system to disrupt the microbial populations. These results obtained confirmed statements made by Schneider *et al.* (1998) who reported that phenotypic fingerprinting was very effective in detecting wastewater treatment upsets. Carbon source utilisation profiles were subsequently also found to be a more sensitive indicator of changes in the microbial community than conventional microbiological methods (Schneider *et al.*, 1998).

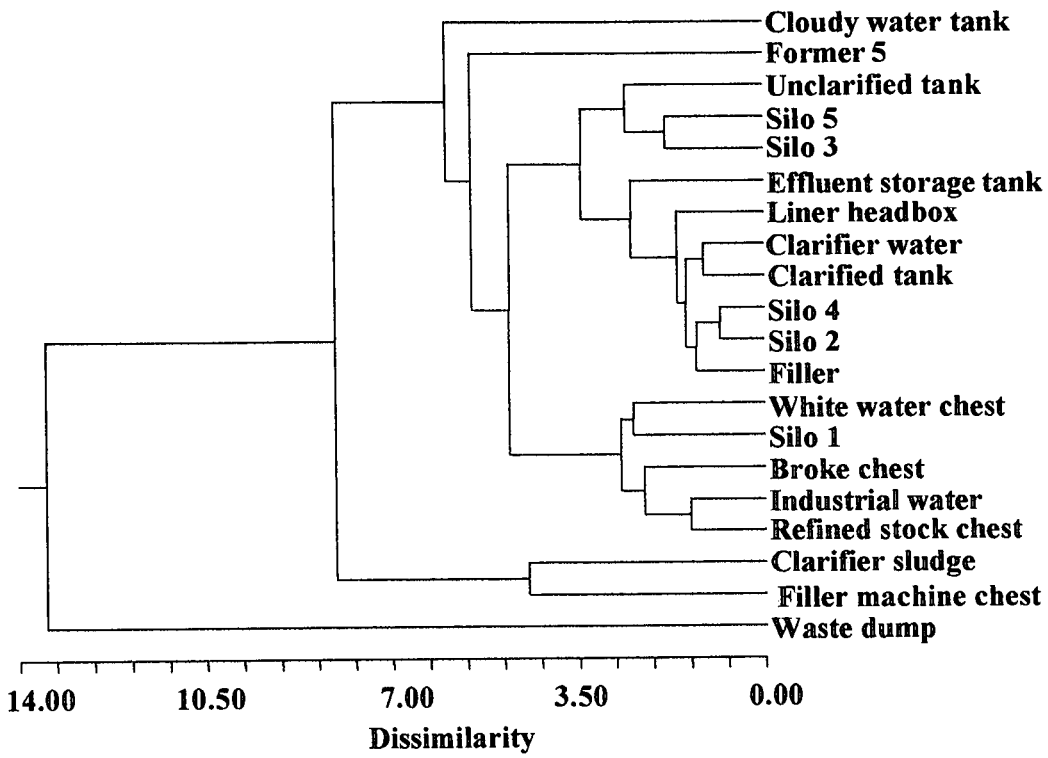

Figure 15. Dendogram obtained from the results of the substrate utilisation as obtained during the second audit using Ward's minimum variance clustering algorithm.

Biodiversity

The Shannon index (*H'*) (1) minimises the degree of substrate utilisation. A high Shannon index would emphasise the range of carbon sources utilised. Numerous studies using conventional microbiological techniques indicated that the microbial diversity of natural ecosystems generally decreases as a result of stress (Atlas and Bartha, 1997). In comparison to the results as obtained during the first audit, it is evident that the diversity of carbon sources utilised generally decreased after the change in the microbial control programme (audit 2) (Figure 16). The observed decrease in the diversity index generally confirmed the assumption that toxification of the water system had occurred, possibly due to the application of the new biocides.

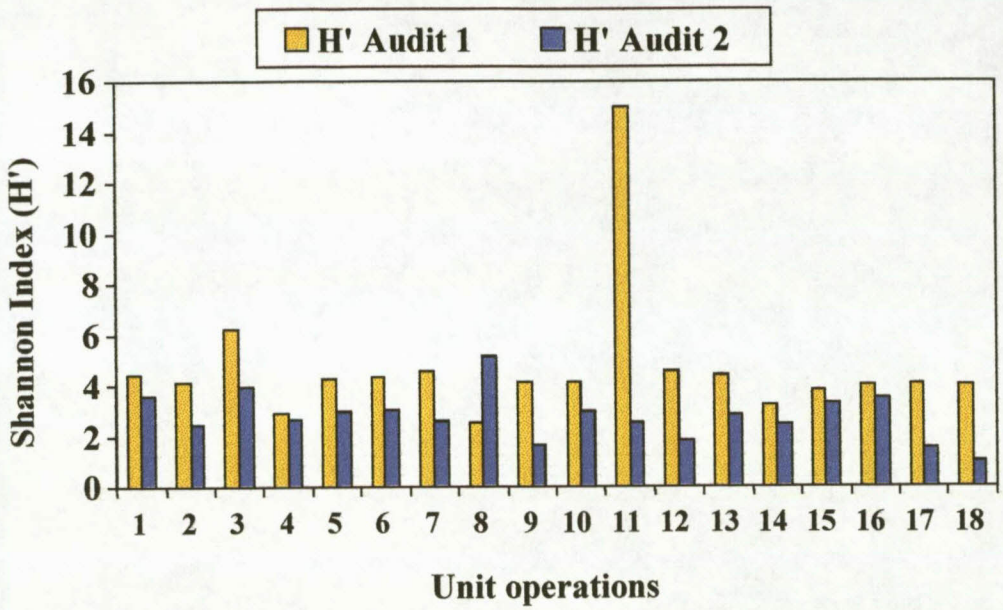

Figure 16. Comparison of the Shannon diversity indices (*H'*) as obtained during the first and second audits.

The Berger-Parker index (*d*) is used to detect the dominance in disrupted communities by guilds or species (Magurran, 1988). Based on the results as obtained during this study, it is evident that the dominance generally increased after the change in the biocide program (Figure 17). These results are indicative of the fact that the environment was disrupted. The increase might also be as a result of one species dominating the system. Therefore, the increase in dominance can be regarded as a reflection of an efficient biocide dosage programme.

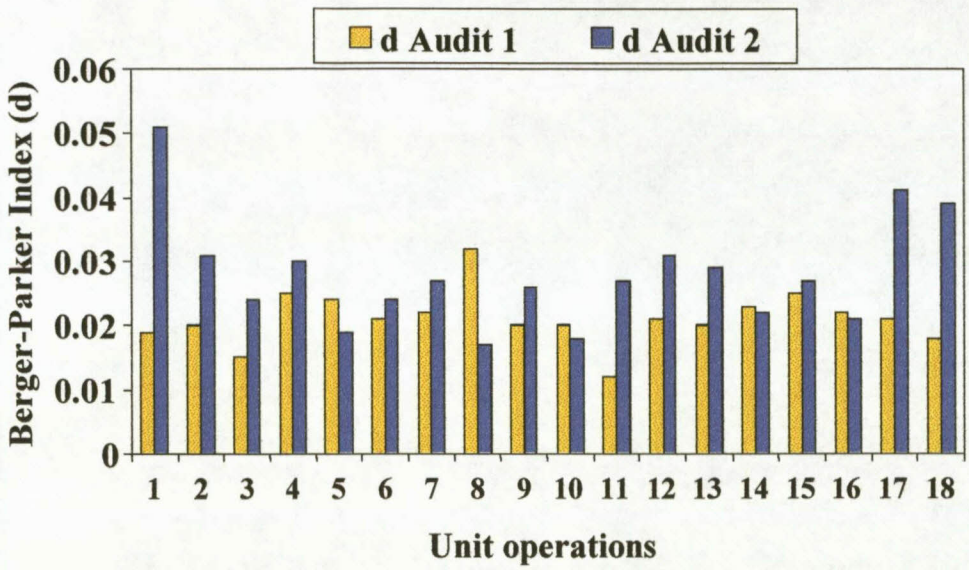

Figure 17. Comparison of the Berger-Parker indices (*d*) as obtained for the first and second audits.

CONCLUSIONS

The first microbiological audit provided information on the problem areas in the water system. Based on the results obtained during this audit, it was evident that the microbial control program was not effective in controlling biofouling at the mill and indications of resistance to the various biocides being applied were observed. Results of the Tra-Cide study indicated that population shifts or microbial resistance had occurred within the water system and alternative biocides were, therefore, recommended. The results of the Tra-Cide analysis as performed during the second audit indicated that the new biocides were more effective in reducing microbial activity and that the control strategy was more successful.

The pH and the temperature values as observed in the various unit operations remained similar, indicating that no major changes had occurred in the mill operation. Lower ORP levels were detected after the change in biocide application indicating that the conditions in the water system had become more oxidative since the first audit. As a result the anaerobic bacteria were easier to control by the improved biocide dosage programme. After the change in the biocide application, the TAB levels and numbers of fungi were generally lower in all the unit operations than observed during the first audit. Reduced numbers of SRB, TAB and fungi indicated a notable improvement in the microbial control programme.

Results obtained from the analysis of the PLFA fraction of the total extracted lipids showed a significant reduction in the Gram negative, Gram positive and fungal communities. However, comparison of the total cell numbers as obtained during the first and second audits, respectively indicated that the cell counts had increased during the second audit. This could possibly be attributed to an increase in the unculturable fraction of the microbial community.

The results of the functional diversity as observed during the first audit indicated that the system was characterised by a diverse microbial community in the various unit operations. New points for biocide application were suggested according to clusters of similar unit operations. The degree of dissimilarity of the clusters obtained after a change in the microbial control programme was much higher than obtained in the first

audit and it was not possible to distinguish any major clusters on the basis of unit operations. Grouping of unit operations for similar biocide treatment was, therefore, not possible. These results reflected the ability of the biocides to disrupt the microbial populations with similar functionalities. The results obtained from the various biodiversity indices confirmed that disruption of the microbial communities had occurred. The substrate diversity indices (H') were generally lower in the larger part of the system after the change in the microbial control programme. The dominance (d) in the system as obtained using the Berger-Parker index had also increased, indicating the disruption in the system, possibly due to the new biocide dosage programme.

In conclusion a considerable improvement in the control of microbial contamination at the mill had been made. Visual inspection of the plant also showed that the general housekeeping practises in the mill had improved considerably. The surveys consequently provided useful information regarding problem areas in the system and supplied a complete understanding of the water system at Cape Kraft. The surveys also provided information to aid in the selection of a biocide dosage programme as well as the management and the closure of the water system.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

In paper mills, microbial biofilms frequently result in the production of odours, paper breakages, spotting, holes and discolouration of paper, resulting in a loss in paper quality (Stoner & King, 1994). Microbial biofilms also play a significant role in microbiologically induced corrosion. Microbial problems subsequently result in poor runnability and lower production rates of the plant that have severe economic implications for a paper mill (Sorelle & Belgard, 1991).

Worldwide, the use of recycled fibre is increasing in all paper and board end products (Anonymous, 1992). However, the use of recycled fibre elevates the microbial counts and consequently the associated problems in paper mill water systems. It has been reported that microbial numbers in recycled fibre can be approximately 1000 times higher than that observed in virgin pulp (Sorelle & Belgard, 1991). The recycled fibre then serves as an inoculum for both bacterial and fungal contamination into a paper mill water system.

Furthermore, the closure of paper mill water systems usually enhances microbial problems. Upon closure of the water system the temperatures are frequently elevated and the nutrient concentrations generally increase (Gudlauski, 1996) contributing to the microbiologically associated problems in wastewater systems (Vaisanen *et al.*, 1994). The closure of water systems generally increases the degree of biofilm formation and subsequent corrosion (Bennett, 1985). Microbial contamination and fouling are thus frequently enhanced in a paper mill, primarily due to the reuse of water and the use of recycled fibre.

Biocides are added to the paper machine water system to reduce the numbers of microorganisms available for attachment and subsequent biofilm formation. The efficacy of biocides is generally measured by conventional methods. Microorganisms can be enumerated using plate counts, adenosine triphosphate measurements and the most probable number method, but these methods have certain disadvantages. Recent

studies have indicated that less than 1 % of all microbes can be cultured (Palojarvi *et al.*, 1997; Vestal & White, 1989) and conventional microbial enumeration techniques (plate counts), therefore, generally underestimate the number of organisms present in a sample (White, 1984). Adenosine triphosphate measurements are generally not recommended since some microorganisms can alter their concentration of ATP with a change in nutritional or physiological conditions (Atlas & Bartha, 1993). The MPN method is based on estimations, and there is consequently opportunity for errors (Melchiorri-Santolini, 1972). It is thus imperative that alternative techniques should be used to monitor microbial numbers in industrial water systems.

Schneider *et al.* (1998) reported that carbon source utilisation patterns were very effective in detecting changes in the toxicity of the effluent at a paper mill. The present study confirmed that carbon source utilisation patterns were a sensitive method for detection of shifts in the microbial community. Carbon source utilisation patterns were used at the Cape Kraft paper mill, Milnerton, Cape Town, South Africa, to detect the influence of the process parameters on the microbial community. The influence on the functional diversity of the microbial community was evaluated for the changes in the production grade, changes in biocide application and changes as a result of cleaning during a shutdown (Chapter 2). It was observed that unique microbial communities developed in the water system when linerboard and fluting were produced. It is thus apparent that different biocides should be used when different board grades are manufactured, but this might not be practical. No trends could be observed in the sessile phase after a shut, possibly due to inefficient cleaning since no boilout was performed during the shutdown period. The microbial communities in the planktonic phase could be differentiated based on the time that had elapsed after a shut. More efficient cleaning methods during the shutdown period, such as a boilout, might solve the problem of biofilm buildup. The addition of biodispersants might be another solution, although biodispersants do not kill the microorganisms. Biocide effectiveness is frequently enhanced (Johnsrud, 1997) since these substances dislodge microorganisms and they will be present in the planktonic phase.

Substrate utilisation profiles were also sensitive enough to detect changes in the microbial community due to a change in the microbial control programme (Chapter 4). The carbon source utilisation approach was successfully used to group unit operations for similar biocide application. The biocide programme was thus changed accordingly. After a change in the microbial control programme no clusters could be distinguished, possibly due to the successful disruption of the microbial community (Chapter 4). Carbon source utilisation patterns can thus successfully be used to determine the most suitable locations for biocide applications and also be used to determine the efficacy of changes in the biocide dosage programme.

Biodiversity indices indicated that the disruption of the microbial communities occurred due to the biocide application. The substrate diversity was generally lower in the system after the change in the microbial control programme. The dominance also increased as a result of the toxic disruption (Magurran, 1988) in the system (Chapter 4). The calculation of biodiversity indices can thus be used as an additional method to evaluate the efficiency of a biocide dosage programme or changes in other process parameters.

Phospholipid fatty acid analysis was found to be efficient in differentiating between different groups of microorganisms (Gillan & Hogg, 1984). Another advantage of PLFA analysis is that bacteria and eukaryotes can be detected in a single analysis (Noble *et al.*, 2000). The analysis of PLFAs also provided a method to determine changes in the overall composition of the microbial community (Frostegard *et al.*, 1997). During the present study changes were observed in the lipids based on the production of different board grades and biocide applications (Chapter 3). The main difference between the two paper grades was the difference in pH during the production. Environmental changes, such as the pH level and exposure to toxic substances influence the PLFA composition of the cell membranes (Frostegard *et al.*, 1997; Dowling *et al.*, 1986). It was concluded that the chemical composition of the water and the biocides influenced the composition of the microbial community in the water system. Werker & Hall (1998) demonstrated the use of fatty acid analysis to distinguish between planktonic and sessile microbial populations based on the relative ratios of different fatty acids. This study confirmed the lower ratio of saturated to

unsaturated C18 fatty acids in the planktonic community and the larger proportion of C18 to C16 fatty acids in the sessile community.

Ratios of the *trans* to *cis* isomers of higher than 0,1 are generally considered to be indicative of exposure to toxins or starvation (Guckert *et al.*, 1991) while ratios of 0,05 or less are generally considered to be indicative of non-stressed microbial communities (White *et al.*, 1996). The ratios of *trans* to *cis* isomers obtained in the present study were indicative of stressed microbial communities. It was concluded that the stress was a result of the biocide application, the change in pH and the chemical composition of the water during a change in the production grade (Chapter 3). The ratios of the *trans/cis* isomers can, therefore, be used as an indication of biocide efficacy.

The same trends were observed in the cell counts as obtained with conventional culturing and with PLFA analysis, although the values obtained with PLFA analysis were much higher (Chapter 3). The higher cell counts obtained with PLFA analysis, stressed the underestimation of microorganisms obtained by conventional microbiological methods. Signature lipid biomarker analysis, can, therefore, provide a better understanding of the effect of biocides on the total microbial community, since only a part of the community can be detected with conventional microbiological culturing techniques. The analysis of PLFAs can also provide a more detailed analysis of the contamination in a paper mill water system and different groups of microorganisms can be detected with a single analysis.

Phospholipid fatty acid analyses provided an overview of the microbial community (White & Macnaughton, 1997) and the data could be used to study changes in the major groups of organisms (Zelles, 1999). Phospholipid fatty acid analysis was used to study the changes in the microbial community before and after a change in the microbial control programme at the Cape Kraft paper mill (Chapter 4). Phospholipid fatty acid analysis showed a significant reduction in the Gram negative, Gram positive and fungal communities. It was evident that the signature lipid biomarker approach was effective in detecting shifts within the microbial community that was not detected with conventional culturing.

The analysis of substrate utilisation patterns facilitated the selection of new biocide application points in the paper mill water system and more comprehensive data were obtained using the signature lipid biomarker approach than with conventional microbiological techniques. This study showed the successful implementation of signature lipid biomarker analyses and carbon source utilisation patterns in a paper mill water system. It is recommended that the analysis of substrate utilisation patterns and signature lipid biomarkers be used in future studies in industrial water systems to monitor the effect of major changes on the microbial communities.

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SUMMARY

KEYWORDS: Paper mill, water system, biofilm, functional diversity, structural diversity, signature lipid biomarkers, Biolog

Microbiological studies of wastewater treatment systems generally rely on methods that are dependent on the culturability of the microorganisms being investigated. However, it has recently been estimated that less than 1 % of all microorganisms are culturable on synthetic media and under artificial conditions. The numbers of microorganisms are, therefore, largely underestimated. Due to the limitation of the conventional microbiological methods, numerous alternative assays have been proposed. These assays include the analysis of the functional and structural diversities of microbial communities *in situ*. A study was, therefore, undertaken at the Sappi Cape Kraft paper mill, Milnerton, Cape Town, South Africa, to evaluate the applicability of these alternative monitoring techniques in industrial systems. Microbial contamination at the plant are substantially enhanced due to the reuse of water and the use of recycled fibre for the production of fluting and linerboard.

The influence of the production of various paper grades, biocide application and monthly shuts on the functional diversity of the microbial communities within the water system was determined. Results obtained during this study indicated that different microbial communities developed during the production of the different paper grades. A difference in the functional diversity of the planktonic and the sessile communities was evident after a change in the production grade. The shift in the functional diversity of the microbial community present in the planktonic phase was evident almost immediately upon a change in the board grade. Although a shift in the functional diversity of the microbial community within the sessile phase was also evident, the response was significantly delayed. The effect of different biocide dosages was also monitored in the planktonic and the sessile phases. No clear trends concerning the period of time after a shutdown could be observed in the planktonic phase, although a change could be observed in the sessile phase. It was concluded that the analysis of the functional diversity was sensitive enough to detect shifts in the community and to differentiate between microbial communities within the same

system. The shifts could be ascribed to changes in the operational parameters in the water system.

The influence of the process parameters on the structural diversity of the microbial community was also determined using signature lipid biomarkers. The ratio of diglyceride fatty acids to phospholipid fatty acids provided an estimate of the non-viable to viable microorganisms in the water system. A high biomass mortality was generally observed throughout the one year period of evaluation. The high ratios of *trans* to *cis* C18:1 obtained during this study were also indicative of stressed microbial communities. Based on the presence of signature lipid biomarkers, it was concluded that a large diversity of microorganisms was present in both the sessile and planktonic phases. The same trends in the number of cultured cells and the counts obtained with PLFA analysis were observed in all samples. Differences in the abundance and groups of specific fatty acids were detected due to the production of different board grades. These results confirmed the shift in the microbial community, as was detected on the basis of substrate utilisation profiles. Based on the results obtained during this study, it is evident that the application of signature lipid biomarkers provided substantially more information on the microbial communities than conventional culturing.

Due to these positive results, the new techniques were applied to characterise the functional and structural diversities of the microbial communities during two microbial audits at the Sappi Cape Kraft paper mill. The first audit was performed to assess the efficiency of the current microbiological control programme at the mill and to make recommendations for the improvement of the microbial control. The second audit was performed to evaluate the effect of subsequent changes made to the microbial control programme. Due to the indication that resistance to the current biocides had developed, alternative biocides were recommended for future use in the system. A large diversity of microorganisms was detected in the various unit operations using phospholipid fatty analysis. Cluster analysis of the functional diversity of the various microbial communities indicated that groups of unit operations could be dosed using separate biocide dosage programmes. It may thus be concluded that the application of these alternative techniques provided substantial

information concerning the microbial function and structure *in situ*. This information assisted in the implementation of an alternative microbiological control programme at the paper mill, which resulted in a significant reduction in the microbiological associated problems.

OPSOMMING

KEYWORDS: Paper mill, water system, biofilm, functional diversity, structural diversity, signature lipid biomarkers, Biolog

Mikrobiologiese studies en evaluasie van programme om watersisteme te behandel maak oor die algemeen staat op metodes wat afhanklik is van die kweekbaarheid van mikroorganismes. Daar is egter onlangs beraam dat minder as 1 % van alle mikroorganismes op sintetiese media en onder kunsmatige toestande gekweek kan word. Die getal mikroorganismes word daarom tot 'n groot mate onderskat. As gevolg van die beperkings waaraan konvensionele mikrobiologiese metodes onderhewig is, is verskeie alternatiewe metodes dus voorgestel. Hierdie metodes sluit die ontleding van die funksionele- en strukturele-diversiteit van mikrobiese gemeenskappe in hulle natuurlike toestand in. 'n Studie om die toepaslikheid van hierdie alternatiewe moniteringstegnieke in indurstriële stelsels te evalueer, is dus by die Sappi Cape Kraft papiermeule te Milnerton, Kaapstad onderneem. Mikrobiese kontaminasie van diè aanleg word aansienlik vererger as gevolg van die hergebruik van water en die gebruik van herwinde vesels in die vervaardiging van riffel- en voering-karton.

Die invloed van verskillende karton produkte, die aanwending van biosiedes en maandelikse sluiting vir instandhouding op die funksionele-diversiteit van die mikrobiese gemeenskappe in die waterstelsel is bepaal. Dit het geblyk dat daar 'n verskil in die funksionele-diversiteit van die planktoniese en sessiele gemeenskappe ingetree het na 'n verandering in die karton graad. Hoewel dit geblyk het dat daar ook 'n verandering in die funksionele-diversiteit van die mikrobiese gemeenskap in die sessiele fase voorgekom het, was die reaksie aansienlik vertraag. Die uitwerking van verskillende biosiedes op die planktoniese en sessiele fases is ook gemonitor. Geen duidelike tendense betreffende die tydsverloop na 'n sluiting kon in die planktoniese fase onderskei word nie, hoewel 'n verandering in die sessile fase waargeneem is. Die gevolgtrekking was dat die ontleding van die funksionele diversiteit sensitief genoeg was om veranderings in die mikrobiese gemeenskap vas te stel en om te onderskei tussen verskillende gemeenskappe in dieselfde stelsel. Die veranderings

kan toegeskryf word aan veranderings in die operasionele parameters in die waterstelsel.

Die invloed van die prosesparameters op die strukturele diversiteit van die mikrobiese gemeenskap is ook bepaal deur gebruik te maak van indikator-lipiede as biomerkers. Die verhouding van digliseried vetsure tot fosfolipied vetsure het dit moontlik gemaak om 'n raming te maak van die lewensvatbaarheid van die mikroorganimses in die waterstelsel. 'n Hoë tempo van afsterwing is waargeneem oor 'n tydperk van een jaar. Die hoë verhouding van *trans* tot *cis* C18:1 wat gedurende hierdie studie waargeneem is, was ook 'n aanduiding van mikrobiese gemeenskappe wat onder omgewings druk was. Die aanwesigheid van spesifieke indikator-lipiede het daarop gedui dat 'n groot diversiteit van mikroorganismes in beide die sessiele en planktoniese fases aanwesig was. Dieselfde tendense in die getal kweekbare selle is verkry as met die getalle wat met behulp van fosfolipied vetsuur analises bepaal is. Verskille wat in die hoeveelhede van spesifieke groepe vetsure vasgestel is, was die gevolg van die vervaardiging van verskillende tipes karton. Hierdie resultate het die verandering in die mikrobiese gemeenskap bevestig, wat bepaal is deur die profiele van substraatbenutting. Uit die resultate wat gedurende die studie verkry is, blyk dit dat die gebruik van indikator-lipiede aansienlik meer inligting verskaf oor die mikrobiese gemeenskappe as wat met konvensionele kweking moontlik is.

Op grond van die positiewe resultate wat verkry is, is hierdie alternatiewe tegnieke gebruik om die funksionele- en strukturele-diversiteit van die mikrobiese gemeenskappe gedurende twee mikrobiese oudits by die Sappi Cape Kraft papiermeule te karakteriseer. Die eerste oudit is uitgevoer om die doeltreffendheid van die huidige mikrobiologiese beheerprogram by die meule te evalueer en aanbevelings te maak om die beheerprogram te verbeter. Die tweede oudit uitgevoer is om die uitwerking van diè veranderinge aan die mikrobiese beheerprogram te evalueer. As gevolg van die aanduidings dat weerstand teen die biosiedes wat aangewend is ontwikkel het, is aanbeveel dat alternatiewe biosiedes in die toekoms toegedien word. 'n Wye verskeidenheid van mikroorganismes is met fosfolipied vetsuuranalises in die verskillende eenheids prosesse aangetref. Trosanalise van die funksionele-diversiteit van die verskillende mikrobiese gemeenskappe het aangedui

dat verskillende prosesse saam gegroepeer kon word om met soortgelyke biosiedprogramme behandel te word. Die gevolgtrekking was dat die aanwending van hierdie alternatiewe tegnieke uiters belangrike inligting betreffende die mikrobiese funksie en stuktuur *in situ* verskaf het. Hierdie inligting het meegehelp om 'n alternatiewe mikrobiologiese beheerprogam by die meule te implementeer, wat aansienlike vermindering in probleme teweeggebring het wat verband hou met mikrobiese kontaminasie.

APPENDICES

Appendix A. Optical density values obtained for the planktonic samples.

	Sample Number																								
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
A02	0,363	0,354	0,174	0,789	0,177	0,209	0,057	0,116	0,186	0,287	0,032	0,283	0,073	0,088	0,432	0,072	0,000	0,232	0,015	0,006	0,000	0,315	0,000	0,000	0,000
A03	0,391	0,646	0,140	0,674	0,953	0,881	0,331	0,361	0,488	0,222	0,506	0,646	0,562	0,777	0,646	0,228	0,393	0,470	0,124	0,222	0,000	0,260	0,000	0,000	0,000
A04	0,544	0,851	0,159	0,435	0,440	0,443	0,462	0,371	0,381	0,635	0,443	0,291	0,460	0,530	0,593	0,022	0,544	0,480	0,409	0,298	0,197	0,172	0,000	0,000	0,000
A05	0,460	0,169	0,422	0,004	0,000	0,000	0,000	0,000	0,046	0,749	0,000	0,508	0,223	0,102	0,326	0,118	0,294	0,389	0,192	0,117	0,100	0,561	0,000	0,000	0,000
A06	0,423	0,215	0,157	0,000	0,000	0,000	0,000	0,000	0,034	0,649	0,000	0,029	0,000	0,000	0,000	0,024	0,215	0,332	0,000	0,000	0,135	0,454	0,000	0,000	0,000
A07	0,662	0,532	1,322	0,008	0,118	0,148	0,711	0,273	0,490	0,017	0,034	0,187	0,121	0,150	0,000	0,000	0,000	0,195	0,473	0,198	0,553	0,575	0,000	0,000	0,000
A08	0,748	0,905	1,589	1,007	1,323	1,212	0,488	0,855	0,724	0,982	1,311	0,706	1,368	1,262	0,538	0,434	0,653	0,741	0,504	0,000	0,000	0,711	0,000	0,000	0,000
A09	0,632	0,405	0,240	0,000	0,793	0,758	0,554	0,445	0,499	0,123	0,135	0,486	0,141	0,321	0,045	0,039	0,000	0,265	0,337	0,211	0,147	0,326	0,000	0,000	0,000
A10	0,044	0,367	0,497	0,889	1,029	0,957	0,411	0,618	0,560	1,125	0,563	0,581	0,779	1,287	0,555	0,366	0,450	0,613	0,195	0,422	0,000	0,000	0,000	0,000	0,289
A11	0,319	0,824	0,377	0,250	0,753	0,791	0,633	0,414	0,539	0,197	0,199	0,579	0,305	0,527	0,095	0,091	0,320	0,000	0,391	0,275	0,037	0,000	0,080	1,070	1,647
A12	0,594	0,885	0,042	1,084	1,224	1,189	0,640	0,472	0,574	0,003	0,727	0,772	0,910	1,393	0,442	0,174	0,160	0,524	0,111	0,000	0,000	0,000	2,385	0,000	0,173
B01	0,092	0,045	0,000	0,000	0,037	0,047	0,324	0,207	0,129	0,135	0,064	0,181	0,081	0,009	0,153	0,000	0,000	0,020	0,192	0,081	0,366	0,498	0,000	0,000	0,000
B02	0,192	0,679	0,575	0,868	1,096	0,993	0,597	0,303	0,372	0,500	1,188	1,030	1,047	1,364	0,641	0,685	0,772	0,580	0,380	0,330	0,039	0,611	0,000	0,000	0,000
B03	0,438	0,529	0,635	0,230	0,284	0,329	0,789	0,240	0,319	0,306	0,193	0,347	0,236	0,403	0,442	0,174	0,310	0,182	0,416	0,294	0,093	0,458	0,000	0,000	0,000
B04	0,724	0,855	0,421	1,089	1,080	1,168	0,851	0,798	0,717	1,112	1,028	1,062	0,813	1,506	0,755	0,552	0,786	0,834	0,402	0,245	0,231	0,644	0,000	0,000	0,000
B05	0,454	0,920	0,220	1,095	1,060	1,155	0,780	0,543	0,561	0,652	0,635	0,727	0,927	1,436	0,615	0,425	0,435	0,627	0,333	0,249	0,000	0,585	0,000	0,000	0,000
B06	0,402	1,005	1,015	0,946	1,120	1,061	1,009	0,250	0,536	0,814	0,931	0,741	1,203	1,548	0,572	0,802	0,668	0,735	0,490	0,260	0,094	0,768	0,000	0,000	0,000
B07	0,517	0,613	0,445	0,543	0,156	0,382	0,789	0,309	0,456	0,937	0,211	0,365	0,177	0,344	0,103	0,066	0,000	0,125	0,730	0,316	0,205	0,285	0,000	0,000	0,000
B08	0,672	0,989	0,410	0,311	0,800	1,307	0,637	0,440	0,613	0,165	0,339	0,295	0,377	1,001	0,808	0,272	0,211	0,545	0,295	0,292	0,308	0,536	0,000	0,000	0,000
B09	0,657	0,936	0,176	0,000	0,049	0,211	0,539	0,390	0,381	0,098	0,157	0,254	0,019	0,163	0,420	0,063	0,119	0,270	0,398	0,139	0,309	0,113	0,000	0,000	0,000
B10	0,444	1,035	0,159	0,906	1,043	1,131	0,789	0,589	0,657	0,556	1,301	0,710	1,284	1,514	0,760	0,491	0,566	0,599	0,433	0,319	0,004	0,000	0,000	0,000	0,185
B11	0,408	0,559	0,000	0,848	1,132	1,172	0,406	0,000	0,546	0,981	0,749	0,655	1,011	1,205	0,454	0,624	0,564	0,322	0,297	0,316	0,000	0,000	0,126	1,153	1,738
B12	0,474	0,967	0,346	1,081	0,967	0,919	0,456	0,473	0,569	0,518	0,511	0,924	0,852	1,250	0,428	0,504	0,410	0,745	0,238	0,065	0,098	0,000	2,501	0,000	0,013
C01	0,474	0,724	0,052	0,899	1,184	1,166	0,738	0,694	0,432	0,171	1,052	0,733	0,938	1,459	0,707	0,496	0,712	0,161	0,357	0,115	0,075	0,629	0,007	0,000	0,062
C02	0,391	0,844	0,000	1,051	1,282	1,170	0,703	0,636	0,564	0,358	1,223	0,827	1,282	1,275	0,604	0,485	0,760	0,576	0,645	0,224	0,255	0,841	0,000	0,000	0,000
C03	0,484	0,514	0,372	0,051	0,361	0,405	0,771	0,354	0,411	0,666	0,209	0,289	0,214	0,437	0,561	0,307	0,102	0,240	0,423	0,290	0,000	0,414	0,000	0,000	0,000

C04	0,520	1,068	0,172	1,038	1,211	1,170	0,894	0,549	0,474	0,295	1,306	0,816	1,089	1,563	0,785	0,450	0,666	0,365	0,530	0,255	0,000	0,714	0,000	0,000	0,000
C05	0,540	0,981	0,502	0,831	0,740	0,833	0,719	0,407	0,447	0,192	0,368	0,874	0,480	0,850	0,487	0,434	0,531	0,348	0,450	0,167	0,256	0,581	0,000	0,000	0,000
C06	0,667	0,927	0,615	1,035	1,227	1,289	0,969	0,813	0,620	0,421	0,720	0,827	0,965	1,310	0,348	0,583	0,598	0,565	0,487	0,317	0,000	0,612	0,000	0,000	0,000
C07	0,622	0,955	0,473	1,063	1,219	1,208	0,983	0,585	0,617	0,701	1,509	0,948	1,649	1,621	0,878	0,690	0,742	0,674	0,450	0,328	0,006	0,646	0,000	0,000	0,000
C08	0,718	1,093	0,242	1,056	1,386	1,393	1,131	0,820	0,704	0,162	1,449	1,015	1,534	1,738	0,880	0,546	0,817	0,603	0,514	0,319	0,171	0,708	0,000	0,000	0,000
C09	0,657	0,901	0,311	0,273	0,784	0,844	0,705	0,398	0,479	0,353	0,117	0,411	0,116	0,256	0,484	0,309	0,291	0,367	0,510	0,320	0,142	0,497	0,000	0,000	0,000
C10	0,444	0,496	0,001	0,128	0,000	0,026	0,565	0,179	0,299	0,385	0,139	0,140	0,096	0,050	0,204	0,002	0,000	0,216	0,447	0,372	0,179	0,000	0,000	0,000	0,040
C11	0,599	0,807	0,003	0,462	0,562	0,611	0,847	0,415	0,582	1,109	0,860	0,732	0,768	0,779	0,759	0,552	0,191	0,564	0,624	0,000	0,341	0,000	0,068	1,016	1,618
C12	0,000	0,092	0,000	0,025	0,000	0,000	0,000	0,000	0,320	0,000	0,056	0,178	0,056	0,078	0,000	0,000	0,000	0,000	0,347	0,000	0,115	0,000	2,428	0,168	0,320
D01	0,015	0,122	0,012	0,017	0,116	0,073	0,000	0,000	0,242	0,000	0,000	0,000	0,024	0,000	0,000	0,000	0,120	0,000	0,000	0,201	0,118	0,319	0,000	0,000	0,026
D02	0,423	0,756	0,432	0,575	0,708	0,762	0,698	0,262	0,595	0,000	0,279	0,496	0,327	0,619	0,588	0,334	0,586	0,325	0,408	0,247	0,572	0,483	0,000	0,098	0,000
D03	0,457	0,516	0,357	0,743	0,549	0,774	0,786	0,734	0,711	0,847	0,417	0,438	0,506	0,801	0,749	0,481	0,570	0,498	0,421	0,000	0,460	0,643	0,000	0,000	0,000
D04	0,408	0,299	0,000	0,000	0,102	0,252	0,415	0,000	0,360	0,565	0,165	0,154	0,008	0,081	0,198	0,000	0,000	0,000	0,183	0,433	0,231	0,017	0,000	0,000	0,000
D05	0,724	0,503	1,186	0,879	0,778	0,974	0,786	0,524	0,696	0,715	0,841	0,425	0,957	1,098	0,729	0,339	0,330	0,368	0,546	0,106	0,309	0,499	0,000	0,000	0,000
D06	0,528	0,484	0,819	0,824	1,130	1,111	0,806	0,623	0,717	0,632	1,089	0,853	1,137	1,188	0,560	0,342	0,381	0,749	0,740	0,315	0,374	0,726	0,000	0,000	0,000
D07	0,328	0,730	0,546	0,930	1,106	1,135	0,719	0,820	0,776	0,870	1,133	1,046	1,194	1,262	0,731	0,515	0,327	0,511	0,468	0,009	0,348	0,641	0,000	0,000	0,000
D08	0,484	0,380	0,441	0,463	0,065	0,187	0,890	0,373	0,519	0,471	0,155	0,255	0,065	0,164	0,178	0,111	0,000	0,220	0,659	0,000	0,290	0,378	0,000	0,000	0,000
D09	0,414	0,485	0,840	0,632	1,068	1,028	0,665	0,728	0,567	0,645	0,865	0,878	0,725	0,985	0,492	0,360	0,359	0,530	0,714	0,383	0,464	0,197	0,000	0,000	0,000
D10	0,402	0,016	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,173	0,166	0,000	0,134	0,329	0,225	0,044	0,000	0,000	0,000	0,420
D11	0,470	0,475	0,000	0,000	0,000	0,000	0,275	0,125	0,381	0,738	0,026	0,276	0,019	0,128	0,097	0,000	0,000	0,000	0,410	0,314	0,325	0,000	0,150	1,195	1,797
D12	0,307	0,000	0,000	0,000	0,000	0,000	0,200	0,096	0,414	0,768	0,035	0,279	0,001	0,000	0,000	0,034	0,000	0,082	0,223	0,028	0,213	0,000	2,623	0,000	0,151
E01	0,229	0,336	0,098	0,257	0,043	0,006	0,383	0,085	0,164	0,000	0,000	0,194	0,006	0,017	0,345	0,000	0,371	0,000	0,357	0,000	0,325	0,452	0,000	0,047	0,092
E02	0,229	0,354	0,100	0,000	0,000	0,000	0,098	0,191	0,390	0,521	0,000	0,351	0,000	0,012	0,149	0,050	0,000	0,000	0,573	0,061	0,126	0,299	0,000	0,079	0,000
E03	0,206	0,067	0,000	0,000	0,000	0,000	0,204	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,078	0,074	0,000	0,000	0,354	0,000	0,000	0,000
E04	0,307	0,734	0,332	1,003	0,103	0,137	0,680	0,339	0,669	0,296	0,090	0,239	0,152	0,297	0,574	0,192	0,173	0,182	0,450	0,187	0,352	0,362	0,000	0,000	0,000
E05	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,051	0,430	0,000	0,204	0,054	0,000	0,000	0,000
E06	0,388	0,719	0,342	0,437	0,276	0,417	0,780	0,423	0,669	0,714	0,229	0,425	0,130	0,255	0,602	0,181	0,294	0,187	0,435	0,212	0,124	0,404	0,000	0,000	0,000
E07	0,119	0,394	0,185	0,442	0,000	0,045	0,319	0,358	0,556	0,387	0,115	0,247	0,020	0,104	0,173	0,106	0,273	0,090	0,352	0,162	0,713	0,381	0,000	0,000	0,000
E08	0,197	0,112	0,163	0,160	0,038	0,080	0,239	0,000	0,011	0,000	0,098	0,155	0,000	0,104	0,000	0,069	0,041	0,207	0,262	0,275	0,573	0,333	0,000	0,000	0,000
E09	0,380	0,592	0,493	0,000	0,075	0,111	0,436	0,234	0,385	0,533	0,074	0,240	0,119	0,148	0,397	0,160	0,207	0,211	0,464	0,276	0,629	0,349	0,000	0,000	0,000

E10	0,369	0,753	0,837	0,650	0,759	0,810	0,819	0,586	0,740	0,777	0,595	0,518	0,491	0,875	0,749	0,456	0,405	0,442	0,415	0,416	0,718	0,000	0,000	0,000	0,221
E11	0,369	0,256	0,000	0,000	0,000	0,000	0,198	0,000	0,455	0,322	0,000	0,071	0,000	0,015	0,022	0,000	0,000	0,000	0,370	0,269	0,505	0,000	0,198	1,246	1,801
E12	0,429	0,235	0,094	0,672	0,237	0,147	0,601	0,157	0,544	0,635	0,256	0,166	0,175	0,105	0,566	0,135	0,075	0,169	0,173	0,000	0,177	0,000	2,577	0,000	0,169
F01	0,158	0,237	0,072	0,000	0,099	0,213	0,631	0,231	0,379	0,851	0,043	0,332	0,086	0,195	0,369	0,238	0,103	0,000	0,392	0,064	0,204	0,372	0,096	0,000	0,124
F02	0,180	0,119	0,663	0,430	0,000	0,042	0,349	0,036	0,254	0,326	0,064	0,251	0,083	0,068	0,478	0,087	0,141	0,000	0,606	0,456	0,519	0,602	0,000	0,092	0,000
F03	0,100	0,251	0,000	0,003	0,008	0,169	0,762	0,404	0,549	0,586	0,407	0,590	0,338	0,493	0,398	0,347	0,344	0,480	0,000	0,008	0,378	0,590	0,000	0,000	0,000
F04	0,331	0,278	0,000	0,056	0,000	0,000	0,128	0,053	0,444	0,495	0,000	0,202	0,000	0,151	0,000	0,062	0,160	0,000	0,455	0,171	0,498	0,597	0,000	0,000	0,000
F05	0,183	0,652	0,757	0,079	0,000	0,000	0,727	0,079	0,386	0,000	0,132	0,286	0,068	0,138	0,000	0,043	0,000	0,098	0,311	0,127	0,664	0,562	0,000	0,000	0,000
F06	0,267	0,229	0,718	0,395	0,000	0,000	0,644	0,095	0,374	0,324	0,000	0,245	0,207	0,095	0,235	0,000	0,265	0,145	0,410	0,217	0,776	0,800	0,000	0,000	0,000
F07	0,210	0,228	0,228	0,352	0,000	0,051	0,309	0,110	0,459	0,431	0,069	0,295	0,081	0,107	0,328	0,000	0,150	0,053	0,437	0,344	0,371	0,659	0,000	0,000	0,000
F08	0,447	0,767	1,414	0,718	0,032	0,124	0,983	0,464	0,479	0,374	0,198	0,458	0,174	0,268	0,697	0,137	0,356	0,207	0,605	0,000	0,458	0,535	0,000	0,000	0,000
F09	0,356	0,152	0,836	0,202	0,000	0,075	0,201	0,358	0,450	0,631	0,115	0,412	0,111	0,241	0,932	0,221	0,249	0,480	0,630	0,391	0,749	0,650	0,000	0,000	0,000
F10	0,353	0,590	1,240	0,383	0,146	0,331	0,941	0,421	0,544	0,589	0,224	0,404	0,265	0,424	0,664	0,157	0,178	0,229	0,512	0,406	0,436	0,110	0,000	0,000	0,101
F11	0,090	0,237	0,000	0,280	0,000	0,000	0,724	0,155	0,416	0,395	0,114	0,363	0,076	0,186	0,205	0,155	0,120	0,004	0,463	0,285	0,715	0,000	0,128	1,160	1,784
F12	0,284	0,155	0,072	0,083	0,000	0,000	0,216	0,052	0,371	0,156	0,126	0,294	0,192	0,140	0,222	0,107	0,043	0,162	0,433	0,042	0,427	0,000	2,521	0,000	0,158
G01	0,180	0,332	0,460	0,620	0,204	0,084	0,484	0,107	0,367	0,882	0,170	0,475	0,137	0,168	0,689	0,233	0,287	0,000	0,499	0,055	0,440	0,507	0,000	0,220	0,172
G02	0,173	0,244	1,074	0,108	0,100	0,006	0,384	0,097	0,374	0,562	0,162	0,263	0,075	0,150	0,300	0,052	0,284	0,000	0,490	0,135	0,546	0,575	0,000	0,195	0,000
G03	0,158	0,297	0,201	0,000	0,000	0,000	0,230	0,000	0,407	0,197	0,000	0,096	0,000	0,022	0,039	0,064	0,063	0,053	0,000	0,000	0,412	0,295	0,000	0,000	0,000
G04	0,311	0,267	0,358	0,009	0,000	0,000	0,154	0,064	0,544	0,537	0,035	0,172	0,018	0,081	0,319	0,070	0,063	0,144	0,463	0,302	0,504	0,877	0,000	0,000	0,000
G05	0,014	0,293	0,000	0,003	0,000	0,000	0,298	0,000	0,402	0,563	0,117	0,103	0,000	0,058	0,125	0,060	0,022	0,341	0,483	0,119	0,419	0,719	0,000	0,000	0,000
G06	0,438	0,859	0,581	0,252	0,120	0,162	0,629	0,096	0,535	0,538	0,240	0,300	0,091	0,199	0,422	0,139	0,349	0,180	0,540	0,119	0,779	0,818	0,000	0,000	0,000
G07	0,215	0,208	0,760	0,238	0,000	0,000	0,493	0,000	0,372	0,588	0,083	0,142	0,000	0,043	0,284	0,032	0,129	0,069	0,644	0,162	0,656	0,791	0,000	0,000	0,000
G08	0,187	0,466	0,835	0,496	0,834	0,934	0,348	0,374	0,288	0,000	0,582	0,530	0,825	0,739	0,609	0,126	0,224	0,181	0,693	0,138	0,361	0,518	0,000	0,000	0,000
G09	0,323	0,473	0,901	0,536	0,367	0,457	0,496	0,360	0,466	0,775	0,000	0,197	0,125	0,343	0,629	0,316	0,725	0,453	0,726	0,197	0,249	0,466	0,000	0,000	0,000
G10	0,163	0,278	0,000	0,000	0,000	0,000	0,107	0,000	0,000	0,364	0,000	0,048	0,000	0,000	0,071	0,037	0,013	0,097	0,152	0,243	0,607	0,000	0,000	0,000	0,326
G11	0,302	0,109	0,217	0,000	0,000	0,000	0,534	0,041	0,415	0,259	0,123	0,215	0,000	0,083	0,076	0,098	0,000	0,000	0,294	0,154	0,483	0,000	0,200	1,290	1,779
G12	0,706	1,192	1,146	0,000	0,118	0,023	0,716	0,216	0,405	0,436	0,240	0,282	0,186	0,175	0,750	0,000	0,059	0,165	0,522	0,049	0,450	0,000	2,577	0,000	0,000
H01	0,187	0,000	0,448	0,171	0,000	0,000	0,503	0,000	0,158	0,215	0,098	0,033	0,067	0,017	0,029	0,000	0,000	0,000	0,404	0,000	0,221	0,497	0,106	0,125	0,152
H02	0,064	0,228	0,579	0,235	0,299	0,340	0,256	0,000	0,244	0,658	0,258	0,642	0,228	0,304	0,391	0,291	0,172	0,000	0,437	0,236	0,686	0,796	0,018	0,210	0,000
H03	0,197	0,495	0,047	0,513	1,003	1,005	0,593	0,534	0,381	0,000	0,365	0,659	0,234	0,477	0,053	0,298	0,339	0,364	0,000	0,244	0,386	0,760	0,065	0,000	0,000

H04	0,369	0,425	0,000	0,000	0,638	0,701	0,591	0,264	0,289	0,000	0,113	0,439	0,182	0,263	0,000	0,111	0,000	0,240	0,457	0,061	0,000	0,757	0,000	0,000	0,000
H05	0,095	0,000	0,000	0,000	0,000	0,000	0,074	0,000	0,143	0,000	0,000	0,000	0,000	0,000	0,000	0,084	0,000	0,103	0,304	0,000	0,000	0,431	0,000	0,000	0,000
H06	0,002	0,023	0,521	0,034	0,000	0,000	0,000	0,000	0,385	0,557	0,115	0,000	0,000	0,085	0,033	0,108	0,000	0,225	0,524	0,122	0,261	0,849	0,000	0,000	0,000
H07	0,219	0,272	0,000	0,000	0,000	0,000	0,000	0,000	0,209	0,723	0,033	0,000	0,000	0,000	0,000	0,000	0,043	0,060	0,498	0,000	0,349	0,828	0,000	0,000	0,000
H08	0,098	0,000	0,000	0,000	0,000	0,000	0,146	0,000	0,130	0,238	0,100	0,070	0,000	0,017	0,000	0,064	0,032	0,197	0,387	0,193	0,410	0,454	0,000	0,000	0,000
H09	0,051	0,000	0,135	0,000	0,000	0,131	0,434	0,312	0,330	0,650	0,099	0,430	0,289	0,508	0,000	0,000	0,000	0,297	0,338	0,130	0,000	0,451	0,000	0,000	0,000
H10	0,120	0,067	0,043	0,146	0,413	0,386	0,388	0,145	0,218	0,752	0,160	0,641	0,273	0,306	0,110	0,110	0,026	0,264	0,233	0,382	0,344	0,288	0,000	0,000	0,096
H11	0,311	0,334	0,386	0,460	0,982	0,875	0,601	0,586	0,430	0,044	0,955	0,548	0,800	1,053	0,384	0,301	0,052	0,319	0,408	0,297	0,358	0,000	0,038	1,117	1,692
H12	0,092	0,079	0,480	0,589	1,064	0,996	0,552	0,648	0,510	0,491	1,175	0,162	1,222	1,123	0,637	0,497	0,000	0,509	0,400	0,137	0,068	0,000	2,439	0,000	0,167

Appendix B. Optical density values obtained for the sessile samples.

	Sample Number																								
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
A02	0,348	0,500	0,815	1,079	0,000	1,045	0,000	0,066	0,234	0,780	0,008	0,169	0,887	0,409	0,560	1,040	1,626	0,897	0,124	0,538	0,427	0,460	0,302	0,614	0,769
A03	0,442	0,599	0,906	1,004	0,594	1,801	0,826	0,770	0,812	1,087	0,588	0,968	0,968	1,165	0,601	1,595	1,650	1,003	0,651	0,841	0,530	0,000	0,292	0,769	0,949
A04	0,447	0,706	0,750	0,725	0,301	1,408	0,360	0,466	0,441	1,200	0,508	0,655	0,866	0,875	0,656	1,255	1,663	1,070	0,562	0,643	0,492	0,000	0,326	0,693	0,943
A05	0,242	0,267	0,668	0,028	0,000	1,115	0,000	0,000	0,000	0,372	0,000	0,000	0,623	0,229	0,123	0,034	0,276	0,324	0,000	0,303	0,332	0,905	0,028	0,130	0,601
A06	0,304	0,169	0,749	0,000	0,000	1,140	0,000	0,000	0,000	0,000	0,016	0,000	0,071	0,034	0,000	0,000	0,600	0,221	0,000	0,112	0,447	1,057	0,000	0,000	0,609
A07	0,170	0,942	0,815	0,682	0,172	2,100	1,154	0,047	0,120	0,489	0,086	0,018	0,845	0,212	0,377	0,000	0,044	0,459	0,299	0,263	0,486	0,000	0,000	0,000	0,809
A08	1,029	0,972	1,742	1,222	1,173	2,535	0,982	1,444	1,217	1,032	1,390	1,518	0,893	1,260	0,674	1,110	1,388	0,916	0,682	0,961	0,510	0,000	0,263	0,000	0,553
A09	0,209	0,988	0,704	0,464	0,840	1,623	0,992	0,662	0,658	1,263	0,262	0,075	0,860	0,148	0,391	0,040	1,278	0,554	0,670	0,655	0,421	0,000	0,257	0,000	0,000
A10	0,211	0,770	0,960	1,034	0,848	1,640	0,916	1,308	0,959	1,379	0,614	0,509	0,988	1,101	0,478	1,394	1,515	0,809	0,551	0,606	0,560	0,507	0,378	0,000	0,000
A11	0,503	0,927	0,792	1,166	0,692	1,421	0,805	0,877	0,835	1,348	0,460	0,582	0,902	0,680	0,357	1,312	1,513	0,739	0,673	0,608	0,448	0,710	0,000	0,402	0,000
A12	0,606	0,874	1,191	1,121	0,989	1,854	1,265	1,491	1,191	1,427	0,777	0,607	0,876	1,275	0,329	0,286	1,488	0,813	0,691	0,739	0,487	0,290	0,341	1,588	0,144
B01	0,000	0,185	0,656	0,000	0,000	1,292	0,000	0,000	0,000	0,050	0,000	0,000	0,505	0,042	0,022	0,000	0,000	0,260	0,126	0,245	0,349	0,135	0,142	0,210	0,049
B02	0,281	0,354	1,247	1,182	0,692	1,739	1,154	1,205	0,777	1,577	0,754	0,783	1,116	1,300	0,850	1,666	1,737	1,143	0,879	0,789	0,631	0,487	0,582	0,991	1,080
B03	0,503	0,571	0,951	0,048	0,472	1,516	0,248	0,611	0,515	1,361	0,343	0,478	0,922	0,493	0,584	0,041	1,484	0,980	0,540	0,487	0,491	0,000	0,358	0,724	0,735
B04	0,863	0,764	1,037	1,359	1,070	1,655	1,208	1,402	1,077	1,538	1,046	0,939	1,136	1,384	0,730	0,000	1,769	1,088	0,888	0,779	0,659	0,000	0,490	0,507	1,043
B05	0,819	0,838	1,145	1,336	0,890	2,208	1,158	1,402	0,717	1,524	0,541	0,501	1,103	1,327	0,677	0,000	1,601	1,035	0,764	0,804	0,596	0,875	0,558	0,175	1,019
B06	0,625	0,827	1,368	1,122	0,886	1,591	1,256	0,787	0,912	1,676	1,300	1,112	1,165	1,612	0,773	1,240	1,762	1,138	0,884	0,894	0,616	0,877	0,550	0,000	0,890
B07	0,746	0,561	0,803	0,093	0,527	1,800	0,384	0,756	0,732	1,235	0,828	0,073	0,786	0,469	0,357	0,490	1,405	0,571	0,611	0,438	0,558	0,000	0,158	0,000	0,753
B08	0,971	1,065	1,187	1,409	0,638	1,863	0,481	1,034	1,045	1,177	0,269	0,051	1,059	1,028	0,769	0,000	0,203	1,162	0,918	0,962	0,655	0,000	0,344	0,000	0,411
B09	0,831	0,612	0,719	0,000	0,353	1,588	0,204	0,118	0,119	0,961	0,345	0,025	0,817	0,055	0,634	0,000	1,385	0,537	0,381	1,065	0,541	0,000	0,336	0,000	0,000
B10	0,867	0,932	1,067	1,277	1,030	1,738	1,194	1,412	1,121	1,579	1,326	0,874	1,127	1,525	0,719	1,525	1,623	1,024	0,755	0,711	0,638	0,484	0,485	0,000	0,000
B11	0,552	0,615	1,045	0,432	1,145	1,611	1,186	1,135	0,779	1,625	0,807	0,621	1,052	1,312	0,563	1,362	1,602	0,820	0,619	0,755	0,502	0,818	0,010	0,489	0,000
B12	0,692	0,569	1,232	1,325	0,864	1,568	1,283	1,401	0,819	1,430	0,703	0,612	0,917	1,238	0,319	0,239	1,396	0,728	0,792	0,683	0,522	0,198	0,292	1,447	0,133
C01	0,608	0,619	1,290	0,966	1,006	1,552	1,161	1,497	1,015	1,707	0,751	0,879	0,958	1,408	0,678	0,254	1,517	1,042	0,695	0,895	0,551	0,647	0,529	0,813	1,048
C02	0,507	0,599	1,633	1,132	0,997	1,745	1,090	1,415	1,027	1,451	1,400	1,246	1,006	1,240	0,676	1,742	1,558	0,986	0,849	0,826	0,584	0,679	0,455	0,892	0,993
C03	0,773	0,712	0,814	0,744	0,533	1,789	0,440	0,205	0,187	0,823	0,292	0,404	1,193	0,511	0,602	0,339	0,901	0,820	0,330	0,526	0,795	0,000	0,463	0,783	0,853

C04	0,695	0,727	1,456	1,148	0,904	1,670	1,198	1,051	1,023	1,547	0,981	0,640	0,972	1,380	0,714	1,133	1,642	0,998	0,721	0,853	0,595	0,000	0,573	0,821	1,005
C05	0,701	0,709	0,915	0,488	0,775	2,027	0,797	0,866	0,842	1,522	0,562	0,667	0,933	0,737	0,642	0,000	1,667	1,038	0,670	0,681	0,493	0,670	0,318	0,418	0,843
C06	1,102	0,904	0,881	1,372	1,156	1,683	1,165	1,535	1,014	1,551	0,854	0,547	1,071	1,265	0,725	1,816	1,667	0,969	0,732	0,792	0,630	1,088	0,545	0,000	0,863
C07	0,804	0,808	1,538	1,365	1,100	1,667	1,229	1,336	1,188	1,594	1,776	1,305	1,104	1,596	0,824	0,545	1,714	1,087	0,838	0,996	0,666	0,000	0,496	0,000	0,978
C08	1,180	0,000	0,876	1,207	1,129	1,341	1,471	1,579	1,381	1,616	1,434	0,907	1,072	1,502	0,801	1,634	1,398	1,110	0,805	0,989	0,743	0,000	0,533	0,000	0,735
C09	0,916	0,887	0,666	0,157	0,823	1,809	0,540	0,531	0,210	0,757	0,301	0,214	0,833	0,290	0,645	0,927	1,491	0,727	0,792	0,826	0,654	0,000	0,475	0,000	0,000
C10	0,740	0,237	0,677	0,104	0,500	1,218	0,000	0,000	0,000	0,454	0,037	0,000	0,691	0,068	0,465	0,002	0,081	0,302	0,185	0,216	0,000	0,629	0,203	0,000	0,000
C11	0,286	0,668	0,791	0,186	0,707	1,466	0,529	0,597	0,535	0,923	0,825	1,049	1,050	0,932	0,583	1,139	1,208	0,412	0,335	0,688	0,429	0,070	0,000	0,318	0,000
C12	0,000	0,000	0,608	0,000	0,000	1,052	0,012	0,126	0,098	0,615	0,166	0,032	0,308	0,190	0,017	0,000	0,171	0,000	0,231	0,285	0,191	0,501	0,000	1,787	0,149
D01	0,180	0,000	0,743	0,000	0,222	0,763	0,000	0,025	0,061	0,305	0,000	0,029	0,114	0,039	0,000	0,000	0,129	0,350	0,000	0,324	0,047	0,000	0,004	0,050	0,313
D02	0,548	0,641	1,116	0,772	0,762	1,664	1,014	0,859	0,568	0,756	0,186	0,156	0,935	0,669	0,541	0,341	1,279	1,112	0,472	0,465	0,470	0,513	0,452	0,764	0,670
D03	0,827	0,684	1,189	0,328	0,746	1,840	0,552	0,851	0,738	0,867	0,433	0,123	0,861	0,756	0,675	0,469	1,413	1,043	0,408	0,843	0,469	0,000	0,261	0,694	1,006
D04	0,356	0,175	0,670	0,000	0,352	1,082	0,000	0,000	0,000	0,454	0,113	0,220	0,757	0,409	0,555	0,000	1,253	0,516	0,336	0,304	0,434	0,000	0,273	0,473	0,809
D05	0,956	0,774	1,525	0,564	0,855	2,444	0,905	0,684	0,897	1,291	0,825	0,239	0,886	1,113	0,707	1,123	1,562	0,981	0,788	0,834	0,510	0,777	0,392	0,291	0,962
D06	0,863	0,819	1,520	0,955	1,192	1,781	0,700	1,252	0,887	0,943	1,242	0,582	0,955	1,166	0,612	1,629	1,485	1,004	0,621	0,836	0,650	1,132	0,428	0,000	0,916
D07	0,893	0,830	1,671	0,939	1,055	1,716	0,850	1,259	1,068	1,413	1,228	1,261	1,017	1,209	0,737	1,568	1,418	0,841	0,750	0,920	0,675	0,000	0,420	0,000	0,862
D08	0,482	0,633	0,676	0,133	0,324	1,444	0,596	0,000	0,000	0,523	0,060	0,067	0,678	0,330	0,523	0,263	0,000	0,372	0,215	0,493	0,488	0,000	0,143	0,000	0,346
D09	0,594	0,715	1,120	0,786	1,088	1,727	0,769	1,151	0,838	1,309	0,917	0,299	0,911	0,942	0,677	1,506	1,474	0,877	0,688	0,837	0,599	0,000	0,442	0,000	0,000
D10	0,198	0,076	0,546	0,000	0,000	0,797	0,000	0,000	0,000	0,000	0,000	0,000	0,079	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,726	0,000	0,000	0,000
D11	0,801	0,387	0,668	0,000	0,080	0,985	0,000	0,012	0,000	0,681	0,035	0,042	0,605	0,175	0,509	0,000	0,083	0,765	0,482	0,402	0,228	0,000	0,000	0,394	0,000
D12	0,016	0,274	0,551	0,000	0,035	0,748	0,000	0,000	0,000	0,273	0,034	0,000	0,004	0,153	0,000	0,000	0,000	0,000	0,000	0,207	0,131	0,040	0,000	1,637	0,169
E01	0,301	0,070	0,785	0,082	0,000	1,169	0,420	0,235	0,128	0,596	0,208	0,120	0,305	0,041	0,000	0,000	0,133	1,050	0,370	0,139	0,314	0,000	0,028	0,085	0,118
E02	0,386	0,144	0,787	0,000	0,051	1,064	0,000	0,000	0,000	0,000	0,000	0,000	0,134	0,000	0,516	0,000	0,268	0,275	0,056	0,000	0,000	0,000	0,000	0,284	0,156
E03	0,000	0,495	0,697	0,000	0,000	1,202	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
E04	0,588	0,900	0,997	0,000	0,420	1,230	0,008	0,073	0,000	0,550	0,168	0,001	0,776	0,552	0,543	0,000	1,080	0,935	0,295	0,237	0,452	0,000	0,389	0,438	0,725
E05	0,000	0,120	0,682	0,000	0,000	0,659	0,000	0,000	0,000	0,000	0,000	0,000	0,018	0,032	0,202	0,000	0,000	0,000	0,000	0,000	0,000	0,706	0,000	0,064	0,000
E06	0,704	0,673	1,145	0,208	0,414	1,548	0,450	0,794	0,587	0,991	0,483	0,221	0,862	0,724	0,574	0,000	1,078	1,101	0,531	0,627	0,561	1,188	0,304	0,000	0,753
E07	0,203	0,244	0,793	0,000	0,327	1,187	0,000	0,000	0,000	0,197	0,176	0,031	0,515	0,155	0,462	0,000	0,692	0,578	0,272	0,362	0,473	0,000	0,000	0,000	0,469
E08	0,613	0,079	0,740	0,000	0,119	0,703	0,000	0,000	0,004	0,204	0,079	0,000	0,125	0,147	0,533	0,190	0,000	0,349	0,100	0,172	0,160	0,000	0,000	0,000	0,294
E09	0,898	0,703	0,902	0,000	0,431	1,264	0,000	0,331	0,173	0,724	0,256	0,069	0,597	0,228	0,579	0,000	1,051	1,281	0,427	0,453	0,504	0,000	0,293	0,000	0,000

E10	0,945	0,624	1,033	0,628	0,866	1,499	0,663	0,919	0,725	0,963	0,499	0,191	0,879	0,789	0,498	0,726	0,828	0,875	0,583	0,673	0,533	0,182	0,334	0,000	0,000
E11	0,000	0,099	0,712	0,000	0,121	0,897	0,000	0,000	0,000	0,000	0,000	0,000	0,095	0,015	0,269	0,000	0,030	0,000	0,000	0,360	0,093	0,682	0,000	0,575	0,000
E12	0,422	0,163	0,825	0,179	0,417	1,062	0,463	0,417	0,422	0,548	0,263	0,109	0,369	0,218	0,247	0,145	0,259	0,790	0,654	0,410	0,482	0,000	0,098	1,652	0,187
F01	0,042	0,137	0,681	0,000	0,120	1,261	0,199	0,294	0,267	0,168	0,304	0,261	0,592	0,204	0,443	0,166	0,751	0,774	0,261	0,386	0,417	0,457	0,191	0,389	0,757
F02	0,134	0,318	0,682	0,525	0,051	1,196	0,000	0,000	0,000	0,029	0,126	0,135	0,183	0,120	0,653	0,206	0,368	0,234	0,593	0,248	0,271	0,263	0,217	0,576	0,686
F03	0,113	0,299	0,616	0,000	0,324	0,989	0,000	0,122	0,053	1,001	0,521	0,611	0,814	0,548	0,711	0,357	0,983	0,855	0,335	0,634	0,502	0,000	0,413	0,723	0,940
F04	0,433	0,541	0,431	0,000	0,031	0,969	0,000	0,000	0,000	0,831	0,144	0,148	0,268	0,278	0,555	0,000	0,671	0,131	0,090	0,000	0,325	0,000	0,224	0,365	0,305
F05	0,524	0,784	0,681	0,000	0,131	1,413	0,000	0,147	0,152	0,408	0,206	0,085	0,349	0,181	0,000	0,000	0,594	0,000	0,406	0,390	0,412	0,616	0,126	0,511	0,393
F06	0,676	0,695	0,646	0,000	0,215	1,677	0,000	0,313	0,000	0,503	0,289	0,114	0,441	0,205	0,586	0,351	0,873	0,724	0,484	0,422	0,359	1,755	0,283	0,000	0,727
F07	0,597	0,471	0,589	0,000	0,120	1,798	0,284	0,215	0,191	0,568	0,338	0,146	0,540	0,187	0,608	0,233	0,908	0,653	0,465	0,475	0,489	0,000	0,087	0,000	0,765
F08	0,902	0,577	0,646	0,000	0,444	1,607	0,738	0,236	0,212	0,349	0,330	0,197	0,883	0,465	0,584	0,000	0,081	1,026	0,637	0,565	0,557	0,000	0,253	0,000	0,700
F09	0,560	0,208	0,563	0,000	0,373	1,030	0,243	0,051	0,000	0,771	0,130	0,103	0,823	0,319	0,583	0,000	1,119	1,138	0,346	0,553	0,495	0,000	0,353	0,000	0,000
F10	0,926	0,739	0,721	0,038	0,556	2,292	0,528	0,489	0,219	0,316	0,199	0,157	0,864	0,281	0,660	0,000	0,532	1,078	0,307	0,382	0,543	0,312	0,327	0,000	0,000
F11	0,157	0,071	0,508	0,000	0,000	1,053	0,000	0,000	0,094	0,366	0,227	0,000	0,473	0,163	0,592	0,000	0,286	0,162	0,190	0,473	0,366	0,511	0,000	0,483	0,000
F12	0,000	0,114	0,435	0,000	0,000	0,713	0,021	0,018	0,044	0,000	0,209	0,036	0,189	0,176	0,234	0,209	0,139	0,205	0,239	0,348	0,295	0,289	0,038	1,489	0,140
G01	0,345	0,233	0,770	0,222	0,145	1,785	0,494	0,262	0,285	0,472	0,450	0,175	0,671	0,124	0,216	0,000	0,343	0,859	0,588	0,144	0,532	0,332	0,164	0,386	0,856
G02	0,368	0,412	0,980	0,089	0,424	1,839	0,018	0,302	0,200	0,000	0,196	0,034	0,018	0,093	0,746	0,394	0,192	0,233	0,623	0,118	0,439	0,262	0,272	0,576	0,000
G03	0,241	0,312	0,592	0,000	0,000	0,775	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,531	0,000	0,132	0,239	0,000	0,197	0,201	0,000	0,168	0,507	0,000
G04	0,231	0,478	0,631	0,000	0,118	1,065	0,000	0,000	0,000	0,000	0,123	0,027	0,357	0,049	0,394	0,000	0,448	0,090	0,103	0,000	0,320	0,000	0,160	0,355	0,182
G05	0,000	0,468	0,577	0,000	0,140	0,857	0,000	0,000	0,000	0,066	0,084	0,000	0,082	0,076	0,501	0,000	0,434	0,000	0,185	0,303	0,201	0,842	0,195	0,321	0,106
G06	0,639	0,362	0,750	0,000	0,465	1,607	0,806	0,380	0,245	1,095	0,420	0,009	0,434	0,232	0,783	0,336	0,971	1,025	0,601	0,579	0,523	1,237	0,237	0,000	0,911
G07	0,174	0,187	0,681	0,000	0,307	0,810	0,000	0,000	0,000	0,000	0,167	0,000	0,177	0,154	0,608	0,000	0,550	0,544	0,000	0,475	0,338	0,000	0,000	0,000	0,631
G08	0,260	0,455	1,022	0,283	0,898	2,353	0,607	0,948	0,719	0,800	0,869	0,400	0,737	0,786	0,577	1,593	1,202	0,731	0,635	0,724	0,509	0,000	0,100	0,000	0,668
G09	0,687	0,549	0,686	0,512	0,680	2,150	0,684	0,067	0,186	0,431	0,107	0,058	0,762	0,400	0,925	0,235	1,524	1,295	0,435	0,699	0,450	0,000	0,296	0,000	0,000
G10	0,000	0,000	0,554	0,000	0,000	0,787	0,000	0,000	0,000	0,000	0,000	0,000	0,626	0,077	0,814	0,159	0,000	0,583	0,000	0,000	0,119	0,562	0,000	0,000	0,000
G11	0,023	0,223	0,576	0,000	0,079	0,712	0,000	0,000	0,001	0,000	0,143	0,000	0,172	0,066	0,631	0,193	0,143	0,000	0,098	0,422	0,226	0,000	0,000	0,676	0,000
G12	0,650	0,777	0,729	0,000	0,273	1,761	0,000	0,541	0,325	0,211	0,154	0,000	0,255	0,129	0,585	0,000	0,297	0,891	0,378	0,200	0,691	0,150	0,111	1,387	0,339
H01	0,120	0,415	0,523	0,000	0,000	1,502	0,461	0,098	0,000	0,000	0,000	0,000	0,000	0,032	0,224	0,000	0,000	0,262	0,041	0,098	0,230	0,028	0,000	0,289	0,002
H02	0,318	0,286	0,627	0,137	0,207	1,398	0,585	0,344	0,146	1,262	0,325	0,151	0,819	0,569	0,760	0,000	0,430	0,894	0,494	0,343	0,531	0,640	0,259	0,746	0,935
H03	0,103	0,411	0,671	0,438	0,852	1,763	0,726	1,286	0,720	1,204	0,661	0,681	0,830	0,545	0,453	0,042	0,749	0,648	0,773	0,647	0,524	0,000	0,260	0,795	1,035

H04	0,000	0,407	0,548	0,189	0,382	1,181	0,314	0,699	0,000	1,292	0,270	0,176	0,719	0,269	0,265	0,034	0,875	0,643	0,741	0,000	0,433	0,000	0,238	0,614	0,824
H05	0,000	0,000	0,405	0,000	0,000	0,544	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,622	0,000	0,234	0,000
H06	0,014	0,000	0,519	0,000	0,589	1,608	0,000	0,004	0,013	0,202	0,182	0,000	0,128	0,064	0,214	0,000	0,233	0,570	0,439	0,260	0,448	1,455	0,070	0,000	0,096
H07	0,000	0,143	0,408	0,000	0,000	0,597	0,000	0,000	0,000	0,220	0,000	0,000	0,000	0,012	0,000	0,000	0,030	0,580	0,000	0,337	0,136	0,000	0,000	0,000	0,000
H08	0,000	0,000	0,419	0,000	0,000	0,532	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,124	0,128	0,046	0,000	0,130	0,000	0,296	0,000	0,000	0,000	0,000	0,310
H09	0,000	0,456	0,618	0,646	0,000	1,375	0,614	0,884	0,642	0,858	0,287	0,329	0,761	0,721	0,359	0,484	1,376	0,790	0,080	0,655	0,378	0,000	0,367	0,000	0,090
H10	0,042	0,069	0,641	0,185	0,290	0,928	0,259	0,439	0,358	0,288	0,522	0,295	0,670	0,450	0,347	0,435	0,254	0,604	0,387	0,228	0,413	0,541	0,239	0,000	0,000
H11	0,404	0,298	1,107	0,625	0,941	1,001	0,549	1,155	0,746	1,042	0,699	0,425	0,712	0,940	0,481	0,389	1,204	0,822	0,577	0,687	0,491	0,471	0,378	0,414	0,000
H12	0,220	0,404	1,288	0,798	0,979	1,026	0,752	1,212	0,850	0,807	1,199	1,269	0,710	1,062	0,542	1,057	1,330	0,912	0,580	0,829	0,294	0,599	0,348	1,539	0,121

Appendix C. Matrix obtained using Sorenson's measure for quantitative data.

Cn	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1		4,548	4,644	4,446	5,014	4,989	4,554	5,100	4,604	5,355	5,586	5,790	4,927	5,529	4,667	4,476	6,009	4,939	4,846	4,675	4,700	3,382	5,309	3,518	4,231
2			4,549	4,179	4,639	4,687	4,246	4,890	4,287	5,222	5,244	5,378	4,824	5,303	4,604	4,178	5,837	4,859	4,684	4,520	4,705	3,491	5,044	3,745	4,183
3				4,097	4,667	4,922	4,087	4,747	4,333	4,891	5,352	5,272	4,622	5,403	4,683	4,106	5,444	4,664	4,815	4,590	4,918	3,430	5,318	3,668	3,969
4					4,424	5,008	4,423	4,751	4,587	4,508	4,799	5,203	4,208	4,413	4,221	4,635	4,527	4,096	4,639	4,175	4,507	3,250	5,322	3,163	3,872
5						5,013	4,296	4,528	4,253	4,980	4,815	5,250	4,569	4,691	4,958	4,402	5,351	4,646	4,717	4,410	4,834	3,387	5,547	3,519	4,101
6							4,941	5,457	5,031	5,790	5,418	5,893	5,186	5,765	4,704	4,621	6,123	5,029	4,984	4,810	4,842	3,576	5,732	3,544	4,306
7								4,722	4,511	4,567	4,809	5,466	4,168	4,528	4,280	4,442	4,697	4,266	4,616	4,303	4,609	3,146	5,469	3,304	3,822
8									4,549	4,724	4,868	5,668	4,652	4,529	4,966	4,532	4,916	4,960	5,195	4,782	5,366	3,536	6,525	3,519	4,187
9										4,859	4,613	5,062	4,347	4,481	4,523	4,766	5,098	4,477	4,563	4,363	4,802	3,327	5,471	3,570	3,965
10											5,661	6,582	4,775	4,897	5,265	4,124	4,585	4,822	5,568	5,000	5,802	3,405	6,885	3,588	4,118
11												4,894	5,257	4,897	5,374	4,650	6,053	5,376	5,132	4,886	5,367	3,637	5,705	3,918	4,696
12													5,706	5,377	5,058	5,417	6,793	5,755	5,489	4,975	5,372	3,482	5,465	3,899	5,021
13														5,075	5,112	4,211	5,196	4,478	5,035	4,637	5,230	3,534	5,955	3,700	3,844
14															5,708	4,307	5,332	5,293	5,419	5,109	5,849	3,929	6,130	3,963	4,559
15																4,555	6,007	4,903	4,601	4,481	4,520	3,372	4,781	3,592	3,946
16																	4,186	4,112	4,718	4,419	4,581	3,362	5,323	3,333	3,737
17																		5,130	6,327	5,612	6,560	3,479	7,563	3,719	4,207
18																			4,929	4,737	5,261	3,220	6,159	3,343	3,879
19																				4,411	4,550	3,367	5,295	3,738	4,117
20																					4,738	3,406	5,062	3,662	4,058
21																						3,553	4,715	3,896	4,377
22																							3,759	3,473	3,129
23																								4,022	5,104
24																									3,967
25																									

Appendix D. Mol % of lipids obtained for the planktonic samples.

Sample	1	2	3	4	5	6	7	8	9	10	11	12
Gram Extracted	0,134	0,493	0,225	0,123	0,449	0,612	0,097	0,426	0,254	0,082	0,150	0,046
Dilution Factor	4	4	4	4	4	4	4	4	4	4	4	4
pmol/g	8,7E+07	2,5E+07	2,8E+07	5,4E+07	4,0E+07	1,0E+07	6,1E+07	1,5E+07	1,5E+07	1,6E+08	1,5E+07	1,3E+08
C14:0	3,25	3,60	3,13	2,97	2,15	3,01	3,03	3,04	4,23	3,73	4,89	5,10
C15:0i	1,28	1,80	1,68	0,00	4,65	0,00	4,08	4,99	0,00	0,00	0,00	0,00
C15:0a	2,66	2,59	2,53	0,00	5,41	0,00	3,89	5,57	0,00	0,00	0,00	0,00
C15:0	2,05	2,08	0,00	0,00	1,63	0,00	1,55	0,00	0,00	0,00	0,00	0,00
C16:0	28,05	28,73	28,34	28,58	27,37	28,19	25,72	33,95	30,33	25,66	29,97	31,19
C16:1	4,69	4,99	3,10	1,99	6,56	3,29	5,69	6,02	4,73	4,01	4,48	5,80
C16:0i	0,00	0,00	0,00	0,00	3,85	0,00	2,98	0,00	0,00	0,00	0,00	0,00
C17:0	13,37	16,30	10,79	12,21	7,45	8,86	10,05	0,00	0,00	1,97	0,00	10,30
C17:1	0,00	1,67	0,00	0,00	1,74	0,00	1,41	0,00	0,00	0,00	0,00	0,00
C18:0	11,28	9,66	13,47	14,68	6,02	13,07	9,21	19,56	9,78	9,48	9,15	26,12
C18:1	27,28	22,59	30,75	33,82	27,93	34,90	26,97	19,94	33,24	37,00	33,94	14,29
C18:2	6,09	6,00	6,22	5,75	5,24	8,67	5,43	6,92	17,70	18,16	17,57	7,20

Sample	13	14	15	16	17	18	19	20	21	22	24	25
Gram Extracted	0,886	0,288	0,426	1,751	0,889	0,505	0,133	0,083	0,104	0,510	0,384	0,703
Dilution Factor	4	4	4	4	4	4	4	4	4	4	4	4
pmol/g	1,1E+07	4,1E+07	2,7E+07	7,3E+06	6,1E+06	2,3E+07	2,7E+07	5,0E+07	3,3E+07	6,3E+06	1,0E+07	8,5E+06
C14:0	3,90	3,15	4,86	4,32	4,33	2,41	5,70	3,89	7,16	3,79	3,48	0,00
C15:0i	2,50	5,24	5,62	2,78	3,67	0,00	0,00	3,00	0,00	2,86	3,47	0,00
C15:0a	2,14	3,56	5,72	3,21	5,05	0,00	0,00	1,81	0,00	1,73	2,68	0,00
C15:0	1,60	1,76	2,38	1,93	0,00	0,00	0,00	0,00	0,00	0,00	2,25	0,00
C16:0	30,58	25,88	35,31	27,98	29,54	21,34	31,41	30,40	25,20	28,16	30,12	37,81
C16:1	4,83	4,02	7,45	7,51	6,75	2,09	4,42	3,79	0,85	6,60	4,80	0,00
C16:0i	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C17:0	3,20	27,41	3,95	4,93	9,32	0,00	0,00	8,55	12,72	6,71	6,11	0,00
C17:1	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:0	18,60	16,05	14,47	18,58	21,70	4,83	40,66	27,24	42,39	23,16	22,90	26,70
C18:1	17,92	8,74	15,40	19,26	14,73	56,35	12,73	14,11	6,90	18,43	17,13	21,54
C18:2	14,74	4,19	4,85	9,50	4,90	12,97	5,07	7,20	4,78	8,56	7,08	13,96

Appendix E. Mol % of lipids obtained for the sessile samples.

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13
Gram Extracted	1,843	1,350	1,294	4,704	2,160	1,044	2,788	1,371	2,255	1,015	2,494	1,993	1,653
Dilution Factor	4	4	4	4	4	4	4	4	4	4	4	4	4
pmol/g	5,7E+07	2,5E+07	6,6E+06	3,1E+06	1,4E+07	1,3E+07	2,1E+07	7,1E+07	1,3E+07	3,1E+07	3,8E+06	3,1E+07	8,4E+07
C14:0	2,91	3,17	2,54	2,16	2,73	2,30	2,84	4,07	4,68	2,76	4,24	3,63	3,54
C15:0i	0,00	6,12	4,55	1,28	7,48	1,98	6,16	11,11	0,00	2,67	3,90	5,71	7,99
C15:0a	0,00	7,32	5,15	1,62	7,77	5,49	12,84	13,66	0,00	3,63	5,16	19,39	14,87
C15:0	0,00	1,90	0,00	0,77	2,21	1,66	2,44	2,12	0,00	1,65	1,96	3,03	2,74
C16:0	32,99	31,58	26,44	18,57	27,30	22,94	27,48	28,73	30,06	31,73	31,91	21,90	25,83
C16:1	3,06	5,83	2,93	3,75	5,93	6,80	6,70	5,72	3,41	6,13	5,59	6,96	12,35
C16:0i	0,00	5,89	0,00	0,00	0,00	1,95	8,40	7,99	0,00	0,00	0,00	5,53	3,54
C17:0	4,04	2,01	4,40	2,82	6,60	7,93	2,94	0,00	1,40	1,14	0,00	1,21	1,30
C17:1	0,00	1,98	0,00	0,00	1,65	1,82	1,85	0,00	0,00	1,03	0,00	2,12	1,53
C18:0	11,81	5,05	11,04	10,69	5,45	7,41	2,82	3,65	10,53	4,55	8,76	3,80	2,61
C18:1	37,49	21,24	34,33	29,16	22,80	32,40	21,93	18,47	32,43	29,35	28,51	24,32	19,36
C18:2	7,71	7,91	8,63	29,18	10,09	7,32	3,60	4,48	17,49	15,37	9,97	2,40	4,33

Sample	14	15	16	17	18	19	20	21	22	23	24	25
Gram Extracted	1,558	1,197	1,751	2,097	1,615	1,589	1,033	2,506	2,934	1,836	3,682	1,970
Dilution Factor	4	4	4	4	4	4	4	4	4	4	4	4
pmol/g	1,5E+07	3,3E+07	2,2E+08	1,3E+07	4,2E+07	6,2E+07	9,1E+06	1,8E+07	8,2E+06	4,5E+07	2,1E+07	3,0E+07
C14:0	6,36	8,27	2,86	5,58	4,53	3,39	2,83	3,61	3,72	2,32	4,01	3,91
C15:0i	0,00	3,06	6,08	0,00	8,33	7,73	6,30	6,45	5,40	1,57	7,05	6,92
C15:0a	0,00	8,67	12,01	0,00	14,44	18,99	4,18	10,48	8,88	3,09	16,82	16,12
C15:0	2,14	3,26	1,95	3,08	2,71	2,96	1,86	2,65	2,42	1,82	2,84	2,77
C16:0	27,58	27,82	26,98	24,52	24,37	23,12	28,20	27,58	27,88	28,83	31,62	31,46
C16:1	3,81	8,31	14,25	9,94	11,80	10,78	6,74	11,00	9,87	5,84	13,20	12,82
C16:0i	0,00	0,00	7,25	0,00	4,71	5,94	4,42	0,00	3,83	0,00	0,00	0,00
C17:0	1,72	7,74	0,81	11,49	1,16	1,16	1,86	1,63	1,33	1,63	1,12	1,25
C17:1	0,00	0,00	3,16	0,00	1,39	1,09	1,38	0,00	1,96	0,00	0,00	0,00
C18:0	8,27	6,37	1,49	7,12	3,16	2,16	12,98	5,50	6,15	11,94	4,30	5,38
C18:1	31,56	21,13	18,67	26,49	19,37	18,28	18,70	26,24	21,75	30,04	12,09	11,96
C18:2	18,54	5,37	4,50	11,78	4,04	4,40	10,56	4,86	6,81	12,91	6,95	7,40

Appendix F. Mol % of lipids obtained during the first audit.

Sample	1	2	3	4	5	6	7	8	9
Gram Extracted	1,802	0,155	0,311	2,448	1,920	1,834	2,254	0,480	3,685
Dilution Factor	4	4	4	4	4	4	4	4	4
pmol/g	4,2E+06	3,8E+07	1,2E+07	1,6E+06	1,4E+06	3,3E+06	3,9E+06	1,6E+07	2,4E+06
C14:0	3,86	4,05	4,96	4,55	4,99	3,33	3,03	3,05	2,84
C15:0i	3,51	4,11	0,00	0,00	0,00	4,37	4,13	5,19	6,06
C15:0a	4,31	3,95	0,00	0,00	0,00	3,24	2,77	3,59	4,71
C15:0	0,00	0,00	0,00	0,00	0,00	0,00	1,77	1,97	2,05
C16:0	29,30	34,06	30,41	29,66	29,45	31,71	30,41	32,64	27,68
C16:1	6,74	7,25	0,00	0,00	0,00	6,02	5,79	5,70	4,89
C17:0	3,68	3,44	6,41	5,40	7,15	2,61	3,28	2,21	2,45
C18:0	21,39	21,84	32,48	28,28	34,67	19,15	17,88	15,24	14,65
C18:1	19,04	12,95	16,08	19,78	15,25	19,12	20,42	19,66	20,23
C18:2	8,17	8,36	9,66	12,34	8,50	10,44	10,51	10,75	14,44

Sample	10	11	12	13	14	15	16	17	18
Gram Extracted	0,522	0,387	0,439	0,748	0,3675	0,1242	0,103	0,435	0,104
Dilution Factor	4	4	4	4	4	4	4	4	4
pmol/g	6,4E+06	1,4E+07	1,3E+07	1,4E+07	2,4E+07	6,7E+07	1,6E+07	1,0E+07	8,4E+07
C14:0	4,42	3,19	3,37	2,96	2,68	3,61	7,93	3,41	5,52
C15:0i	0,00	2,94	3,06	4,15	4,45	6,34	0,00	5,83	1,77
C15:0a	0,00	2,00	2,14	2,67	3,11	4,52	0,00	3,64	1,25
C15:0	0,00	0,00	0,00	1,83	1,67	0,47	0,00	0,00	1,32
C16:0	29,54	27,50	28,94	31,95	29,56	36,79	30,00	31,04	28,66
C16:1	5,64	5,40	5,49	5,60	5,61	8,68	0,00	7,42	5,66
C17:0	6,92	4,28	3,88	2,10	1,89	2,99	11,99	3,71	2,44
C18:0	28,18	20,05	18,85	15,10	13,74	16,01	50,08	17,05	12,25
C18:1	15,85	20,01	20,11	19,96	28,05	12,86	0,00	17,96	25,09
C18:2	9,45	14,64	14,15	13,67	9,25	7,73	0,00	9,95	16,04

Appendix G. Mol % of lipids obtained during the second audit.

Sample	1	2	3	4	5	6	7	8	9
Gram Extracted	0,664	2,228	1,628	1,061	1,843	1,950	0,493	0,425	0,422
Dilution Factor	4	4	4	4	4	4	4	4	4
pmol/g	2,4E+07	3,6E+06	6,5E+06	9,8E+06	5,1E+06	3,8E+06	1,8E+07	1,9E+07	1,6E+07
C14:0	4,54	9,33	5,15	4,55	5,25	4,22	3,18	5,49	5,29
C15:0i	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C15:0a	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C16:0	27,06	39,09	29,21	34,67	28,60	29,32	29,04	29,37	28,81
C16:1	2,97	0,00	3,03	3,65	2,54	3,54	3,18	3,23	2,97
C17:0	17,94	0,00	16,15	12,45	19,06	18,93	17,35	16,95	18,11
C18:0	38,26	29,10	33,91	29,72	33,66	32,62	35,70	35,15	33,79
C18:1	6,82	22,48	10,36	12,42	8,66	8,38	8,23	8,08	8,87
C18:2	2,40	0,00	2,19	2,54	2,22	2,98	3,33	1,73	2,15

Sample	10	11	12	13	14	15	16	17	18
Gram Extracted	0,467	0,539	0,478	0,337	0,1912	0,0317	0,194	1,631	0,656
Dilution Factor	4	4	4	4	4	4	4	4	4
pmol/g	2,2E+07	1,3E+07	1,2E+07	2,2E+07	3,5E+07	1,8E+08	3,1E+07	9,0E+06	1,4E+07
C14:0	5,09	4,88	5,10	4,92	4,56	4,78	4,60	4,98	4,34
C15:0i	0,00	0,00	0,00	0,00	0,00	0,00	0,00	8,52	4,18
C15:0a	0,00	0,00	0,00	0,00	0,00	0,00	0,00	7,31	0,40
C16:0	28,14	27,10	29,23	28,49	25,78	26,91	26,52	33,91	33,65
C16:1	2,82	3,14	2,92	3,15	2,63	3,08	2,36	4,31	4,84
C17:0	22,51	19,95	18,18	20,47	22,69	21,21	21,96	7,34	10,91
C18:0	32,08	33,48	33,08	34,20	35,60	36,00	36,05	13,76	20,99
C18:1	7,92	8,27	8,90	6,71	6,16	5,73	6,34	14,04	14,69
C18:2	1,44	3,19	2,58	2,07	2,58	2,30	2,16	5,83	5,99

Appendix H. Optical density values obtained during the first audit.

	Sample Number																	
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
A02	0,191	0,013	0,445	0,127	0,095	0,095	0,366	0,000	0,000	0,138	2,389	0,000	0,287	0,000	0,158	0,000	0,371	0,000
A03	0,198	0,311	0,626	0,103	0,127	0,220	0,450	0,060	0,086	0,245	2,435	0,311	0,622	0,255	0,266	0,000	0,240	0,340
A04	0,167	0,439	0,289	0,092	0,171	0,227	0,500	0,000	0,172	0,381	2,408	0,361	0,423	0,240	0,322	0,311	0,416	0,536
A05	0,513	0,429	0,859	0,385	0,335	0,484	0,452	0,215	0,457	0,421	2,410	0,525	0,547	0,338	0,409	0,273	0,440	0,295
A06	0,536	0,208	0,796	0,081	0,396	0,175	0,498	0,160	0,452	0,007	2,609	0,449	0,355	0,261	0,071	0,280	0,328	0,386
A07	0,523	0,340	0,878	0,294	0,542	0,370	0,567	0,013	0,449	0,430	2,690	0,528	0,626	0,388	0,237	0,354	0,164	0,330
A08	0,286	0,348	0,691	0,274	0,441	0,452	0,953	0,050	0,336	0,378	2,643	0,525	0,492	0,212	0,381	0,349	0,508	0,519
A09	0,441	0,177	0,465	0,041	0,338	0,125	0,717	0,000	0,264	0,173	2,401	0,176	0,391	0,219	0,123	0,266	0,294	0,067
A10	0,466	0,231	0,611	0,133	0,175	0,317	0,276	0,031	0,290	0,191	2,449	0,271	0,370	0,189	0,077	0,169	0,106	0,369
A11	0,369	0,161	0,673	0,026	0,382	0,199	0,738	0,035	0,181	0,241	2,306	0,355	0,329	0,201	0,000	0,160	0,229	0,221
A12	0,360	0,112	0,516	0,074	0,000	0,388	0,727	0,032	0,000	0,000	2,401	0,158	0,329	0,000	0,000	0,116	0,328	0,279
B01	0,000	0,007	0,722	0,127	0,145	0,162	0,000	0,000	0,545	0,153	2,685	0,045	0,257	0,217	0,142	0,356	0,371	0,197
B02	0,512	0,319	0,782	0,132	0,317	0,490	0,201	0,256	0,321	0,348	2,451	0,270	0,510	0,333	0,406	0,211	0,414	0,408
B03	0,575	0,479	0,854	0,199	0,359	0,418	0,468	0,211	0,394	0,447	2,434	0,394	0,313	0,371	0,314	0,286	0,648	0,290
B04	0,527	0,356	0,887	0,439	0,608	0,608	0,761	0,166	0,442	0,480	2,707	0,407	0,654	0,541	0,444	0,376	0,392	0,521
B05	0,591	0,357	0,806	0,253	0,512	0,349	0,680	0,112	0,233	0,423	2,667	0,530	0,642	0,332	0,556	0,525	0,274	0,392
B06	0,623	0,352	0,772	0,292	0,530	0,512	0,496	0,000	0,309	0,460	2,525	0,423	0,383	0,458	0,464	0,276	0,518	0,462
B07	0,714	0,457	0,848	0,339	0,476	0,495	0,797	0,210	0,670	0,489	1,947	0,595	0,578	0,319	0,332	0,367	0,624	0,512
B08	0,503	0,326	0,704	0,278	0,428	0,650	0,901	0,224	0,394	0,449	2,554	0,544	0,519	0,334	0,431	0,437	0,233	0,411
B09	0,653	0,541	0,763	0,324	0,412	0,513	0,717	0,216	0,346	0,363	2,638	0,578	0,649	0,290	0,412	0,316	0,455	0,438
B10	0,543	0,348	0,701	0,203	0,380	0,526	0,765	0,161	0,295	0,287	2,554	0,564	0,455	0,229	0,477	0,329	0,526	0,198
B11	0,531	0,377	0,552	0,072	0,282	0,342	0,646	0,027	0,253	0,207	2,468	0,331	0,230	0,104	0,340	0,205	0,268	0,294
B12	0,505	0,274	0,495	0,000	0,373	0,382	0,590	0,008	0,148	0,194	2,276	0,301	0,156	0,045	0,142	0,053	0,213	0,239
C01	0,144	0,344	0,733	0,378	0,381	0,430	0,494	0,193	0,380	0,371	2,558	0,363	0,409	0,336	0,227	0,438	0,587	0,274
C02	0,390	0,318	0,732	0,280	0,441	0,381	0,414	0,270	0,255	0,540	2,627	0,354	0,479	0,362	0,106	0,250	0,341	0,174
C03	0,317	0,460	0,860	0,268	0,363	0,309	0,777	0,170	0,376	0,449	2,607	0,562	0,442	0,406	0,445	0,371	0,630	0,351

C04	0,517	0,396	0,793	0,306	0,437	0,561	0,587	0,356	0,373	0,497	2,595	0,841	0,598	0,328	0,378	0,330	0,547	0,430
C05	0,759	0,405	0,879	0,497	0,638	0,636	0,717	0,196	0,308	0,372	2,533	0,538	0,556	0,360	0,262	0,297	0,687	0,474
C06	0,347	0,328	0,893	0,468	0,646	0,611	1,011	0,292	0,432	0,339	2,594	0,626	0,653	0,442	0,162	0,356	0,646	0,454
C07	0,537	0,400	0,897	0,277	0,499	0,560	0,757	0,226	0,384	0,398	2,520	0,430	0,626	0,327	0,212	0,336	0,565	0,338
C08	0,634	0,432	0,947	0,442	0,646	0,660	1,110	0,148	0,356	0,521	2,648	0,624	0,500	0,397	0,428	0,389	0,733	0,552
C09	0,496	0,324	0,788	0,247	0,563	0,491	0,819	0,281	0,277	0,596	2,546	0,696	0,768	0,403	0,283	0,429	0,591	0,333
C10	0,686	0,485	0,896	0,205	0,469	0,367	0,965	0,393	0,477	0,518	2,656	0,682	0,542	0,363	0,455	0,489	0,042	0,441
C11	0,663	0,375	0,863	0,339	0,521	0,247	0,652	0,153	0,284	0,371	2,545	0,374	0,261	0,253	0,388	0,340	0,425	0,421
C12	0,486	0,383	0,660	0,000	0,047	0,034	0,000	0,198	0,315	0,440	2,278	0,383	0,280	0,366	0,303	0,215	0,337	0,460
D01	0,268	0,427	0,724	0,144	0,145	0,088	0,187	0,271	0,547	0,526	2,394	0,663	0,600	0,374	0,539	0,513	0,000	0,511
D02	0,608	0,644	0,812	0,199	0,278	0,324	0,509	0,590	0,776	0,847	2,804	0,667	0,573	0,639	0,800	0,686	0,697	0,517
D03	0,451	0,602	0,679	0,426	0,260	0,497	0,781	0,445	0,630	0,721	2,747	0,753	0,337	0,526	0,790	0,549	0,560	0,672
D04	0,363	0,619	0,725	0,000	0,070	0,000	0,439	0,299	0,759	0,583	2,358	0,655	0,391	0,469	0,578	0,655	0,000	0,633
D05	0,367	0,539	0,876	0,386	0,396	0,550	0,886	0,465	0,438	0,480	2,626	0,487	0,573	0,344	0,652	0,298	0,582	0,303
D06	0,643	0,434	0,715	0,173	0,507	0,516	0,781	0,190	0,282	0,376	2,401	0,432	0,451	0,338	0,335	0,541	0,332	0,487
D07	0,428	0,407	0,574	0,274	0,473	0,426	0,789	0,186	0,515	0,291	2,453	0,278	0,469	0,318	0,284	0,294	0,526	0,418
D08	0,926	0,694	0,988	0,575	0,714	0,775	0,637	0,443	0,639	0,708	2,726	0,564	0,812	0,498	0,803	0,682	0,608	0,674
D09	0,581	0,554	0,751	0,264	0,460	0,545	0,577	0,000	0,371	0,127	2,512	0,276	0,459	0,236	0,300	0,356	0,201	0,302
D10	0,378	0,287	0,729	0,174	0,272	0,263	0,569	0,257	0,407	0,335	2,345	0,514	0,327	0,206	0,473	0,365	0,000	0,348
D11	0,534	0,471	0,609	0,000	0,172	0,270	0,907	0,172	0,325	0,389	2,372	0,424	0,220	0,173	0,532	0,380	0,314	0,415
D12	0,366	0,275	0,396	0,009	0,300	0,326	0,392	0,038	0,283	0,267	2,333	0,295	0,151	0,000	0,357	0,213	0,183	0,306
E01	0,169	0,536	0,685	0,379	0,450	0,396	0,417	0,356	0,595	0,450	2,559	0,689	0,478	0,443	0,575	0,437	0,370	0,557
E02	0,445	0,528	0,891	0,066	0,449	0,565	0,412	0,423	0,640	0,618	2,542	0,586	0,448	0,574	0,674	0,540	0,365	0,659
E03	0,345	0,262	0,972	0,283	0,454	0,344	0,345	0,248	0,516	0,409	2,486	0,501	0,149	0,233	0,517	0,513	0,000	0,429
E04	0,544	0,502	0,911	0,086	0,424	0,339	0,592	0,452	0,645	0,711	2,579	0,714	0,501	0,591	0,720	0,580	0,662	0,659
E05	0,533	0,308	0,614	0,037	0,356	0,332	0,000	0,287	0,517	0,406	2,239	0,376	0,344	0,390	0,268	0,561	0,000	0,426
E06	0,402	0,490	0,564	0,117	0,296	0,385	0,696	0,313	0,555	0,494	2,552	0,468	0,449	0,396	0,494	0,437	0,461	0,377
E07	0,668	0,624	0,899	0,302	0,617	0,567	0,290	0,257	0,499	0,618	2,582	0,678	0,344	0,395	0,644	0,529	0,581	0,561
E08	0,318	0,664	0,505	0,329	0,521	0,421	0,667	0,278	0,623	0,492	2,688	0,746	0,641	0,148	0,722	0,622	0,295	0,507
E09	0,525	0,601	0,727	0,301	0,439	0,538	0,896	0,176	0,566	0,470	2,588	0,486	0,516	0,265	0,432	0,515	0,427	0,463

E10	0,537	0,413	0,715	0,301	0,530	0,543	0,861	0,365	0,473	0,474	2,610	0,474	0,524	0,325	0,495	0,539	0,412	0,471
E11	0,525	0,117	0,590	0,103	0,311	0,466	0,220	0,000	0,160	0,014	2,287	0,189	0,000	0,121	0,481	0,000	0,234	0,268
E12	0,292	0,294	0,247	0,000	0,228	0,304	0,622	0,046	0,173	0,177	2,205	0,137	0,098	0,021	0,257	0,128	0,204	0,215
F01	0,102	0,571	0,647	0,000	0,533	0,378	0,280	0,270	0,686	0,451	2,686	0,496	0,604	0,352	0,467	0,615	0,542	0,000
F02	0,501	0,522	1,015	0,544	0,671	0,817	0,282	0,518	0,671	0,772	2,622	0,791	0,548	0,567	0,706	0,713	0,485	0,611
F03	0,678	0,790	0,907	0,326	0,777	0,751	0,395	0,461	0,515	0,542	2,677	0,730	0,651	0,347	0,599	0,535	0,616	0,559
F04	0,647	0,554	0,929	0,748	1,096	0,870	0,781	0,421	0,705	0,733	2,965	0,762	0,878	0,451	0,546	0,442	0,368	0,532
F05	0,649	0,654	1,049	0,677	0,000	0,911	0,505	0,795	0,730	0,841	3,062	0,901	0,785	0,456	0,497	0,846	0,904	0,755
F06	0,647	0,679	1,002	0,736	0,881	0,528	0,649	0,512	0,803	0,866	3,033	0,931	0,893	0,494	1,006	0,565	0,706	0,608
F07	0,849	0,825	1,007	0,748	0,862	0,695	0,686	0,461	0,684	0,753	2,982	1,063	0,809	0,370	0,563	0,815	0,568	0,496
F08	0,747	0,733	0,931	0,602	0,552	0,587	1,110	0,571	0,762	0,644	3,004	0,764	0,939	0,468	0,809	0,927	0,810	0,711
F09	0,766	0,653	0,988	0,487	0,843	0,518	0,676	0,683	0,680	0,873	2,984	0,961	0,911	0,745	0,871	0,938	0,763	0,421
F10	0,846	0,668	1,019	0,632	0,792	0,628	0,918	0,610	0,773	0,873	2,947	0,960	0,850	0,756	0,980	0,827	0,751	0,598
F11	0,807	0,648	0,942	0,600	0,907	0,815	0,330	0,605	0,519	0,733	2,874	0,479	0,681	0,454	0,390	0,624	0,506	0,660
F12	0,456	0,402	0,664	0,377	0,495	0,565	0,352	0,300	0,406	0,512	2,627	0,510	0,279	0,332	0,449	0,389	0,557	0,422
G01	0,244	0,627	0,922	0,527	0,902	0,167	0,405	0,379	0,625	0,746	2,795	0,801	0,851	0,344	0,146	0,529	0,699	0,402
G02	0,762	0,480	0,875	0,575	0,893	0,512	0,374	0,051	0,625	0,515	2,905	0,741	0,667	0,462	0,384	0,633	0,721	0,000
G03	0,608	0,743	1,090	0,616	0,000	0,846	0,482	0,627	0,759	0,855	3,022	0,844	0,404	0,595	0,673	0,613	0,695	0,596
G04	0,727	0,643	0,932	0,679	0,834	0,948	0,560	0,134	0,795	0,213	2,969	0,829	0,749	0,623	0,621	0,665	0,752	0,682
G05	0,924	0,745	0,859	0,498	0,718	0,805	0,271	0,533	0,660	0,655	2,620	0,458	0,653	0,536	0,685	0,670	0,441	0,758
G06	0,876	0,870	1,098	0,685	0,936	0,881	0,757	0,429	0,640	0,743	2,890	0,618	0,736	0,426	0,668	0,744	0,907	0,495
G07	0,653	0,631	0,889	0,718	0,901	0,671	0,496	0,508	0,736	0,694	2,915	0,722	0,712	0,498	0,784	0,643	0,573	0,590
G08	0,505	0,521	0,698	0,454	0,639	0,553	0,405	0,112	0,474	0,632	2,945	0,442	0,606	0,312	0,337	0,312	0,281	0,416
G09	0,825	0,524	0,863	0,573	0,730	0,744	0,557	0,475	0,359	0,771	2,804	0,633	0,595	0,258	0,513	0,661	0,724	0,559
G10	0,765	0,649	0,828	0,426	0,646	0,588	0,152	0,394	0,524	0,288	2,532	0,567	0,577	0,383	0,375	0,534	0,228	0,666
G11	0,604	0,505	0,618	0,075	0,418	0,521	0,327	0,236	0,436	0,426	2,530	0,529	0,331	0,279	0,348	0,310	0,469	0,420
G12	0,489	0,427	0,503	0,111	0,473	0,544	0,865	0,107	0,156	0,299	2,469	0,375	0,265	0,089	0,260	0,218	0,305	0,204
H01	0,170	0,376	0,555	0,253	0,802	0,754	0,352	0,140	0,350	0,359	2,665	0,584	0,618	0,340	0,000	0,543	0,588	0,601
H02	0,495	0,834	0,582	0,669	0,761	0,543	0,363	0,747	0,851	0,433	3,035	0,777	0,820	0,141	0,676	0,831	0,729	0,593
H03	0,508	0,501	0,863	0,374	0,416	0,518	0,319	0,320	0,656	0,522	2,770	0,841	0,263	0,290	0,564	0,770	0,712	0,498

H04	0,452	0,513	0,859	0,429	0,657	0,607	0,273	0,205	0,056	0,256	2,630	0,642	0,255	0,386	0,357	0,593	0,675	0,315
H05	0,497	0,431	1,020	0,440	0,659	0,622	0,000	0,293	0,564	0,512	2,789	0,681	0,493	0,496	0,508	0,589	0,051	0,623
H06	0,783	0,355	1,094	0,566	0,896	0,685	0,329	0,553	0,574	0,611	2,745	0,768	0,750	0,402	0,469	0,630	0,308	0,657
H07	0,605	0,824	0,847	0,699	0,970	0,855	0,000	0,330	0,664	0,687	2,821	0,678	0,713	0,254	0,491	0,638	0,758	0,654
H08	0,490	0,346	0,588	0,316	0,541	0,523	0,000	0,219	0,454	0,490	2,390	0,404	0,361	0,248	0,254	0,413	0,733	0,301
H09	0,116	0,236	0,794	0,102	0,235	0,344	0,172	0,224	0,332	0,357	2,401	0,000	0,428	0,189	0,233	0,316	0,411	0,372
H10	0,370	0,513	0,690	0,204	0,467	0,415	0,210	0,077	0,349	0,113	2,398	0,297	0,168	0,260	0,128	0,397	0,596	0,320
H11	0,344	0,261	0,672	0,193	0,373	0,380	0,423	0,193	0,429	0,170	2,083	0,000	0,182	0,167	0,101	0,308	0,376	0,581
H12	0,000	0,401	0,367	0,000	0,245	0,125	0,219	0,000	0,133	0,279	2,285	0,093	0,084	0,250	0,000	0,323	0,203	0,333

Appendix I. Optical density values obtained during the second audit.

	Sample Number																	
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
A02	0,000	0,788	0,000	0,430	0,814	0,382	0,232	0,987	1,312	0,058	0,547	0,258	0,565	0,000	1,427	0,294	0,000	1,396
A03	0,000	2,338	1,783	0,403	0,923	1,646	1,252	0,869	0,585	0,838	1,327	0,452	0,131	0,000	0,000	1,254	0,000	1,935
A04	0,000	0,374	1,492	0,263	1,182	1,363	1,243	1,268	1,729	0,706	0,700	1,226	1,448	1,656	0,790	1,269	1,273	0,250
A05	0,000	0,019	0,962	0,000	1,462	1,319	1,354	0,920	1,182	0,860	0,827	0,295	1,155	0,742	1,193	1,285	1,543	0,347
A06	0,000	0,000	0,992	2,220	1,220	0,939	1,079	1,044	0,716	0,868	1,220	0,000	1,486	1,096	0,700	0,602	0,551	0,000
A07	1,477	2,894	1,167	0,993	1,261	0,712	1,076	0,591	1,247	0,739	0,933	0,959	0,655	0,117	0,000	1,077	0,654	0,000
A08	2,459	2,532	1,849	2,271	1,702	1,159	1,163	0,683	0,320	1,137	1,167	1,415	1,273	0,886	0,000	0,747	0,723	0,270
A09	3,999	0,621	0,858	1,018	0,762	1,054	0,962	0,820	0,466	0,494	0,673	1,752	0,191	1,491	0,000	1,048	1,755	0,568
A10	4,550	2,319	1,546	1,320	1,506	0,905	1,119	0,617	1,279	0,909	1,157	1,715	0,524	0,739	1,674	1,019	0,172	0,000
A11	1,369	1,156	1,219	1,596	0,915	1,162	1,367	0,697	1,832	0,478	0,570	1,378	0,382	0,622	1,252	0,743	0,000	0,867
A12	0,367	2,427	1,804	2,111	1,566	0,256	1,070	0,946	0,000	1,008	1,783	1,295	0,000	0,756	0,550	0,823	2,867	1,772
B01	0,000	0,297	0,000	0,070	0,278	0,233	0,000	1,214	1,816	0,483	0,477	0,000	0,057	0,450	0,477	0,297	0,436	0,000
B02	0,000	2,436	0,000	1,096	1,179	0,877	1,824	0,967	1,285	1,431	1,940	0,973	1,560	0,625	1,842	1,454	0,000	3,562
B03	0,000	0,297	0,994	0,058	0,901	1,499	0,758	0,928	1,626	1,071	1,223	1,272	1,120	0,429	0,000	1,791	2,913	1,290
B04	0,000	2,000	2,270	0,000	1,198	0,850	1,750	1,212	1,518	1,508	1,790	1,143	2,046	1,089	0,999	2,001	0,596	0,241
B05	0,000	2,897	2,040	0,627	1,201	1,583	1,070	1,113	1,366	1,379	1,143	0,000	1,275	1,481	0,787	1,562	1,755	0,000
B06	0,000	2,801	1,782	2,844	1,100	1,499	1,456	0,939	1,399	1,266	0,827	2,955	0,767	1,075	1,789	1,649	2,024	1,290
B07	0,461	0,281	0,732	0,957	1,590	1,426	1,902	1,182	1,979	1,175	1,600	0,862	0,524	0,151	1,179	1,095	2,552	0,626
B08	1,898	2,777	1,893	1,151	1,503	1,306	1,023	1,126	0,721	1,236	0,000	1,415	0,467	0,477	1,024	1,903	1,646	2,080
B09	3,216	0,615	1,087	1,387	0,920	2,316	1,438	1,103	0,732	0,851	0,303	1,037	1,887	1,185	0,000	1,432	1,795	0,000
B10	4,507	2,891	2,298	1,832	1,332	1,863	1,546	1,088	1,382	1,046	1,610	2,369	1,188	1,285	1,331	1,111	1,239	0,000
B11	1,387	2,987	1,808	2,262	1,800	1,334	1,008	0,895	0,493	0,791	0,970	1,954	0,333	1,226	1,087	1,093	2,885	1,367
B12	0,385	1,911	1,930	2,511	0,866	0,568	0,371	0,738	1,274	0,780	1,040	1,595	0,759	1,116	1,301	0,957	1,388	0,568
C01	0,138	2,294	2,263	1,120	1,217	1,125	0,179	1,119	2,364	1,362	1,843	1,383	0,407	1,511	2,258	1,338	2,695	2,320
C02	0,000	2,338	0,000	1,245	1,410	1,149	0,458	1,042	2,065	1,609	1,917	1,051	0,983	0,900	1,167	1,115	1,709	1,473
C03	0,000	0,708	1,066	0,000	0,978	0,445	1,330	1,057	0,884	0,898	1,153	1,051	1,281	1,034	0,154	1,274	2,518	0,568

C04	0,000	2,619	1,778	0,000	1,160	1,371	1,735	1,151	1,453	1,126	1,640	1,051	1,161	1,566	1,565	1,874	0,682	0,000
C05	0,000	0,714	0,292	0,194	0,705	1,499	1,512	0,701	0,965	0,442	0,973	0,120	0,150	1,257	1,441	0,569	2,552	0,000
C06	0,000	2,511	1,822	2,108	1,536	0,586	1,540	0,799	1,442	1,661	1,907	2,918	1,188	0,948	1,517	1,586	1,118	1,772
C07	0,370	2,743	2,196	1,326	1,658	1,096	2,022	0,973	1,751	1,005	1,193	2,692	0,808	1,302	1,996	1,151	1,308	0,664
C08	1,479	2,845	2,021	1,084	1,122	0,934	1,478	1,056	2,450	1,700	1,360	2,913	1,090	1,587	1,377	1,646	1,422	3,042
C09	2,959	0,705	0,894	0,978	1,225	1,777	1,166	1,123	0,770	0,772	1,077	2,199	0,989	1,384	0,302	1,234	2,822	1,194
C10	4,482	0,025	0,513	0,966	1,007	1,714	1,048	0,934	0,390	0,983	1,050	0,562	1,133	1,230	1,563	0,812	0,677	1,088
C11	1,495	2,239	1,347	1,163	1,092	1,290	0,974	1,154	1,046	1,052	1,113	1,420	1,357	1,329	0,833	1,408	2,260	1,232
C12	0,336	0,059	0,494	0,848	1,514	0,233	1,497	1,001	0,000	1,044	0,187	0,023	0,000	0,890	0,610	0,172	0,493	0,443
D01	0,082	1,231	0,656	1,769	1,402	0,809	0,000	1,310	0,900	1,066	1,113	0,000	0,494	0,835	2,026	1,483	2,110	2,099
D02	0,000	2,294	1,440	0,869	1,263	0,644	0,921	1,238	0,699	0,755	1,380	0,880	1,109	2,106	1,800	1,423	0,000	0,895
D03	0,000	1,500	1,549	0,000	1,125	0,701	0,000	1,110	0,000	1,354	0,473	0,341	1,420	1,374	0,000	1,227	0,000	0,000
D04	0,000	1,691	0,435	0,000	1,127	0,958	0,436	1,093	0,683	0,670	0,030	0,286	1,655	1,288	1,648	0,961	3,120	0,125
D05	0,000	2,260	1,458	0,491	1,247	1,138	2,004	0,897	0,173	0,755	1,467	1,231	1,000	1,010	2,037	1,376	1,015	0,568
D06	0,000	2,433	1,977	2,647	1,511	0,973	2,551	0,721	1,708	1,296	1,647	1,844	0,677	0,776	1,059	0,919	0,717	1,849
D07	0,334	2,922	1,796	1,211	1,372	1,570	1,905	1,054	1,138	1,650	0,847	1,563	1,169	1,134	1,149	0,000	0,671	3,524
D08	1,627	0,371	0,754	0,960	0,871	0,785	1,490	1,200	1,653	1,077	1,167	1,111	0,934	1,814	1,117	1,796	2,363	0,000
D09	3,036	1,963	1,813	1,823	0,852	1,941	1,351	0,956	1,903	1,063	0,983	2,263	1,000	1,010	1,153	1,216	0,986	2,205
D10	4,801	0,000	0,000	0,824	1,062	1,345	1,039	1,156	0,672	0,827	0,573	0,088	0,896	1,010	1,278	0,419	1,434	0,510
D11	1,625	0,000	0,754	1,287	1,135	1,884	0,801	0,794	0,569	0,645	0,847	0,525	0,000	0,670	0,992	0,765	0,946	1,473
D12	0,579	0,105	0,459	1,272	0,999	0,861	0,495	1,135	0,000	0,324	0,417	0,189	0,000	1,017	0,000	0,426	0,000	0,000
E01	0,000	0,043	0,630	0,875	0,692	0,827	0,099	1,324	0,000	1,274	0,353	0,793	0,000	0,807	1,112	0,531	0,740	1,916
E02	0,000	0,000	0,331	0,727	0,746	1,303	0,000	1,005	0,000	0,882	0,993	0,000	1,316	1,000	0,090	1,283	0,000	0,039
E03	0,000	0,000	0,052	0,000	1,081	0,638	0,260	0,000	0,000	1,011	0,000	0,000	0,666	1,072	0,000	0,384	0,000	0,000
E04	0,000	0,702	1,079	0,542	0,705	0,636	1,036	1,147	0,732	1,068	0,847	0,000	1,863	0,818	2,606	1,321	0,000	2,243
E05	0,000	0,000	0,000	0,615	1,462	0,693	0,000	0,799	0,510	0,000	0,607	0,000	1,084	1,357	0,000	0,464	1,244	0,000
E06	0,000	0,433	1,159	2,020	0,874	1,379	0,012	1,270	0,347	1,142	1,003	0,466	1,117	0,704	0,843	1,338	0,000	0,000
E07	0,482	0,133	0,626	0,615	1,029	1,664	1,756	1,194	1,632	0,934	1,243	0,466	0,929	1,010	0,962	1,383	0,975	1,252
E08	1,781	0,810	0,483	1,345	1,391	1,264	1,466	1,238	0,949	0,945	0,513	0,996	1,478	1,498	1,715	1,849	1,485	0,000
E09	2,967	0,077	0,797	1,066	0,259	1,102	1,308	0,962	0,575	1,011	0,237	0,963	0,909	1,982	0,716	0,589	1,273	0,000

E10	4,701	1,373	1,497	1,766	0,664	1,447	1,224	0,974	0,184	0,838	1,527	2,074	1,213	0,605	1,105	1,506	0,401	1,935
E11	1,526	0,000	0,357	1,151	0,732	1,311	0,603	0,706	0,000	0,590	0,177	0,000	0,869	1,092	0,739	0,000	0,000	0,000
E12	0,507	0,686	1,183	1,018	1,018	1,353	0,572	0,984	0,000	0,450	0,577	0,429	0,000	0,000	0,518	0,562	0,000	0,000
F01	0,260	0,476	0,479	0,781	0,487	0,790	0,000	1,389	0,976	0,750	0,637	0,000	1,248	1,168	1,328	0,825	0,000	0,000
F02	0,000	0,235	0,602	0,878	0,719	0,879	1,054	1,043	0,965	0,687	0,260	0,000	1,355	1,347	0,992	0,825	0,000	0,000
F03	0,000	0,903	0,529	0,569	1,552	0,403	0,000	0,823	0,439	1,307	0,827	0,129	1,489	0,976	0,147	1,227	0,000	0,000
F04	0,000	0,000	0,251	0,000	0,893	0,918	0,000	1,033	0,553	1,077	0,477	0,816	1,543	1,381	1,565	0,578	3,693	0,558
F05	0,000	0,000	0,126	0,000	0,455	0,798	0,000	0,808	0,960	1,359	0,660	0,673	1,628	1,185	1,766	0,794	0,103	0,000
F06	0,000	0,000	0,591	0,951	1,035	0,788	1,704	0,822	1,361	1,510	0,457	1,705	1,767	0,447	1,814	1,082	0,000	0,655
F07	0,209	0,124	0,884	0,860	0,626	0,641	0,603	1,002	0,672	0,626	1,030	1,401	1,188	0,934	0,965	0,712	0,149	2,590
F08	1,605	0,393	1,412	0,918	0,901	1,070	0,087	1,214	0,846	1,140	1,337	2,452	2,327	1,343	1,103	1,943	0,000	0,703
F09	2,619	0,365	1,167	0,851	0,703	0,945	1,382	1,004	1,279	1,549	0,850	1,996	1,745	1,470	1,655	1,669	0,327	1,435
F10	4,466	0,816	1,182	0,754	0,727	1,824	1,509	0,972	1,881	1,186	1,493	2,083	2,786	1,590	0,965	0,959	0,304	1,175
F11	1,550	0,121	0,708	0,969	0,373	1,185	1,673	1,014	1,046	1,343	0,700	1,203	0,000	0,866	0,953	0,451	0,717	0,231
F12	0,600	0,767	0,752	1,205	0,956	1,193	1,258	1,040	0,157	0,945	0,920	0,899	1,226	0,646	1,027	0,602	0,711	0,924
G01	0,466	0,331	0,734	1,011	1,672	0,262	0,000	1,319	1,220	0,511	1,537	0,254	1,778	1,817	1,727	1,562	2,581	0,000
G02	0,000	0,189	0,985	1,015	0,114	0,288	0,238	1,186	0,916	1,082	0,940	0,327	0,661	1,127	2,095	0,901	0,321	0,000
G03	0,000	0,000	0,117	0,000	0,095	1,068	0,074	1,004	0,266	0,769	0,510	0,562	0,590	1,333	0,000	0,754	0,000	0,000
G04	0,000	0,000	0,485	0,015	0,286	0,581	0,000	1,023	1,035	1,167	0,423	1,249	0,000	0,635	1,706	1,093	0,000	1,281
G05	0,000	0,553	0,344	0,000	0,313	1,138	1,020	0,993	1,355	1,002	0,880	0,175	0,000	1,872	0,541	0,471	0,126	0,597
G06	0,000	0,238	0,908	1,420	0,523	0,740	0,807	0,977	0,992	1,222	1,333	1,281	1,469	1,130	1,973	1,392	0,000	3,100
G07	0,322	0,000	0,658	0,803	0,713	0,672	1,605	1,074	1,653	1,329	0,850	1,821	1,639	1,336	0,932	1,428	0,505	2,234
G08	1,421	1,320	1,269	1,136	1,056	1,238	1,722	1,259	0,000	0,980	0,510	1,180	1,275	1,223	0,866	0,718	0,000	1,502
G09	2,706	1,234	1,893	1,033	1,100	1,321	1,463	1,233	1,046	0,920	0,000	1,632	1,281	0,979	1,059	1,169	0,000	1,772
G10	4,418	0,000	0,730	0,170	0,506	1,994	1,113	1,061	1,203	0,769	0,490	0,000	0,587	1,213	0,173	0,970	0,849	0,655
G11	1,520	0,000	0,810	1,429	1,498	1,157	0,646	0,714	1,171	0,706	0,050	0,627	0,429	0,790	0,297	0,560	0,000	0,356
G12	0,421	0,176	1,458	1,357	0,692	0,536	0,504	0,542	0,396	0,871	1,013	1,337	1,117	0,000	0,382	0,801	0,000	2,060
H01	0,706	0,000	0,240	0,957	0,280	0,000	0,000	0,909	0,558	1,120	2,160	0,000	0,890	1,494	0,000	0,000	0,000	0,000
H02	0,000	0,798	1,211	1,299	0,599	0,018	1,166	1,375	1,849	1,499	1,977	2,203	1,699	0,000	1,588	1,093	0,000	1,675
H03	0,000	0,751	0,838	0,206	0,727	0,795	2,084	1,141	1,962	1,167	2,607	2,185	0,563	0,543	1,883	0,930	0,499	2,513

H04	0,000	0,108	0,600	0,905	1,293	0,301	0,000	0,745	2,331	1,247	1,307	1,060	0,000	1,786	1,344	1,153	0,688	2,070
H05	0,000	0,000	0,216	0,000	0,506	0,183	1,868	0,036	1,296	1,274	1,910	0,631	2,666	1,065	1,625	1,651	2,380	0,510
H06	0,000	0,804	1,468	2,838	0,583	0,487	1,713	0,966	1,832	1,395	1,013	0,410	1,833	0,725	0,000	0,000	1,331	0,578
H07	0,072	0,000	0,256	0,569	1,182	0,829	1,252	0,890	0,862	1,011	0,450	0,424	1,814	1,371	0,000	0,000	0,000	2,349
H08	1,153	0,009	0,268	0,951	1,016	0,557	1,441	1,617	0,564	1,282	0,733	0,000	0,000	0,752	0,000	0,000	2,988	1,348
H09	2,329	0,000	0,721	1,072	1,146	0,853	0,989	1,278	1,534	1,722	1,943	0,000	1,945	0,000	0,242	0,647	0,000	3,716
H10	4,181	0,974	0,052	0,905	0,683	0,560	1,345	1,054	0,320	0,330	1,670	1,074	0,918	0,117	0,672	0,462	2,105	2,263
H11	1,862	2,647	1,915	1,808	1,133	0,730	1,738	1,063	1,496	0,873	1,390	2,102	0,489	0,000	1,404	0,886	0,241	0,770
H12	0,730	2,183	1,665	2,023	1,427	0,560	0,612	1,054	1,138	0,882	1,400	0,899	0,915	0,000	1,361	0,785	3,888	2,523